ZOOLOGICAL SCIENCE **23**: 383–391 (2006) 

# Preparation of Two Recombinant Crustacean Hyperglycemic Hormones from the Giant Freshwater Prawn, *Macrobrachium rosenbergii*, and Their Hyperglycemic Activities

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**Crustacean hyperglycemic hormone (CHH) is released from the X-organ/sinus gland complex located in the eyestalks, and regulates glucose levels in the hemolymph. In the giant freshwater prawn (*Macrobrachium rosenbergii*), two cDNAs encoding different CHH molecules were previously cloned by other workers. One of these (Mar-CHH-2) was expressed only in the eyestalks, whereas the other (Mar-CHH-L) was expressed in the heart, gills, antennal gland, and thoracic ganglion, but not in the eyestalks. However, their biological activities had not yet been characterized. Therefore, in this study, recombinant Mar-CHH-2 (rMar-CHH-2) and Mar-CHH-L (rMar-CHH-L) were produced using an *E. coli* expression system, by expression in bacterial cells and recovery in the insoluble fraction. Thereafter, rMar-CHH-2 and rMar-CHH-L were subjected to refolding and were subsequently purified by reversed-phase HPLC. The rMar-CHH-2 and rMar-CHH-L thus obtained exhibited the same disulfide bond arrangements as those of other CHHs reported previously, indicative of natural conformation. In *in vivo* bioassay, rMar-CHH-2 showed significant hyperglycemic activity, whereas rMar-CHH-L had no effect. These results indicate that Mar-CHH-L does not function as a CHH, but may have some other, unknown function.**

**Key words:** Crustacea, eyestalk hormone, hyperglycemic activity, recombinant peptide, sinus gland, X-organ

## INTRODUCTION

In crustaceans, hemolymph glucose levels are regulated by a neuropeptide, crustacean hyperglycemic hormone (CHH), which is produced by the X-organ/sinus gland complex in the eyestalks. Vitellogenesis-inhibiting hormone (VIH), molt-inhibiting hormone (MIH), and mandibular organ-inhibiting hormone (MOIH) are also synthesized in, and released from, the X-organ/sinus gland complex. Most of these neuropeptides consist of 72–83 amino acid residues and share similar amino acid sequences. Therefore, these peptides form a peptide family referred to as the CHH-family (Keller, 1992). The CHH-family peptides commonly have six cysteine residues, which form three intramolecular disulfide bonds.

CHH-family peptides are divided into two subtypes based on the absence (Type I) or presence (Type II) of a glycine residue at position 12 in the mature peptide (Yang *et al.,* 1997). CHH itself has been isolated from many crustacean species, and all of these have been classified as type I peptides. Most CHHs are amidated at the C-terminus, except for PO-CHH isolated from the pericardial organs in the green shore crab (*Carcinus maenas*) (Dircksen *et al.,* 2001) and two C-terminally truncated CHHs, lacking seven C-terminal amino acid residues, purified from the sinus glands in the African spiny lobster (*Jasus lalandii*) (Marco *et al.,* 2000). The PO-CHH and two truncated CHHs, all of which have a free C-terminus, showed no hyperglycemic activity in *in vivo* bioassays. Recombinant CHHs in the kuruma prawn (*Marsupenaeus japonicus*) showed much lower hyperglycemic activity in the absence of the C-terminal amide (Katayama *et al.,* 2002; Ohira *et al.,* 2003). All these results indicate that the C-terminal amide in type I peptides is essential for conferring biological activity.

In addition to the regulation of glucose levels in the hemolymph, CHH is also involved in the control of other physiological phenomena, such as molting and reproduction. The American lobster (*Homarus americanus*) CHH-A (Hoa-CHH-A) possesses both hyperglycemic and molt-inhibiting activities (Chang *et al.,* 1990), and its contents in the sinus gland changed depending on molt stage (Chang *et al.,* 2001). These findings suggest that Hoa-CHH-A may be associated with the regulation of molting. Six CHHs in *M. japonicus* (Maj-CHHs) inhibited protein and mRNA syntheses in the ovary (Khayat *et al.,* 1998), indicating that the

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doi:10.2108/zsj.23.383

Maj-CHHs may regulate ovarian physiology in addition to carbohydrate metabolism. CHHs in the spider crab (*Libinia emarginata*) (Lie-CHHs) inhibited the synthesis of methyl farnesoate (MF) in the mandibular organ (Liu and Laufer, 1996). It has been thought that MF may be involved in regulating reproduction (Laufer *et al.,* 1998) and molting (Tamone and Chang, 1993) in crustaceans; therefore, Lie-CHH may control these phenomena through the modulation of MF synthesis.

Several CHH-family peptides are expressed in non-eyestalk tissues. mRNAs corresponding to *M. japonicus* MIH-B (Maj-MIH-B) and *Metapenaeus ensis* MIH-B (Mee-MIH-B) were expressed in the central nervous system in addition to the eyestalks (Gu *et al.,* 2002; Ohira *et al.,* 2005). Molt-inhibiting activities of both Maj-MIH-B and Mee-MIH-B were weaker than those of Maj-MIH-A and Mee-MIH-A, indicating that the two MIH-B molecules do not function as MIHs, but may have unknown physiological functions (Gu *et al.,* 2002; Ohira *et al.,* 2005). In *C. maenas*, a CHH referred to as SG-CHH was detected in the gut and was identical to that expressed in the eyestalks (Chung *et al.,* 1999). This report suggested that CHH in the gut may possibly function to regulate water and ion uptake during the postmolt stages. Another type of *C. maenas* CHH, PO-CHH, was expressed in the pericardial organs (Dircksen *et al.,* 2001). PO-CHH and SG-CHH arise by alternative splicing, and share an identical N-terminal sequence at positions 1–40, but the remaining C-terminal sequences differ considerably (Dircksen *et al.,* 2001). PO-CHH showed neither hyperglycemic activity nor molt-inhibiting activity (Dircksen *et al.,* 2001); therefore, the function of this molecule remains unclear.

