Expression of a Recombinant Molt-Inhibiting Hormone of the Kuruma Prawn *P. japonicus* in *Escherichia coli*

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The crustacean molt-inhibiting hormone (MIH) suppresses ecdysteroid synthesis by the Y-organ. The MIH of the kuruma prawn *P. japonicus* has recently been isolated and its cDNA cloned. In this study, we expressed the MIH in *Escherichia coli* to obtain a large quantity of this hormone with biological activity. The MIH cDNA was processed and ligated into an expression plasmid. *E. coli* was transformed with this plasmid, and then the recombinant MIH (r-MIH) was expressed. The r-MIH was put through the refolding reaction and was purified by reverse-phase HPLC. N-terminal amino acid sequence and time-of-flight mass spectral analyses supported the idea that the r-MIH had the entire sequence. By *in vitro* bioassay using the *Y*-organ of the crayfish, the r-MIH was found to be comparable to natural MIH in inhibiting ecdysteroid synthesis.

**Key words:** molt-inhibiting hormone; molting; crustacean; *P. japonicus*; bacterial expression

In crustaceans, molt-inhibiting hormone (MIH) is released from the X-organ sinus gland complex which is located in the medulla terminalis of the eyestalk. MIHs have been isolated from the lobster *Homarus americanus*, the shore crab *Carcinus maenas*, the kuruma prawn *P. japonicus*, the Mexican crayfish *Procambarus bouvieri* and the American crayfish *Procambarus clarkii*. These hormones consist of 75-77 amino acid residues and have similar amino acid sequences with six conserved Cys residues. They belong to a peptide family called the crustacean hyperglycemic hormone (CHH) family. MIHs are presumed to inhibit molting through suppressing synthesis and/or secretion of the molting hormone, ecdysteroids, by the Y-organ. Along this line, the MIHs in *C. maenas*, *P. japonicus*, *P. bouvieri* and *P. clarkii* have been shown to suppress ecdysteroid secretion by the *Y*-organ in *in vitro*. By contrast, *in vivo* biological activity of MIH was reported only in *H. americanus* and knowledge about the mechanism of molt inhibition is therefore very limited. The main reason may be due to the difficulty in obtaining a large quantity of MIH, because it needs many experimental animals and much time to purify MIH from the sinus glands. To solve this problem, we attempted to express the *P. japonicus* MIH in *Escherichia coli* to obtain a large quantity of this hormone with biological activity.

**Materials and Methods**

*Construction of the expression plasmid.* Isolation and characterization of a cDNA encoding MIH of the kuruma prawn *P. japonicus* (Pej-MIH) have been described previously. A cDNA encoding Pej-MIH with two additional amino acid residues, Met-Ala, extended at the N-terminus was inserted into an expression plasmid pET-28a (Novagen) by the following method. Two oligonucleotide primers were designed based on the nucleotide sequence of the Pej-MIH cDNA. The N-terminal primer (5'-CCATGGAACGCTATAGACCA-3') contained the *NcoI* site (italics), two nucleotide residues (bold characters), and 20 nucleotide residues encoding the N-terminal 7 amino acid residues of the Pej-MIH (Fig. 1). The sequence of the *NcoI* site, CCATGG, includes a G residue (bold character) following the initiation codon ATG (italics) so that two more nucleotide residues, C and A, were inserted next to the *NcoI* site in order to adjust the reading frame (Fig. 1). The C-terminal primer (5'-GGGGCGACCTAGCGCAGGTCAAG-3') contained the *NsiI* site (italics), stop codon (bold characters), and 20 nucleotide residues encoding the C-terminal 7 amino acid residues of the Pej-MIH (Fig. 1). The polymerase chain reaction (PCR) was done with these primers using a plasmid containing the Pej-MIH cDNA as template. The amplified PCR product was cloned into the *EcoRV* site of pBluescript SK- (Strategene). To check the nucleotide

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**Abbreviations:** MIH, molt-inhibiting hormone; CHH, crustacean hyperglycemic hormone; Pej, *P. japonicus*; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TOF, time-of-flight; GSH, reduced glutathione; GSSG, oxidized glutathione; LMWP, low molecular weight protein
sequence of the PCR product, both strands were sequenced on a Model 373A DNA sequencer using dye terminator cycle sequencing kits (Applied Biosystems). Subsequently, the Pej-MIH insert was released from pBluescript SK" by NcoI/NotI digestion, and then ligated into the NcoI/NotI site of the expression plasmid pET-28a (Fig. 1).

