Short Communication

Isolation and Amino Acid Sequence of a Molt-inhibiting Hormone from the American Crayfish, Procambarus clarkii

Hiromichi NAGASAWA,† Wei-Jun YANG,* Hidetsugu SHIMIZU,**† Katsumi AIDA,* HibiKi TSUTSUMI,**† Akiko TERAUCHI,**† and Haruyuki SONOBE,**†

Ocean Research Institute, The University of Tokyo, Nakanoku, Tokyo 164, Japan
* Department of Fisheries, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
** School of Hygiene Science, Kita-Satou University, Sagamihara, Kanagawa 228, Japan
** Department of Biology, Konan University, Higashinada-ku, Kobe, Hyogo 658, Japan
Received September 6, 1995

A molt-inhibiting hormone (Pc-MIH) was isolated from the sinus glands of the American crayfish, Procambarus clarkii, and its amino acid sequence was determined. It comprised 75 amino acid residues and had an amidated carboxyl terminus. The amino acid sequence was much more similar to MIH of the shore crab (Cam-MIH) than to MIH of the American lobster (HoA-MIH).

Key words: crayfish, Procambarus clarkii; molt-inhibiting hormone; molting; ecdysteroid

Molting in crustaceans is known to be regulated endocrinologically, as it is in insects. Although the molting hormone, ecdysteroid, is common in these two groups of arthropods, the control system for the ecdysteroid-producing gland, the Y-organs in crustaceans and the prothoracic gland in insects, is completely different; the former is inhibited by the molt-inhibiting hormone (MIH) not to produce ecdysteroids, while the latter is stimulated by the prothoracicotropic hormone (PTTH) to produce ecdysteroids. These two hormones, MIH and PTTH, have recently been chemically characterized, both revealing them to be completely different from each other in both molecular size and amino acid sequence. Up to now, two MIHs have been isolated and sequenced, one (HoA-MIH) from the American lobster, Homarus americanus, and the other (Cam-MIH) from the shore crab, Carcinos maenas. Both MIHs are members of the peptide family, called the CHH family or the CHH MIH VH family, which comprises the crustacean hyperglycemic hormone (CHH), MIH, and the vitellogenesis-inhibiting hormone (VII). They are produced in the X-organ and released from the sinus gland into the hemolymph. However, it seems strange that HoA-MIH is structurally more similar to CHHs of various species than to Cam-MIH. In order to get more information on the structure-activity relationship of MIH, we tried to characterize an MIH from the American crayfish, Procambarus clarkii.

Five hundred sinus glands were dissected from live crayfish, P. clarkii, obtained from a local dealer. They were homogenized in 0.5 ml of 30% acetonitrile containing 0.9% sodium chloride on ice, and the homogenate was centrifuged at 15,000 rpm and 4°C for 5 min. The precipitate was suspended in 0.5 ml of the same solution and centrifuged again under the same conditions. The two supernatants were combined and subjected to bioassay for MIH, which used an in vitro culture of Y-organs and subsequent determination of ecdysteroids in the culture medium by a radioimmunoassay. Since the extracts were found to have high MIH activity, they were subjected to reverse-phase high performance liquid chromatography (HPLC), using a column of Asahipak ODP-50 (4.6 × 250 mm; Showa Denko, Tokyo). The materials were eluted with a 65-min linear gradient of acetonitrile from 0% to 65% in 0.05% trifluoroacetic acid (TFA). The elution was monitored by UV absorbance at both 225 and 280 nm. Fractions were manually collected peak by peak at 225 nm and were numbered in the order of elution (Fig. 1). Each fraction was subjected to a bioassay for MIH, extremely high activity being found in fraction No. 29, and low activity being observed in some fractions including No. 34. Fraction No. 34 was further separated into two isomeric peptides (Pc-CHH-I and -II) by using a TSKgel ODS 120T column (Tosoh), both of these peptides having already been characterized by Yasuda et al. The isomeric peptides had the same amino acid sequence, but differed from each other only in the configuration of Phe at position 3: L-Phe in Pc-CHH-I and d-Phe in Pc-CHH-II. Interestingly, Pc-CHH-II exhibited higher MIH activity than Pc-CHH-I. In our present experiment, these two peptides showed MIH activity, but were almost equally potent. The maximum level of inhibition by each of these peptides, about 30%, was attained at the approximate concentration of 1 sin gland equivalent (SGE) in 500 μl of medium. In contrast, fraction No. 29 exhibited much higher activity than Pc-CHH-I and -II: the maximum level of inhibition was about 50%, which was caused by 0.125–0.5 SGE in 500 μl of medium, while 30% inhibition could be attained by as little as 0.01 SGE in 500 μl of medium. The dose-response relationship of fraction No. 29 was nearly identical to that of the crude extract (data not shown), indicating that only fraction No. 29 could explain the activity of the crude extracts. Thus, we designated the peptide from fraction No. 29 as Pc-MIH and tried to determine its amino acid sequence. The yield of Pc-MIH from 500 sin glands was about 17 μg (2 nmol).