In the giant freshwater prawn (*Macrobrachium rosenbergii*) four CHH molecules have been identified to date. Two of these, Mar-CHH and Mar-CHH-1, were isolated from the sinus glands, and their hyperglycemic activities were characterized by *in vivo* injection assays (Lin *et al.,* 1998; Sithigorngul *et al.,* 1999a). Only 25 N-terminal amino acid residues were sequenced for Mar-CHH (Lin *et al.,* 1998), while Mar-CHH-1 was completely characterized as a peptide consisting of 72 amino acid residues with a C-terminal amide (Sithigorngul *et al.,* 1999a, b). The two CHHs differed by 6 amino acids within their 25 N-terminal residues, indicating that Mar-CHH and Mar-CHH-1 are different molecular species. On the other hand, the two other CHH molecules were identified as cDNA clones referred to as *Mar-chh* and *Mar-chh-L* (Chen *et al.,* 2004). In order to avoid confusion among these various CHHs and for purposes of expediency in discussion, we refer to the gene product of *Mar-chh* as Mar-CHH-2 and that of *Mar-chh-L* as Mar-CHH-L in this report. The amino acid sequence of mature Mar-CHH-2, consisting of 72 residues with a C-terminal amide, is similar to that of Mar-CHH-1, but differs by two amino acid residues at positions 41 and 61. Mar-CHH-L is a variant of Mar-CHH-2 which results by alternative splicing in the same manner as does PO-CHH (Chen *et al.,* 2004; Dircksen *et al.,* 2001). Mar-CHH-L consists of 73 amino acid residues having a free C-terminus. Therefore, these four CHHs (Mar-CHH, Mar-CHH-1, Mar-CHH-2, and Mar-CHH-L) are considered to be distinct molecular species.

At present, knowledge concerning the role of non-eyestalk CHH is very limited. Mar-CHH-L is the first example of a CHH expressed in the gills and antennal glands but not in

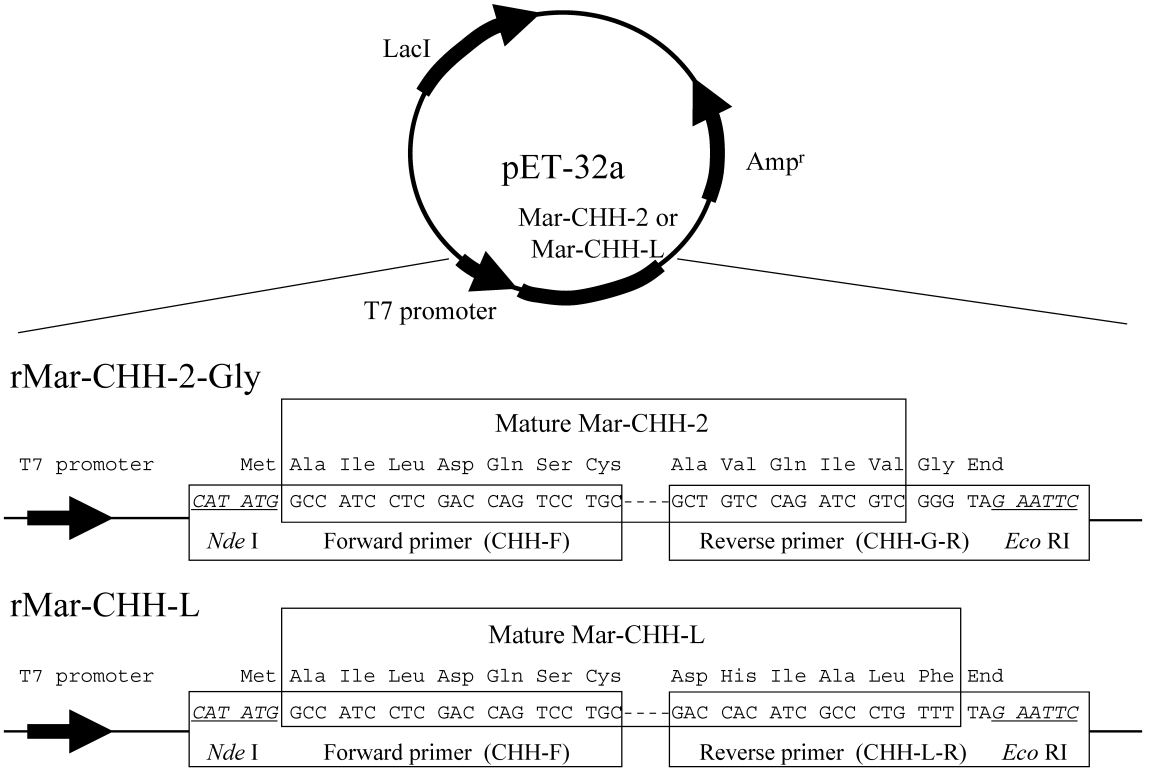


**Fig. 1.** Construction of the expression plasmid for rMar-CHH-2-Gly and rMar-CHH-L. LacI, *lac*I gene encoding lac repressor protein; Amp$^r$, β-lactamase gene conferring ampicillin resistance.

the eyestalks (Chen *et al.,* 2004); however, the biological activity of this non-eyestalk CHH had not yet been characterized. Here we report the production of recombinant Mar-CHH-L (rMar-CHH-L) using the *Escherichia coli* expression system as well as the examination of its hyperglycemic activity. In addition, an eyestalk-type recombinant Mar-CHH-2 (rMar-CHH-2) was also prepared in order to compare its hyperglycemic activity with that of Mar-CHH-L.

