A Recombinant Molt-inhibiting Hormone of the Kuruma Prawn Has a Similar Secondary Structure to a Native Hormone: Determination of Disulfide Bond Arrangement and Measurements of Circular Dichroism Spectra

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In crustaceans, molt-inhibiting hormone (MIH) is presumed to regulate molting through suppressing synthesis and/or secretion of ecdysteroids by the Y-organ. Recently, a recombinant MIH of the kuruma prawn Penaeus japonicus was produced in E. coli. To approximate the secondary structure of native and recombinant MIH of P. japonicus containing six cysteine residues, the arrangements of disulfide bridges in both MIHs were determined by characterizing their enzymatic digests, and their circular dichroism spectra were measured. The arrangements of disulfide bonds in both MIHs were determined to be identical, and they were linked between Cys15 and Cys44, Cys24 and Cys49, and Cys27 and Cys53. The circular dichroism spectra of both MIHs were very close, and demonstrated that they were rich in α-helix. α-Helix contents in native and recombinant MIHs were calculated to be 49.3% and 46.0%, respectively. All these results strongly suggested that the recombinant MIH was folded in the same manner as the native MIH.

Key words: molt-inhibiting hormone; crustacean hyperglycemic hormone; Penaeus japonicus; disulfide bond arrangement; secondary structure

In crustaceans, various kinds of neuropeptides are produced in the X-organ and transferred to the sinus gland, from where they are released into hemolymph.1) They include crustacean hyperglycemic hormone (CHH), molt-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIIH), and mandibular organ-inhibiting hormone (MOIH), and have been identified in many crustaceans. MIH is presumed to regulate molting through suppression of synthesis and/or secretion of molting hormone, ecdysteroids, by the Y-organ in vivo. MIHs have similar amino acid sequences to CHHs, VIIHs, and MOIHs, and therefore all of them form a peptide family referred to as the CHH family.1,2) Members of this family have also been identified from other arthropods; an ion transport peptide (ITP) from the locust, Schistocerca gregaria,3) a low molecular weight protein (LMWP) from the black widow spider, Latroductes mactans tredecimguttatus,4) and BmCHHL (Bombyx mori CHH-like peptide) from the silkworm, Bombyx mori.5) The CHH family peptides consist of 70 to 83, mostly 72 to 78, amino acid residues and have six conserved cysteine residues.

The CHH family is classified into type I and type II depending on the primary structure of mature hormones.6) The carboxyl-terminus of most type I peptides is amidated, while that of most type II peptides is free. A glycine residue is inserted at position 12 in type II peptides, resulting in a longer sequence by one residue between the first and second cysteine residues than type I peptides. All CHHs are classified into type I and most MIHs, VIIHs, and MOIHs are classified into type II. However, there have been some exceptions: MIH of Procambarus bouvieri nevertheless classified into type I, showed MIH activity but no CHH activity,7) CHH of Homarus americanus, classified into type I, showed both CHH and MIH activities,8) and MOIH of Libinia emarginata, classified into type I, showed both CHH and MOIH activities.9) Thus, the classification by amino acid sequences does not always agree with that by biological activities.

The arrangements of three disulfide bridges in some type I peptides were determined to be identical; CHH of Carcinus maenas10) CHH and MIH of Procambarus bouvieri,11) CHH of Armadillidium vulgare,12) CHH of Procambarus clarkii,13) LMWP of L. mactans tredecimguttatus,4) and Pej-SGP-III (SGP: sinus gland peptide of Penaeus japonicus).14)
Secondary structures were estimated by circular dichroism (CD) spectra in type I peptides such as CHH and MIH of *P. bouvieri*, and in LMWP of *L. mactans tredecimguttatus*. In type II, the disulfide bridge arrangement of only one peptide, MIH of *P. clarkii* (Prc-MIH), was analyzed using Prc-MIH synthesized chemically. Therefore, there has been no precedent of determination of disulfide bridge arrangements in natural type II peptides of the CHH family. Moreover, no CD spectra of any type II peptides of the CHH family have been recorded up to now, so the difference in the secondary structure between type I and type II peptides remains unclear.