Expression of recombinant Pej-MIH (r-Pej-MIH). E. coli BL21(DE3) competent cells (Novagen) were transformed with the expression plasmid pET-28a containing the Pej-MIH insert and selected on LB-Kan plates (kanamycin 30 μg/ml). An isolated colony was grown by shaking at 37°C overnight in an LB-Kan medium (kanamycin 30 μg/ml) and then diluted 50-fold in the same medium. The diluted medium was incubated with shaking at 37°C for 2 h. Isopropyl-β-D-thiogalactoside (IPTG) was added to the culture to a final concentration of 1 mM and the incubation was continued for another 2 h. Then, the bacteria were harvested by centrifugation and suspended in 1/20 culture volume of phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.5) containing 0.02% (w/v) lysozyme (Wako). The cells were disrupted by sonication on ice two times for 20 s each using Handy Sonic model UR-20P (Tomy Seiko), the diameter of its probe being 2 mm. Triton X-100 was added to the homogenate to a final concentration of 2% (v/v) and the resulting mixture was stirred at room temperature overnight. The supernatant was removed and the pellet was suspended in PBS. Both the supernatant and the suspended insoluble material were put on 15% SDS/polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. As a negative control, E. coli BL21(DE3) cells transformed with the pET-28a not containing the MIH insert were grown and the fractions prepared as above were run on SDS-PAGE similarly.

Purification of r-Pej-MIH. The insoluble material after cell breakage was solubilized in 6 M guanidine-HCl/10 mM Tris-HCl (pH 8.0). The resulting solution was
put on a reverse-phase HPLC on a TSKgel Octadecyl-4PW column (4.6 × 150 mm, Tosoh). Separation was done with a 1-min linear gradient of 0–10% acetonitrile in 0.1% trifluoroacetic acid (TFA), a 10-min holding at 10% acetonitrile in 0.1% TFA, and a 40-min linear gradient of 10–60% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The elution was monitored by measuring the absorbance at 280 nm and the yield of each peak was calculated as reported previously. Each peak material was run on SDS-PAGE under the same conditions as described above.

Amino acid sequence analysis. N-terminal amino acid sequences of the purified peaks were analyzed on an Applied Biosystems model 476A protein sequencer in the pulsed-liquid mode.

Mass spectral analysis. Mass spectra were measured on a time-of-flight (TOF) mass spectrometer (Voyager-DESTR, PerSeptive Biosystems) in the positive ion mode with 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix.

Refrolding of the isomers of r-Pej-MIH. Four fractions each containing at least one isomer of r-Pej-MIH obtained by reverse-phase HPLC were combined and lyophilized. Then this was dissolved in 8 M urea/50 mM Tris-HCl (pH 8.0) at a concentration of 50 μg/ml. To this solution, five volumes of a dilution buffer (50 mM Tris-HCl, 1.3 mM reduced glutathione, pH 8.0) chilled on ice were added slowly. The resulting solution, which contained 1 mM reduced glutathione, was stirred gently for 10 min at 4°C. Then, oxidized glutathione was added to this solution to a final concentration of 1 mM and the solution was stirred gently overnight at 4°C. The reoxidized r-Pej-MIH was purified by reverse-phase HPLC under the same conditions as described above and run on SDS-PAGE, N-terminal amino acid sequence and TOF mass analyses.

Bioassay for molt-inhibiting hormone (MIH) activity. The MIH activity was measured by using an in vitro culture of the crayfish Procambarus clarkii and a subsequent ecdyssostoid radioimmunoassay (RIA) as reported previously.