## MATERIALS AND METHODS

### Construction of expression plasmids

The nucleotide sequences of cDNAs encoding Mar-CHH-2 and Mar-CHH-L have been described previously (Chen *et al.,* 2004). cDNAs encoding Mar-CHH-2 having an additional glycine residue at the C-terminus (Mar-CHH-2-Gly) and normal Mar-CHH-L were inserted into pET-32a expression plasmids (Novagen, USA) according to the following method (Fig. 1). Three oligonucleotide primers were designed based on the nucleotide sequences of the Mar-CHH-2 and Mar-CHH-L cDNAs. The forward primer (CHH-F: 5'-*CATATG*-GCCATCCTCGACCAGTCCTGC-3') contained the *Nde* I site (italics) and 21 nucleotide residues encoding the 7 N-terminal amino acid residues of mature Mar-CHH-2 and Mar-CHH-L. The reverse primer for Mar-CHH-2 (CHH-G-R: 5'-*GAATT**CTA**CCCGACGAT-CTGGACAGC-3') contained the *Eco*R I site (italics), stop codon (bold characters), three additional residues (underlined) encoding an amidating donor residue, glycine, and 15 nucleotide residues encoding the 5 C-terminal amino acid residues of mature Mar-CHH-2. The reverse primer for Mar-CHH-L (CHH-L-R: 5'-*GAATT**CTA**AAA-CAGGGCGATGTGGTC-3') contained the *Eco*R I site (italics), stop codon (bold characters), and 18 nucleotide residues encoding the 6 C-terminal amino acid residues of mature Mar-CHH-L. Polymerase chain reaction (PCR) was conducted with CHH-F and CHH-G-R, or CHH-F and CHH-L-R, using the corresponding cDNA encoding Mar-CHH-2 or Mar-CHH-L as the template (Chen *et al.,* 2004). The two amplified cDNA inserts were subcloned into pCR2.1-TOPO plasmid (Invitrogen, USA) and their nucleotide sequences were confirmed. Subsequently, the inserts were released from pCR2.1-TOPO by *Nde* I/*Eco*R I digestion, and then ligated into the *Nde* I/*Eco*R I site of the expression plasmid pET-32a. The expression plasmids thus obtained were designated as Mar-CHH-2-Gly+pET-32a and Mar-CHH-L+pET-32a, respectively.

### Expression of recombinant Mar-CHH-2-Gly (rMar-CHH-2-Gly) and Mar-CHH-L (rMar-CHH-L)

*Escherichia coli* BL21 Star (DE3) competent cells (Invitrogen) were transformed separately with the Mar-CHH-2-Gly+pET-32a and Mar-CHH-L+pET-32a plasmids, and subsequently their transformants were selected on LB plates containing ampicillin (50 μg/ml). Bacterial cells from a single colony were grown at 37°C overnight in LB medium containing ampicillin (50 μg/ml), and then diluted 50-fold with the same medium. The diluted medium was incubated at 37°C for 2 h, and then isopropyl-β-D-thiogalactoside (IPTG) was added to the culture to a final concentration of 1 mM. After additional incubation for 2 h, bacterial cells that expressed rMar-CHH-2-Gly and rMar-CHH-L were harvested by centrifugation. The bacterial cell bodies were then suspended in Laemmli sample buffer (Bio-Rad, USA) containing β-mercaptoethanol and subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 15% gels. As a negative control, *E. coli* BL21 Star (DE3) cells transformed with pET-32a lacking the insert were grown, and the sample prepared by the same method was similarly subjected to SDS-PAGE.

### Refolding reaction and purification of rMar-CHH-2-Gly and rMar-CHH-L

Refolding reactions for rMar-CHH-2-Gly and rMar-CHH-L were performed according to the methods described previously (Ohira *et al.,* 2005). The products thus obtained were subjected to a Sep-Pak $C_{18}$ Cartridge (Waters, USA) and eluted with 60% acetonitrile in 0.05% trifluoroacetic acid (TFA). After concentrating the eluants, rMar-CHH-2-Gly and rMar-CHH-L were purified separately by RP-HPLC on a Shodex Asahipak ODP-50 column (4.6×250 mm, Showa Denko, Tokyo) with a 40-min linear gradient of 10-60% acetonitrile in 0.05% TFA at a flow rate of 1.0 ml/min.

### Trypsin digestion

The rMar-CHH-2-Gly (100 μg) obtained after the refolding reaction was dissolved in 0.1 M Tris-HCl, pH 9.0. To this solution, 1 μl of trypsin (Wako, Japan) solution (5 mg/ml) was added, and the mixture was incubated at 37°C for 2 h. The reaction product was applied to a Sep-Pak $C_{18}$ Cartridge (Waters) and eluted with 60% acetonitrile in 0.05% TFA. After concentration, the digests were separated by RP-HPLC on a Capcell-Pak $C_{18}$ column (2.0×250 mm, Shiseido, Japan) with a 40-min linear gradient of 0-40% acetonitrile in 0.05% TFA at a flow rate of 0.5 ml/min.

### Lysyl endopeptidase digestion

The rMar-CHH-L (100 μg) obtained after refolding reaction was dissolved in 0.1 M Tris-HCl, pH 9.0. To this solution, 2.5 μl of lysyl endopeptidase (Wako) solution (2 mg/ml) was added, and the mixture was incubated at 37°C overnight. Purification was performed under the same conditions as described in the preceding section.

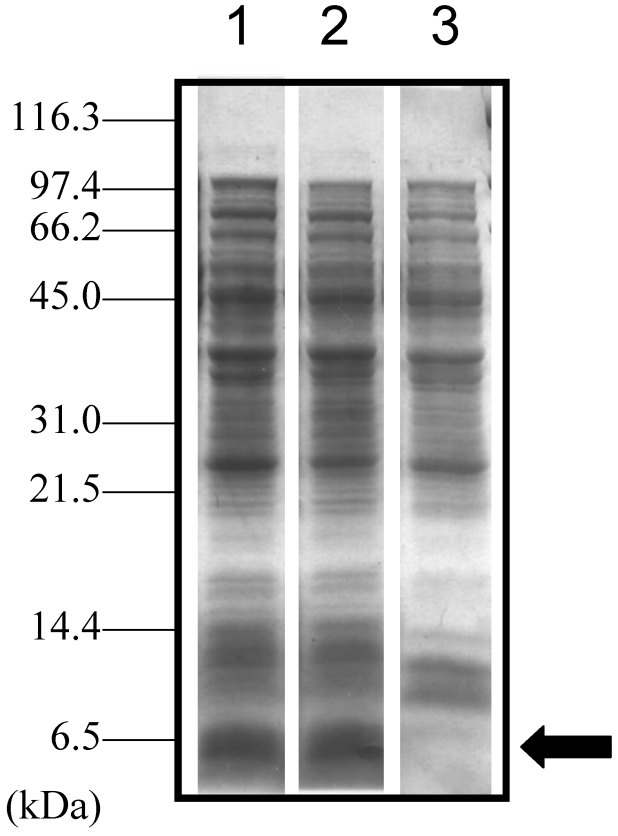


**Fig. 2.** SDS-PAGE of bacterial cell bodies. Lane 1, cells carrying an expression plasmid containing the Mar-CHH-2-Gly insert; lane 2, cells carrying an expression plasmid containing the Mar-CHH-L insert; lane 3, cells carrying an expression plasmid lacking the insert. Arrow indicates the position of rMar-CHH-2-Gly and rMar-CHH-L. Positions of eight molecular weight markers (116.3, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4, and 6.5 kDa) are indicated.