In the kuruma prawn, *P. japonicus*, seven CHH-family peptides were isolated, and designated Pej-SGP-I to -VII. Six of them, Pej-SGP-I, -II, -III, -V, -VI, and -VII, classified into type I, have CHH activity, while Pej-SGP-IV, classified into type II, does not have CHH activity *in vivo* but has high MIH activity *in vitro*. Therefore, Pej-SGP-IV is presumed to be an MIH, and the six other peptides are presumed to be CHHs. A cDNA encoding the MIH of *P. japonicus* (Pej-MIH) was cloned, and its recombinant peptide was expressed in *E. coli*. Since the recombinant peptide was inactive, it was then subjected to a refolding reaction, resulting in its displaying biological activity comparable to that of the natural peptide.

In this paper, in order to find if the recombinant MIH has a similar structure to the native MIH, we determined disulfide bridge arrangements of native and recombinant Pej-MIHs and recorded their CD spectra.

**Materials and Methods**

**Animals.** Live kuruma prawns *P. japonicus* were obtained at a fish market in Tokyo, Japan. Sinus glands were dissected out of the eyestalks of anesthetized prawns and kept in a solution of 0.9% NaCl containing 2 M urea and 30% acetonitrile on ice, and then peptides were extracted from them immediately.

**Extraction and purification of native MIH and other CHH family peptides.** Extraction and purification of CHH family peptides were done essentially according to the method described previously with slight modifications. Sinus glands were homogenized in 0.9% NaCl containing 2 M urea and 30% acetonitrile on ice. After centrifugation at 12,000 g and 4°C for 10 min, the pellet was treated in the same manner as described above. The two supernatants were combined, and put through reverse-phase chromatography on a Sep-Pak Vac C18 cartridge (100 mg, Waters). CHH family peptides were eluted with 50% acetonitrile/0.1% trifluoroacetic acid (TFA), and after concentration, the resultant solution was put through reverse-phase HPLC on an Asahi-Pack ODP-50 column (4.6 × 250 mm, Showa Denko, Tokyo). Elution was done with a 60-min linear gradient of 0–40% acetonitrile in 0.05% TFA at a flow rate of 0.6 ml/min. The elution was monitored by measuring the absorbances at 225 and 280 nm. Each CHH family peptide fraction was then put through the second reverse-phase HPLC in the same manner as in the first HPLC except for the use of 0.05% heptafluorobutyric acid (HFBA) instead of 0.05% TFA. The amount of each peptide was calculated based on the absorbance at 280 nm.

**Expression and purification of recombinant MIH.** Recombinant MIH was expressed in *E. coli* as described previously. Purification of recombinant MIH and subsequent refolding reaction were done essentially according to the method described previously with some modifications. The insoluble material after cell breakage was solubilized in 6 M guanidine-HCl/10 mM Tris-HCl buffer (pH 8.0). The resulting solution was put on a Vydac Proteins C4 column (22 × 50 mm, Vydac). The column was washed with 10% acetonitrile in 0.1% TFA and recombinant MIH was eluted with 50% acetonitrile in 0.1% TFA. The eluate was lyophilized and the lyophilized material was dissolved in 8 M urea in 0.5 M Tris-HCl buffer (pH 8.0) at the concentration of 40 µg/ml. To this solution, five volumes of dilution buffer (0.5 M Tris-HCl buffer (pH 8.0) containing 9.6% glycerol and 2.7 M reduced form of glutathione) chilled on ice were added slowly. The resulting solution was stirred gently for 10 min at 4°C. Then, oxidized form of glutathione was added to this solution to a final concentration of 1 mM and this solution was stirred gently for 40 h at 4°C. The resulting peptide was purified by reverse-phase HPLC on a Senshu Pak PEGASIL-300 ODS column (10 × 250 mm, Senshu Kagaku, Tokyo). Separation was done with a 5-min holding at 20% acetonitrile in 0.05% TFA, and a 30-min linear gradient of 20–60% acetonitrile in 0.05% TFA at a flow rate of 3 ml/min. The elution was monitored by measuring the absorbance at 280 nm.

**Amino acid sequence analyses.** Amino-terminal amino acid sequences of a native peptide and enzymatic digests were analyzed on an Applied Biosystems model 476A protein sequencer in the pulsed-liquid mode.

**Mass spectral analyses.** Mass spectra were measured on a matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE STR, Applied Biosystems) with α-cyano-4-hydroxycinnamic acid as a matrix in the positive ion mode.