Results and Discussion

Expression of r-Pej-MIH

After E. coli cells were cultured, harvested, and sonicated, soluble and insoluble fractions were obtained. A heavily stained band of a candidate for r-Pej-MIH was detected only in the insoluble fraction by SDS-PAGE (Fig. 2, lane 2), but the band was not detectable in the soluble fraction (Fig. 2, lane 1) nor in control cells carrying an expression plasmid without the Pej-MIH insert (Fig. 2, lanes 3 and 4). This result indicated that the r-Pej-MIH was accumulated in inclusion bodies. Formation of inclusion bodies have been reported in case of high protein expression in E. coli.

Purification of r-Pej-MIH

In most cases, inclusion bodies are insoluble and the proteins in them are inactive. Denaturants such as SDS, Triton X-100, urea, and guanidine-HCl are usually used for solubilization of recombinant proteins from inclusion bodies. In this study, the inclusion bodies of r-Pej-MIH were solubilized in 6 M guanidine-HCl/10 mM Tris-HCl (pH 8.0), because guanidine-HCl solubilizes inclusion bodies at higher concentrations than the other denaturants. This solution was put on reverse-phase HPLC, which gave four major peaks (Fig. 3, Peaks 1-4). In SDS-PAGE of the four peak materials, bands were detected at the same mobility for all of the four peak materials (Fig. 3).

Chemical characterization of the four peak materials

Sequence analyses of the four peak materials identified N-terminal 3 amino acid residues, Ala-Ser-Phe-, which were identical. Their N-terminal sequences were the same as that of natural Pej-MIH except for the addition of an Ala residue. In the TOF mass spectra of the four peak materials, protonated molecular ion peaks were observed at m/z 9140 (Peak 1), 9136 (Peak 2), 9133 (Peak 3), and 9133 (Peak 4). These values coincided well with the theoretical value of 9136 for (M + H)+, which was calculated from a natural Pej-MIH plus an Ala residue. These results indicated that the four peak
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Fig. 3. Reverse-phase HPLC Elution Profile of Insoluble Materials from 100-ml Culture.

Chromatographic conditions are detailed in Materials and Methods. The concentration of acetonitrile is indicated by the line. SDS/polyacrylamide gel electrophoresis of major four peak materials (Peaks 1–4, 5 μg per each sample) is shown as inset. Positions of three molecular weight markers (10.7, 8.2, and 6.2 kDa) are indicated.

Fig. 4. Reverse-phase HPLC Elution Profile of Samples after Refolding Reaction.

Chromatographic conditions are the same as in Fig. 3. SDS/polyacrylamide gel electrophoresis of the shaded fraction (5 μg) is shown as inset. Positions of three molecular weight markers (10.7, 8.2, and 6.2 kDa) are indicated.

Materials had the same sequence as that of natural Pej-MIH except for the N-terminal Ala residue. If the six Cys residues form three intramolecular disulfide bonds, fifteen disulfide bond isomers would be theoretically possible for MIH. Therefore, it cannot be ruled out that each peak consisted of more than two disulfide bond isomers.

In this study, an expression plasmid carrying the Pej-MIH insert was constructed by adding Met-Ala residues to the N-terminus of natural Pej-MIH (see Materials and Methods). The r-Pej-MIH, however, lacked the initiation Met residue. These results indicated that the r-Pej-MIH was correctly translated and the N-terminal Met residue was removed, possibly by a methionine aminopeptidase in *E. coli*.16,17