The amino-terminal amino acid sequence analysis of Pc-MIH by a protein sequencer (Applied Biosystems, model 476A) resulted in the identification of residues up to position 60, except for 10 residues at positions 7, 24, 27, 40, 44, 46,
Fig. 1. Elution Profile of Sinus Gland Extracts from Reverse-phase HPLC.

Columns: Asahi Pak ODS-30 (4.6 × 250 mm); elution: a linear gradient of 0.65%, acetonitrile in 0.05% TFA in 65 min; temperature, 40 °C; flow rate, 1 ml min; detection, absorbance at 225 nm. The dotted line indicates the concentration of acetonitrile.

Fig. 2. Summary of the Sequence Analysis of Pre-MIH.
(a) Amino-terminal sequence analysis; the broken line indicates the identified residue, while * indicates unidentified residues. (b) Fragments (K2, K3) generated by lysyl endopeptidase digestion. (c) Fragments (E1, E2) generated by endoproteinase Glu-C digestion.

47, 48, 53, and 58 (Fig. 2(a)). To determine these unidentified residues and those beyond position 60, the intact peptide (500 pmol) was digested with lysyl endopeptidase. This digestion generated three peptide fragments named K1, K2, and K3, which were separated by reverse-phase HPLC on a column of μBondapak Phenyl (3.9 × 250 mm, Waters) with the same gradient conditions as those used for the purification of the intact peptide. An aliquot of each peptide fragment was subjected to the sequence analysis. By comparing the sequences of the fragments with the aminoterminal sequence, K2 and K3 were each found to consist of two peptide chains connected by disulfide bond(s), while K1 was presumed to be a carboxyl-terminal peptide (Fig. 2(b)). Reductive carboxymethylation of both K2 and K3 resulted in the separation of the two peptides, K2-a and K2-b from K2, and K3-a and K3-b from K3. Sequence analyses of these peptides determined all the residues that could not be identified by amino-terminal sequencing, as well as the sequence from positions 61 to 67. The direct connection of K3-b and K1 was established by the fragment peptide, E2, which was obtained by endoproteinase Glu-C digestion of intact Pre-MIH (Fig. 2(c)). Thus, the entire sequence consisting of 75 amino acid residues was determined. The carboxyl-terminal structure of this peptide was determined by a high-resolution FAB mass spectral analysis of K1. The protonated molecular ion peak of K1 1 was observed at m/z 941.58, which agrees with the amidated structure (calculated value of 941.54). In order to confirm this, we synthesized two peptides with the same amino acid sequence as K1, one of which had an amide group at the carboxyl terminus, and the other had a free carboxyl group. K1 was eluted at the same retention time as that of the synthetic peptide with an amide group by reverse-phase HPLC, establishing the amide structure of K1.

Pre-MIH had six cysteine residues, whose positions were well-conserved in the peptides of the CHH MIH VII family. The amino acid sequence of Pre-MIH was much more similar to that of Cam-MIH (48%) than to those of Hoa-MIH (29%) or to Pre-CHH-I and -II (25% each). The similarity between Pre-MIH and Cam-MIH was found in the overall sequence, while that between Pre-MIH and Hoa-MIH was confined to the central part of the sequence from Cys' to Leu 53. Pre-MIH was dissimilar to Pre-CHH-I or -II in almost the same extent as Pre-MIH to Hoa-MIH. Further study is needed to understand the structure–activity relationship of MIH more deeply.

Acknowledgments. We are grateful to Dr. J. Nakayama of Faculty of
Agriculture at The University of Tokyo for measuring the high-resolution FAB mass spectrum. This work was partly supported by Grants-in-Aid for Scientific Research (No. 07450054 and No. 07406009) from the Ministry of Education, Science, and Culture of Japan.

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