**Amidating reaction**

The rMar-CHH-2-Gly was amidated at the C-terminus according to the methods described previously (Katayama *et al.,* 2002). The reaction product was applied to a Sep-Pak $C_{18}$ Cartridge (Waters), and rMar-CHH-2 amidated at the C-terminus (rMar-CHH-2) was purified by RP-HPLC on a Shodex Asahipak ODP-50 column (4.6×250 mm, Showa Denko) with a 60-min linear gradient of 10–60% acetonitrile in 10 mM ammonium acetate (pH 9.1) at a flow rate of 1 ml/min.

**Amino acid sequence analysis**

N-terminal amino acid sequences of peptides were analyzed on an Applied Biosystems model 491cLC protein sequencer (Applied Biosystems, USA) in the pulsed-liquid mode. For the analysis of the arrangement of disulfide bonds, the phenylthiohydantoin derivative of cystine (PTH-cystine) was detected on the same sequencer in the pulsed-liquid mode using a shallower HPLC gradient program to separate it from PTH-Tyr.

**Mass spectral analysis**

Mass spectra were measured on a matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE™ STR, Applied Biosystems) with 3,5-dimethoxy-4-hydroxycinnamic acid or α-cyano-4-hydroxycinnamic acid as a matrix in the positive ion mode.

**Bioassay for hyperglycemic activity *in vivo***

Adult giant freshwater prawns (*Macrobrachium rosenbergii*) weighing 15–25 g were obtained from a commercial source in China. Animals were maintained at 28°C under recirculating freshwater during the experiment. A bioassay for hyperglycemic activity was performed according to the methods described previously (Katayama *et al.,* 2002; Yang *et al.,* 1995). Preparation of sinus gland extracts from *M. rosenbergii* for use as a positive control was done using the same methods described previously (Yang *et al.,* 1995).

## RESULTS

### Expression of recombinant Mar-CHH-2-Gly and Mar-CHH-L

Recombinant Mar-CHH-2 having a glycine residue at the C-terminus (rMar-CHH-2-Gly) and recombinant Mar-CHH-L (rMar-CHH-L) were expressed in *E. coli*. Bacterial cells that expressed rMar-CHH-2-Gly and rMar-CHH-L were directly subjected to SDS-PAGE on 15% gels (Fig. 2). Bands corresponding to rMar-CHH-2-Gly and rMar-CHH-L were detected (Fig. 2, lanes 1 and 2), but no band was detectable in control cells carrying an expression plasmid lacking the insert (Fig. 2, lane 3). After sonication, rMar-CHH-2-Gly and rMar-CHH-L were recovered in insoluble inclusion bodies. Therefore, the refolding reaction was thought to be necessary in order to obtain rMar-CHH-2-Gly and rMar-CHH-L in their natural conformations.

### Refolding reaction

We conducted refolding reactions for rMar-CHH-2-Gly and rMar-CHH-L in the same redox buffer solution as used in our previous study (Ohira *et al.,* 2005), and obtained refolded rMar-CHH-2-Gly and rMar-CHH-L by RP-HPLC (Fig. 3A, B). Amino acid sequence analysis revealed that both peptides had ten identical amino acid residues, i.e., Ala-Ile-Leu-Asp-Gln-Ser-Xaa-Lys-Gly-Ile, at their N-terminals. The absence of N-terminal initiation Met residues was probably due to the enzymatic activity of methionine aminopeptidase in *E. coli* (Ben-Bassat and Bauer, 1987; Ben-Bassat *et al.,* 1987). The seventh amino acid residue (Xaa) could not be identified, as it was likely a cysteine residue. These sequences were the same as those of mature Mar-