Refolding of the four peak materials of r-Pej-MIH

The four peak materials obtained by reverse-phase HPLC were bioassayed in *vitro*. Only peak 1 showed molt-inhibiting activity on the first run of the bioassay. But on the second run of the bioassay, only peak 2 showed molt-inhibiting activity, while peak 1 lost the activity (data not shown). We examined the activity of the four peak materials several times, but at every time the results were not reproducible, although natural MIH (sinus gland extracts) showed activity at every time. These results indicated that the four peak materials would be structurally unstable, possibly due to having different ar-
rangements of the three disulfide bonds, or the three disulfide bonds being partially reduced. So we thought that it was necessary to refold these materials. Refolding of proteins involves several interdependent steps, solubilization, reduction, oxidative refolding, and withdrawal of denaturants. Guanidine-HCl is generally more effective than urea in solubilizing inclusion bodies. However, it was reported that the recovery of biological activity from guanidine-HCl solution was much lower than that from urea-containing buffer, and that guanidine-HCl might interfere with the refolding process. For these reasons, we used a urea-containing buffer for refolding. Reduced and oxidized forms of glutathione (GSH, GSSG) have been used as redox reagents to increase the rate and yield of correct disulfide bond formation, and to recover biological activity more effectively than dithiothreitol or β-mercaptoethanol. A mixture of the four peak materials was therefore reduced and reoxidized using a buffer solution containing 1.3 M urea, 1 mM GSH, and 1 mM GSSG. This procedure resulted in obtaining only one peak in the elution profile on reverse-phase HPLC (Fig. 4). The retention time of this peak was different from that of any of the four peaks. SDS-PAGE of this peak material gave a single band (Fig. 4). Sequence analysis of this peak material identified the N-terminal 5 amino acid residues, Ala-Ser-Phe-Ile-Asp. In the TOF mass spectrum of this peak material, a protonated molecular ion peak was observed at m/z 9135, indicating that it had a full sequence. The yield of the refolded, active r-Pej-MIH was about 1 mg from 1 liter of culture.

Molt-inhibiting activity of the r-Pej-MIH

The dose-response relationship of the refolded and purified r-Pej-MIH was examined. As shown in Fig. 5, the lowest inhibition (12%) was observed at a concentration of 2.0 × 10^{-10} - 1.0 × 10^{-9} M. The inhibition was enhanced with increased dosage, and reached a maximum (41%) at the concentration of 1.0 × 10^{-8} - 2.0 × 10^{-8} M. These values are almost comparable to those of natural Pej-MIH, lowest inhibition (10%, 8.6 × 10^{-10} M) and maximum inhibition (45%, 8.6 × 10^{-9} - 4.3 × 10^{-8} M), using the same bioassay system. The r-Pej-MIH had an extra Ala residue at the N-terminus of natural MIH. Thus, the extension of one residue, Ala, at the N-terminus does not cause a significant change in biological activity.

Many efforts would be required to obtain a large quantity of MIH from the natural source; the yield of natural MIH is only about 80 ng from one prawn. This has been overcome by this experiment, in which we succeeded in expressing a large quantity of the r-Pej-MIH with full biological activity using the E. coli expression system. The r-Pej-MIH will be useful not only for in vivo biological study but also for analysis of its three-dimensional structure, which would give us very important information for understanding the structure-activity relationship of MIH.

![Fig. 5. Dose-response Relationship of r-Pej-MIH to Inhibit the Synthesis of Ecdysteroids by the Procumbarum clarkii Y-Organ Cultured in Vitro.](image)

The Y-organs were incubated in 0.5 ml of culture medium at the indicated dose (pmol) for 6 h. Ecdysteroids in the medium were measured by RIA. Points represent percent inhibition of ecdysteroids secretion measured by comparison with the RIA activities in untreated control cultures. Results are expressed as mean ± S.E. Numbers of independent bioassays are shown in parentheses.
Low molecular weight protein (LMWP), which was purified from the venom of the black widow spider *Latrodectus mactans tredecimguttatus* and the amino acid sequence of which was similar to crustacean hyperglycemic hormone (CHH) family peptides, has previously been expressed in *E. coli.* This is the only one example that a CHH-family peptide was synthesized with a bacterial expression system. However, there was no data on the biological or pharmacological activity of LMWP. In addition, there was not enough data on the sterical similarity between recombinant LMWP and the natural one. Therefore, this is the first report that describes the production of a CHH-family peptide with activity comparable to the natural peptide in a bacterial expression system.

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