**Enzymatic digestion and separation of the digests.**
Thermolysin digestion. Native and recombinant MIH (10 μg each) were dissolved separately in 500 μl of 25 mM HEPES buffer (pH 6.8) containing 50 mM CaCl\(_2\) and digested with 1 μg of thermolysin (Nacalai Tesque, Kyoto) at 37°C for 24 h. The digests were separated by reverse-phase HPLC on a Capcell Pak C\(_{18}\) column (2.0 × 250 mm, Shiseido, Tokyo) with a 30-min linear gradient of 0–40% acetonitrile in 0.05% TFA at a flow rate of 0.2 ml/min. The elution was monitored by measuring the absorbances at 225 and 280 nm.

Trypsin digestion. Each disulfide-containing fragment peptide obtained by thermolysin digestion of native and recombinant MIHs was dissolved in 300 μl of 25 mM HEPES buffer (pH 6.8) and incubated with 1 μg of modified trypsin (Promega) at 37°C for 24 h. The digests were separated under the same conditions as used in the separation of thermolysin digests.

Endoproteinase Asp-N digestion. Each disulfide-containing fragment peptide obtained by tryptic digestion described above was dissolved in 250 μl of 25 mM HEPES buffer (pH 6.8) and incubated with 0.1 μg of endoproteinase Asp-N (Boehringer Mannheim) at 37°C for 24 h. The digests were separated under the same conditions as used in the separation of thermolysin digests.

CD spectral analyses. CD spectra were recorded on a JASCO J-720 spectropolarimeter. Native and recombinant MIHs were separately dissolved in 25 mM phosphate buffer (pH 6.8) at the concentration of 6.6 μM. Pej-SGP-I and -V were dissolved in the same buffer at the concentration of 13.1 μM and 7.2 μM, respectively. CD spectra were recorded from 200 to 260 nm using a 1-mm path length cell at room temperature. α-Helix contents were calculated by the method reported previously.\(^{22}\)

Results

Preparation of native CHH family peptides and recombinant MIH
Native CHH family peptides and recombinant MIH were purified as described previously\(^{16,17}\) with slight modifications. All peptides were fully purified (for native and recombinant MIHs, Fig. 1; for native Pej-SGP-I and -V, data not shown), and were used in the following experiments. Recombinant MIH had an additional alanine residue at the amino-terminus of native MIH.\(^{20}\)

Location of disulfide bridges in native and recombinant MIHs
Pej-MIH contains six cysteine residues, so 15 patterns of disulfide bridge arrangement are theoretically possible. In order to determine the arrangements of disulfide bridges in native and recombinant MIHs, three steps of enzymatic digestions were performed.

First, native MIH was digested with thermolysin and the resulting digest was separated with reverse-phase HPLC. Six major peaks were observed (Fig. 2A), and the peak materials, Th1-6, were subjected to amino acid sequencing and mass spectral analyses. Sequence analysis of Th3 found that this peptide consisted of four peptide chains, suggesting that Th3 included all three disulfide bonds. A quasi molecular ion peak of Th3 was observed at \(m/z\) 2857.1 ((M + H)\(^+\)). These results indicated that Th3 was a fragment consisting of the four peptides, Ile\(^3\)-Gly\(^5\), Val\(^2\)-Asn\(^9\), Leu\(^16\)-Cys\(^4\) and Ile\(^12\)-Cys\(^5\), and its calculated mass number was 2857.0 ((M + H)\(^+\)). All the other fragments were found not to include disulfide bridges (Table 1).

Secondly, in order to digest two arginyl bonds at positions 41 and 43 in Th3, it was digested with trypsin and the tryptic peptides were separated by reverse-phase HPLC. Three fragments, Tr1-3, were recovered (Fig. 3A), and their sequences and mass spectra
were analyzed. Amino acid sequence analysis of Tr1 showed that it consisted of two peptide chains, suggesting that Tr1 included one disulfide bond. In the mass spectrum of Tr1, however, two quasi molecular ion peaks were observed at m/z 1113.5 ((M+H)+) and m/z 1169.6 ((M+H)+). These results indicated that Tr1 was a mixture of two double chain peptides, each consisting of Ile2-Arg8 and Asn42-Cys44, and Ile4-Gly9 and Asn42-Cys44, the calculated mass numbers of which were 1111.2 ((M+H)+) and 1168.2 ((M+H)+), respectively. Therefore, one disulfide bond was established to be connected between Cys7 and Cys44. Tr2 and Tr3 were found to have identical amino acid sequences, Val3-Asn39, Leu36-Arg41 and Ile51-Cys53, though they showed different mass spectra; m/z 1724.3 ((M+H)+) for Tr2 and m/z 1709.2 ((M+H)+) for Tr3. These results strongly suggested that Tr2 was an oxidized form of Tr3, the Met59 residue of which was oxidized to methionine sulfoxide in Tr2.