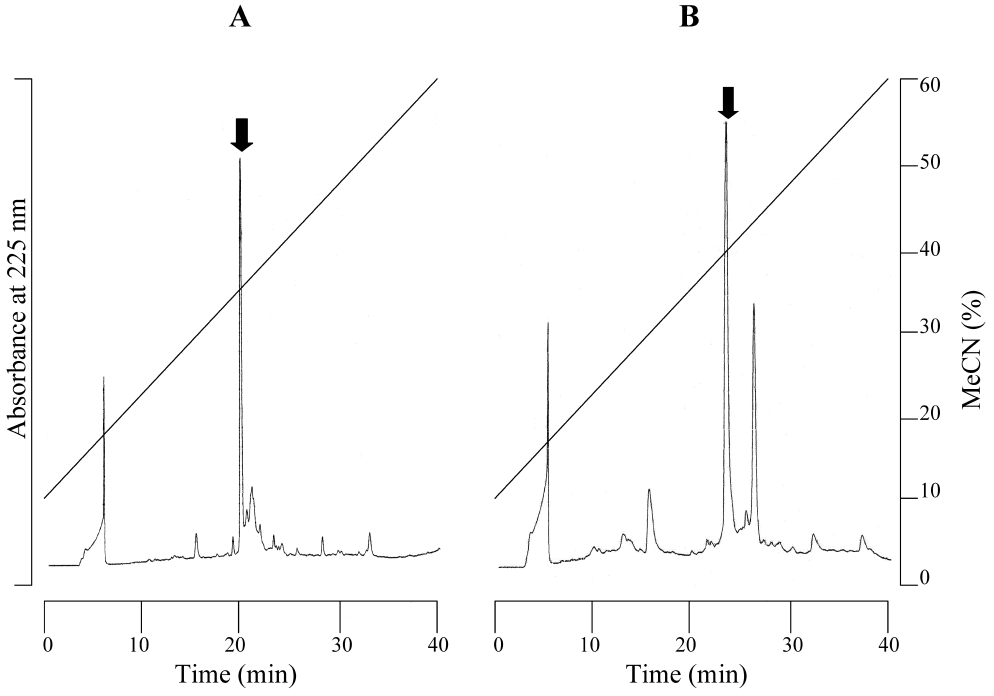


**Fig. 3.** RP-HPLC elution profiles of rMar-CHH-2-Gly (A) and rMar-CHH-L (B) after the refolding reaction. Chromatographic conditions are detailed in the Materials and Methods section. Concentration of acetonitrile is indicated by the solid line. Arrows indicate rMar-CHH-2-Gly and rMar-CHH-L peaks, respectively.

CHH-2 and Mar-CHH-L. In MALDI-TOF mass spectra of the purified rMar-CHH-2-Gly and rMar-CHH-L, protonated molecular ion peaks were observed at *m/z* 8582.2 and 8657.8, respectively. These values coincided well with the theoretical values of 8583.8 and 8658.9 for $(M+H)^+$, respectively. Yields of the refolded rMar-CHH-2-Gly and rMar-CHH-L were each about 1 mg from one liter of culture.

**Determination of the arrangement of disulfide bonds**

In order to examine whether the products of the refolding reaction possess native conformation, we analyzed the arrangements of disulfide bonds in rMar-CHH-2-Gly and rMar-CHH-L. rMar-CHH-2-Gly and rMar-CHH-L were digested with trypsin and lysyl endopeptidase, respectively, and the digests were separated by RP-HPLC (Fig. 4A, B). In MALDI-TOF mass spectral analysis, four internal fragments (Peaks 1-4 in Fig. 4), which likely contained single or double disulfide bonds, were identified (Table 1). Other major peaks excluding Peaks 1-4 were unexpected and therefore we determined their N-terminal amino acid sequences. The results showed that the other major peaks consisted of partially digested and non-digested fragments, some of which were more dominant than expected fragments (Fig. 4). These results indicate that both of the two recombinant CHHs appear to be highly resistant to peptidase digestion.

The results of N-terminal amino acid sequence and MALDI-TOF mass spectral analyses of the four peak materials (Fig. 4, Peaks 1-4) are shown in Table 1. Peak 1 consisted of two peptide chains, $Ala^1$-$Lys^8$ and $Glu^{41}$-$Arg^{50}$, with one disulfide bond between $Cys^7$-$Cys^{43}$ in rMar-CHH-2-Gly (Fig. 5). Peak 2 comprised three peptide chains, $Val^{22}$-$Arg^{31}$, $Lys^{32}$-$Arg^{40}$, and $Gln^{51}$-$Gly^{73}$,with two disulfide bonds. PTH-cystine was detected at cycles 5 and 8 by amino acid sequence analysis of peak 2, establishing the presence of

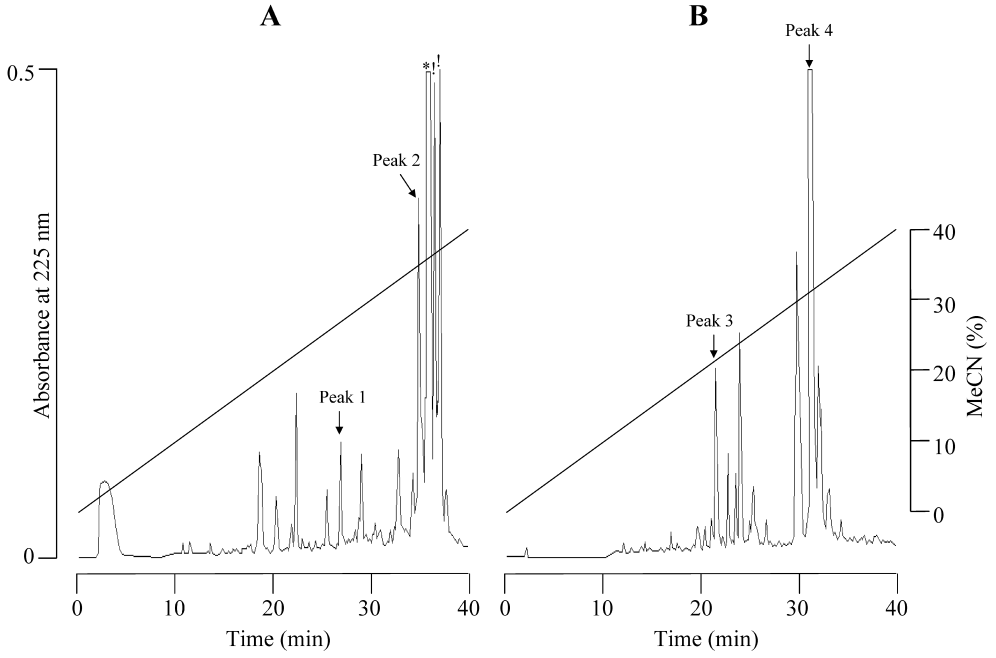


**Fig. 4.** RP-HPLC elution profiles of trypsin digests of rMar-CHH-2-Gly (A) and lysyl endopeptidase digests of rMar-CHH-L (B). Chromatographic conditions are detailed in the Materials and Methods section. Concentration of acetonitrile is indicated by the solid line. Asterisk (*) indicates non-digested rMar-CHH-2-Gly and exclamation marks (!) represent partially digested products.

**Table 1** Amino acid sequence and MALDI-TOF mass spectral analyses of trypsin digested fragments of rMar-CHH-2-Gly and lysyl endopeptidase digested fragments of rMar-CHH-L.

| Peak no. | Sequence | Molecular ion peak (*m/z* $(M+H)^+$) | |
|---|---|---|---|
| | | Observed | Theoretical |
| 1 | AILDQSCK | 2104.6 | 2104.4 |
| | EGCYQNLVFR | | |
| 2 | VCDDCYNLYR | 4931.4 | 4932.6 |
| | KPYVAIDCR | | |
| | QCIQDLQLMDQLDEYANAVQIVG | | |
| 3 | AILDQSCK | 1546.3 | 1545.8 |
| | DCFGTK | | |
| 4 | LDRVCDDCYNLYRK | 4857.3 | 4856.5 |
| | PYVAIDCRK | | |
| | TFGHCVEDLLLDQTHYK | | |

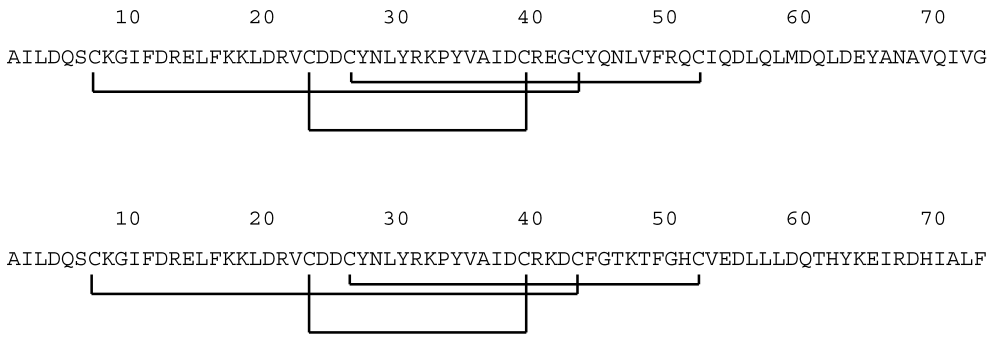


**Fig. 5.** Complete primary structures and disulfide bond arrangements of rMar-CHH-2-Gly (upper) and rMar-CHH-L (lower).

two disulfide bonds between $Cys^{23}$-$Cys^{39}$ and $Cys^{26}$-$Cys^{52}$ in rMar-CHH-2-Gly (Fig. 5). Peak 3 consisted of two peptide chains, $Ala^{1}$-$Lys^{8}$ and $Asp^{42}$-$Lys^{47}$, with one disulfide bond between $Cys^{7}$-$Cys^{43}$ in rMar-CHH-L (Fig. 5). Peak 4 contained three peptide chains, $Leu^{19}$-$Lys^{32}$, $Pro^{33}$-$Lys^{41}$, and $Thr^{48}$-$Lys^{64}$, with two disulfide bonds. PTH-cystine was detected at cycles 7 and 8 during the sequencing of peak 4, establishing the presence of two disulfide bonds between $Cys^{23}$-$Cys^{39}$ and $Cys^{26}$-$Cys^{52}$ in rMar-CHH-L (Fig. 5).

### Amidating reaction

In order to obtain a C-terminally amidated rMar-CHH-2 (rMar-CHH-2), rMar-CHH-2-Gly was treated with peptidylglycine $\alpha$-amidating enzyme. The resultant peptide was purified by RP-HPLC under weakly alkaline conditions using 10 mM ammonium acetate (pH 9.1), which yielded a peak containing rMar-CHH-2 (Fig. 6). In MALDI-TOF mass spectral analysis of the purified rMar-CHH-2, a protonated molecular ion peak was observed at *m/z* 8522.5, the value of which agreed well with the theoretical value of 8525.8 for $(M+H)^{+}$.

### Bioassay for hyperglycemic activity

The hyperglycemic activities of rMar-CHH-2 and rMar-CHH-L were examined by an *in vivo* bioassay (Fig. 7). Injections of 3 and 0.3 μg of rMar-CHH-2 increased hemolymph glucose levels by 210.4±58.2 μg/ml and 96.7±45.0 μg/ml, respectively. In contrast, rMar-CHH-L showed no effect on hemolymph glucose levels, which were 0.5±4.4 μg/ml (3 μg injection) and 6.1±6.4 μg/ml (0.3 μg injection). As a positive control, crude sinus gland extracts of *M. rosenbergii* were also assayed (Fig. 7). Injection of the extracts at a concen-

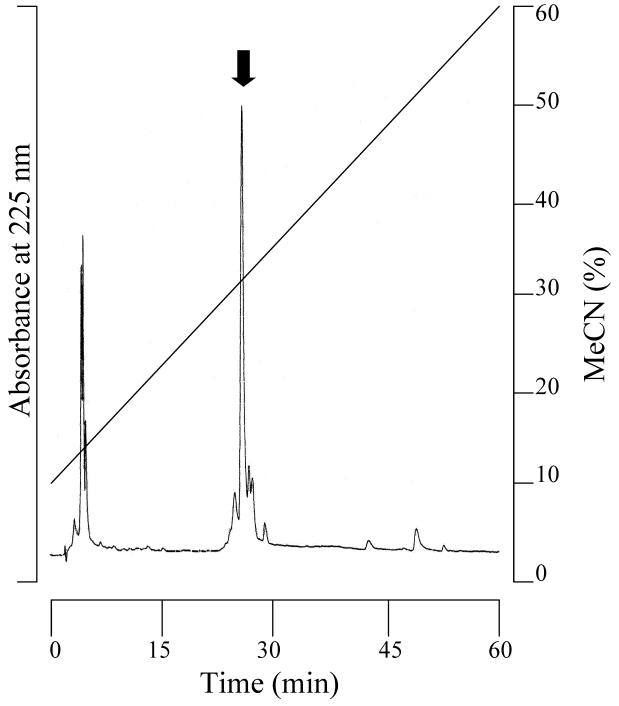


**Fig. 6.** RP-HPLC elution profile after C-terminal amidation of rMar-CHH-2-Gly. Chromatographic conditions are detailed in the Materials and Methods section. Concentration of acetonitrile is indicated by the solid line. Arrow indicates rMar-CHH-2 peak.