Thirdly, to digest the peptide bond of the N-termi-
that the arrangement of disulfide bridge of recombinant MIH was the same as that of native MIH (Fig. 5).

**Secondary structure analysis**

Figure 6A shows CD spectra of native and recombinant MIHs recorded at room temperature. The two spectra demonstrated a similar pattern and showed two negative troughs centered at 208 and 222 nm. These spectra suggested that both native and recombinant MIHs are rich in \( \alpha \)-helix. The \( \alpha \)-helix contents of native and recombinant MIHs were calculated to be 49.3\% and 46.0\%, respectively. CD spectra of Pej-SGP-I and -V were also measured (Fig. 6B). The CD spectra of these peptides classified into type I of the CHH family were similar to MIHs classified into type II. \( \alpha \)-Helix contents of Pej-SGP-I and -V were calculated to be 49.1\% and 48.3\%, respectively.

**Discussion**

The most popular strategy for determining arrangements of disulfide bonds is to analyze disulfide bond containing peptides obtained by proteolytic digestion. In the preliminary experiment, we first examined trypsin digestion using recombinant MIH, but the yields of expected fragment peptides were very low due to incomplete digestion, which resulted in obtaining various fragment peptides (data not shown). Considering the limited amount of the native peptide available, digestion with trypsin at the first step was thought to be inappropriate. The incomplete digestion suggested that some arginine and/or lysine residues take part in maintaining tertiary structure in MIH. By contrast, thermolysin digestion gave much better results than trypsin digestion and we used thermolysin at the first step of digestion.

The arrangement of disulfide bridge in Pej-SGP-III, one of the CHHs of *P. japonicus*, was determined previously.\(^{10}\) In this case, the peptide bonds in the sequence, Cys\(^23\)-Asp\(^24\)-Asp\(^25\)-Cys\(^26\), could not be digested by endoprotease Asp-N or endoprotease Glu-C, so the location of disulfide bonds including Cys\(^23\) and Cys\(^26\) was determined by identification of a phenylthiohydantoin derivative of cystine in sequencing.\(^{14}\) Fortunately, in this experiment, since the peptide bond, Glu\(^23\)-Asp\(^26\), in the corresponding sequence, Cys\(^24\)-Glu\(^25\)-Asp\(^26\)-Cys\(^27\), in MIH of *P. japonicus* was digested by endoprotease Asp-N, three fragment peptides each containing a single disulfide bond were obtained and the arrangement was determined straightforwardly. As expected, the ar-

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**Table 1. Amino Acid Sequences of Enzymatic Digests**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction No.</th>
<th>Amino-terminal sequences</th>
<th>Observed mass number (Theoretical mass number)</th>
<th>Position</th>
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</thead>
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<tr>
<td>Thermolysin</td>
<td>Th1</td>
<td>FR</td>
<td></td>
<td>31-32 and/or 66-67</td>
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<tr>
<td></td>
<td>Th2</td>
<td>FYN</td>
<td></td>
<td>16-19</td>
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<tr>
<td></td>
<td></td>
<td>IDNTCRG</td>
<td></td>
<td>45-47</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3-9</td>
</tr>
<tr>
<td></td>
<td>Th3</td>
<td>LDGMCRNRRC</td>
<td>2857.1 (2857.0)</td>
<td>36-44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VCEDCTN</td>
<td></td>
<td>23-29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IC</td>
<td></td>
<td>52-53</td>
</tr>
<tr>
<td></td>
<td>Th4</td>
<td>VW</td>
<td></td>
<td>68-69</td>
</tr>
<tr>
<td></td>
<td>Th5</td>
<td>IFRLPG</td>
<td></td>
<td>30-35</td>
</tr>
<tr>
<td></td>
<td>Th6</td>
<td>FYNEW</td>
<td></td>
<td>45-49</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Tr1</td>
<td>IDNTCR(G)</td>
<td>1113.5 and 1169.6 (1111.2 and 1168.2)</td>
<td>3-8 (3-9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRC</td>
<td></td>
<td>42-44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LDGM*CR</td>
<td></td>
<td>36-41</td>
</tr>
<tr>
<td></td>
<td>Tr2 &amp; Tr3</td>
<td>VCEDCTN</td>
<td>1724.3 and 1709.2 (1723.8 and 1707.8)</td>
<td>23-29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IC</td>
<td></td>
<td>52-53</td>
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<tr>
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<td>A1</td>
<td>DCTN</td>
<td></td>
<td>26-29</td>
</tr>
<tr>
<td>Asp-N</td>
<td>A2 &amp; A3</td>
<td>VCE</td>
<td>N. D. and 1059.2 (1042.1 and 1058.1)</td>
<td>23-25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LDGM*CR</td>
<td></td>
<td>36-41</td>
</tr>
</tbody>
</table>