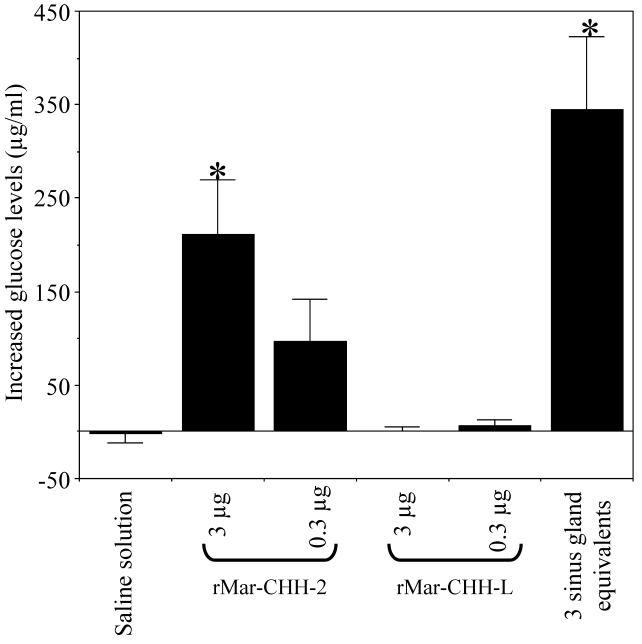


**Fig. 7.** Comparison of hyperglycemic activities of rMar-CHH-2, rMar-CHH-L, and sinus gland extracts. The saline solution column represents the injection of saline solution only as a negative control. Results are expressed as the mean±SE of eight or nine independent bioassays. Points with asterisks (*) indicate values significantly different from that of saline solution ($P$<0.05, Dunnett's multiple comparison test).

tration of 3 sinus gland equivalents significantly increased hemolymph glucose levels by 344.5±77.6 μg/ml.

## DISCUSSION

In this study, recombinant Mar-CHH-2 (rMar-CHH-2) and Mar-CHH-L (rMar-CHH-L) were produced using an *E. coli* expression system, and their hyperglycemic activities were assessed by *in vivo* injection assays. rMar-CHH-2 significantly induced glucose levels in the hemolymph, while rMar-CHH-L had no effect. It has been previously shown that Mar-CHH-2 is expressed in the eyestalks, whereas Mar-CHH-L is expressed in other tissues such as the heart, thoracic ganglion, antennal glands, and gills (Chen *et al.,* 2004). Therefore, Mar-CHH-2 and Mar-CHH-L are considered to be eyestalk and non-eyestalk forms, respectively. These results coincide well with that of Dircksen *et al.* (2001) in *C. maenas*, which showed that eyestalk CHH (SG-CHH) had hyperglycemic activity, whereas non-eyestalk CHH (PO-CHH) did not. Other eyestalk CHHs in *M. rosenbergii*, Mar-CHH and Mar-CHH-1, also showed significant hyperglycemic activity (Lin *et al.,* 1998; Sithigorngul *et al.,* 1999a), indicating that eyestalk CHHs including Mar-CHH-2 possibly regulate glucose levels in the hemolymph.

The arrangements of the three disulfide bonds in rMar-CHH-2 and rMar-CHH-L were determined to be identical, and the disulfide bonds were connected between $Cys^{7}$-$Cys^{43}$, $Cys^{23}$-$Cys^{39}$ and $Cys^{26}$-$Cys^{52}$. This arrangement is identical to that of other CHH-family peptides determined thus far (Aguilar *et al*., 1996; Huberman *et al.,* 1993; Katayama *et al.,* 2001, 2002; Kawakami *et al.,* 2000; Kegel *et al.,* 1989; Martin *et al.,* 1993; Nagasawa *et al.,* 1999; Ohira *et al.,* 2005; Yasuda *et al.,* 1994), and therefore, it is likely that this arrangement of disulfide bonds is also the same as in natural Mar-CHH-2 and Mar-CHH-L. These results indicate that the difference in hyperglycemic activity between rMar-CHH-2 and rMar-CHH-L is not caused by variations in disulfide bond arrangement. mRNAs for Mar-CHH-2 and Mar-CHH-L are transcribed from the same gene by alternative splicing and share the same sequence of 40 N-terminal amino acids in their mature peptides (Chen *et al.,* 2004). The remaining C-terminal regions are derived from different exons and therefore differ considerably (Chen *et al.,* 2004). These facts indicate that the C-terminal region is important for conferring hyperglycemic activity. In *M. japonicus*, the presence of the C-terminal amide moiety in CHH is important for the folding of the molecule to its natural conformation and is consequently required for conferring its hyperglycemic activity (Katayama *et al.,* 2002). This is consistent with our present results in that rMar-CHH-2 having a C-terminal amide showed hyperglycemic activity, whereas rMar-CHH-L with a free C-terminus had no activity.

In generally, CHH content in the sinus glands is not very high. In the case of *M. japonicus*, six CHHs have been purified from the sinus glands, and the yield of the most abundant CHH (Pej-SGP-III) was only about 80 ng from one sinus gland (Yang *et al.,* 1995). Mar-CHH-2 showed definite hyperglycemic activity in the *in vivo* bioassay, although its efficacy at even a concentration of 3 μg was lower than that of extracts from three sinus glands. Previous studies have shown that the sinus gland of *M. rosenbergii* contains at least two other CHHs, Mar-CHH and Mar-CHH-1 (Lin *et al.,* 1998; Sithigorngul *et al.,* 1999a). In addition, there is the possibility of the existence of other new CHHs which have not yet been characterized. The high hyperglycemic activity of the sinus gland extracts observed in this study may be due to the presence of CHHs other than Mar-CHH-2 that have higher activity than that of Mar-CHH-2 from the sinus gland.

Mar-CHH-L and PO-CHH are non-eyestalk molecules which arise via alternative splicing in the same manner (Chen *et al.,* 2004; Dircksen *et al.,* 2001). Moreover, they consist of 73 amino acid residues and have a free C-terminus (Chen *et al.,* 2004; Dircksen *et al.,* 2001). These results suggest that Mar-CHH-L and PO-CHH molecules may have evolved along the same pathway and have a common function. This study has demonstrated that Mar-CHH-L does not possess hyperglycemic activity. In a previous study, PO-CHH did not induce increases in glucose levels in the hemolymph and did not inhibit ecdysteroid synthesis in the Y-organ (Dircksen *et al.,* 2001). At present, therefore, the functions of Mar-CHH-L and PO-CHH are still unclear. Chen *et al.* (2004) proposed that Mar-CHH-L may be involved in ionic and osmotic regulation, volume control, or acid-basic balance, since Mar-CHH-L is expressed in the gill and antennal glands. Further experiments are required in order to fully elucidate its biological roles.