* Methionine residue was oxidized to methionine sulfoxide residue in Tr2 and A2.
N. D.: not determined.
arrangement of disulfide bridges in Pej-MIH was made in the same manner as that in other type I CHH family peptides thus far determined. This is the first demonstration of the disulfide bridge arrangement in natural type II peptides of the CHH-family.

The CD spectra of native and recombinant MIHs were similar to those of Pej-SGP-I and -V, and also similar to those of other CHH-family peptides reported before. Nevertheless α-helix contents of their CHH family peptides were reported to be very low (10–20%), while those of the peptides belonging to both type I and II calculated in this study were about 50%. The possible reasons for the differences in α-helix contents may be due to the method for estimation of peptide concentrations. The calculation method for α-helix content may also cause the difference. We are trying to determine the tertiary structure of recombinant MIH by NMR, and preliminary results showed that MIH is rich in α-helix, which is consistent with the α-helix contents obtained in this study.

Identity of the arrangements of disulfide bridges and similarity of CD spectra between native and recombinant MIHs strongly suggest that the recombinant MIH structure is the same as that of native MIH.

Fig. 4 Reverse-phase HPLC Elution Profiles of the Endoproteinase Asp-N Digests of Tryptic Digests.
(A) Endoproteinase Asp-N digests of Tr2 and Tr3 fragments. Tr2 and Tr3 fragments (see Fig. 3) were digested with endoproteinase Asp-N (0.1 µg) in 25 mM HEPES buffer (pH 6.8) at 37°C for 24 h. (B) Endoproteinase Asp-N digests of r-Tr2 and r-Tr3 fragments. r-Tr2 and r-Tr3 fragments (see Fig. 3) were digested with endoproteinase Asp-N (0.1 µg) under the same conditions as in (A). The digests were separated under the same conditions as used in the separation of thermolysin digests (see Fig. 2).

Fig. 5 Location of Disulfide Bridges in Native MIH.
Recombinant MIH with an additional alanine residue at the amino terminus was found to have the same arrangement of disulfide bridges as that of native MIH.

Fig. 6 CD Spectra of CHH Family Peptides.
(A) CD spectra of (a) native and (b) recombinant MIHs dissolved in 25 mM phosphate buffer (pH 6.8) at the concentration of 6.6 µM were recorded on a JASCO J-720 spectropolarimeter at room temperature. (B) CD spectra of (c) Pej-SGP-I and (d) Pej-SGP-V dissolved in 25 mM phosphate buffer (pH 6.8) at the concentration of 13.1 µM and 7.2 µM, respectively, were recorded in the same manner as in (A).
binant MIH has a similar tertiary structure to the native MIH. These findings on the structure are consistent with the previous result that recombinant MIH was as active as native MIH.\(^{39}\)

As described above, the locations of disulfide bridges in CHH family peptides are same between type I and type II, and CD spectra of CHH family peptides are also similar, suggesting that CHH family peptides are folded to form a similar secondary and tertiary structure irrespective of type. The variation of biological activity of CHH family peptides therefore may arise from differences in the surface structure such as distribution of external charges and hydrophobic side chains. Such fine structural differences may cause the differences of binding affinity to their receptors which directly participate in biological activities. Based on the finding obtained in this experiment that recombinant MIH was folded in the same manner as native MIH, the analysis of tertiary structure of recombinant MIH is now in progress.

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References


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