Until now, it has been difficult to obtain Mar-CHH-L from natural sources, as natural Mar-CHH-L was scarcely detectable in the gill and antennal glands (Chen *et al.,* 2004). This obstacle has been overcome in the present investigation, in which we have succeeded in producing a large quantity of rMar-CHH-L having the correct arrangement of three intramolecular disulfide bridges, by employing an *E. coli* expression system and a refolding reaction. Recombinant Mar-CHH-L prepared in this study will be a useful tool for elucidating the full range of its functioning. Recently, recombinant proteins for crustacean neuropeptides have been utilized in various studies, such as the raising of antibodies (Edomi *et al.,* 2002; Gu *et al.,* 2001), prediction of secondary structure (Katayama *et al.,* 2001), determination of three-dimensional structure (Katayama *et al.,* 2003), analysis of structure-activity relationship (Katayama *et al.,* 2004), and characterization of receptors (Asazuma *et al.,* 2005). Utilization of rMar-CHH-L in such physiological studies is expected to yield important information necessary for achieving a better understanding of the roles of non-eyestalk CHH in crustaceans.

## ACKNOWLEDGMENTS

We are grateful to Dr. V. Jayasankar of the Japan International Research Center for Agricultural Sciences for helpful suggestions. The amidating enzyme was a gift provided by Drs. K. Ohsue and K. Furukawa of Daiichi Suntory Pharma Co. Ltd. The first author (T.O.) was supported by the Cooperative System for Supporting Priority Research of the Japan Science and Technology Corporation. This work was supported by a Research and Development Program for New Bio-industry Initiatives of the Bio-oriented Technology Research Advancement Institution (BRAIN) of Japan, by a Grant-in-Aid for Scientific Research (No. 15208011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Fisheries Research Agency project of the Government of Japan.

## REFERENCES


Aguilar MB, Falchetto R, Shabanowitz J, Hunt DF, Huberman A (1996) Complete primary structure of the molt-inhibiting hormone (MIH) of the Mexican crayfish *Procambarus bouvieri* (Ortmann). Peptides 17: 367–374

Asazuma H, Nagata S, Katayama H, Ohira T, Nagasawa H (2005) Characterization of a molt-inhibiting hormone (MIH) receptor in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*. Ann N Y Acad Sci 1040: 215–218

Ben-Bassat A, Bauer K (1987) Amino-terminal processing of proteins. Nature 326: 315

Ben-Bassat A, Bauer K, Chang SY, Myambo K, Boosman A, Chang S (1987) Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine amino-peptidase and its gene structure. J Bacteriol 169: 751–757

Chang ES, Prestwich GD, Bruce MJ (1990) Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. Biochem Biophys Res Commun 171: 818–826

Chang ES, Chang SA, Mulder EP (2001) Hormones in the live of crustaceans: an overview. Am Zool 41: 1090–1097

Chen SH, Lin CY, Kuo CM (2004) Cloning of two crustacean hyperglycemic hormone isoforms in freshwater giant prawn (*Macrobrachium rosenbergii*): evidence of alternative splicing. Mar Biotechnol 6: 83–94

Chung JS, Dircksen H, Webster SG (1999) A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proc Natl Acad Sci USA 96: 13103–13107

Dircksen H, Bocking D, Heyn U, Mandel C, Chung JS, Baggerman G, Verhaert P, Daufeldt S, Plosch T, Jaros PP, Waelkens E, Keller R, Webster SG (2001) Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas*, are putatively spliced and modified products of multiple genes. Biochem J 356: 159–170

Edomi P, Azzoni E, Mettulio R, Pandolfelli N, Ferrero EA, Giulianini PG (2002) Gonad-inhibiting hormone of the Norway lobster (*Nephrops norvegicus*): cDNA cloning, expression, recombinant protein production, and immunolocalization. Gene 284: 93–102

Gu PL, Chu KH, Chan SM (2001) Bacterial expression of the shrimp molt-inhibiting hormone (MIH): antibody production, immunocytochemical study and biological assay. Cell Tissue Res 303: 129–136

Gu PL, Tobe SS, Chow BK, Chu KH, He JG, Chan SM (2002) Characterization of an additional molt inhibiting hormone-like neuropeptide from the shrimp *Metapenaeus ensis*. Peptides 23: 1875–1883

Huberman A, Aguilar MB, Brew K, Shabanowitz J, Hunt DF (1993) Primary structure of the major isomorph of the crustacean hyperglycemic hormone (CHH-I) from the sinus gland of the Mexican crayfish *Procambarus bouvieri* (Ortmann): Interspecies comparison. Peptides 14: 7–16

Katayama H, Ohira T, Nagata K, Nagasawa H (2001) 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. Biosci Biotechnol Biochem 65: 1832–1839

Katayama H, Ohira T, Aida K, Nagasawa H (2002) Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone. Peptides 23: 1537–1546

Katayama H,Nagata K, Ohira T, Yumoto F, Tanokura M, Nagasawa H (2003) The solution structure of molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*. J Biol Chem 278: 9620–9623

Katayama H, Ohira T, Nagata S, Nagasawa H (2004) Structure-activity relationship of crustacean molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*. Biochemistry 43: 9629–9635

Kawakami T, Toda C, Akaji K, Nishimura T, Nakatsuji T, Ueno K, Sonobe M, Sonobe H, Aimoto S (2000) Synthesis of a molt-inhibiting hormone of the American crayfish, *Procambarus clarkii*, and determination of the location of its disulfide linkages. J Biochem 128: 455–461

Kegel G, Reichwein B, Weese S, Gaus G, Peter-Katalinic J, Keller R (1989) Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. FEBS Lett 255: 10–14

Keller R (1992) Crustacean neuropeptides: Structures, functions and comparative aspects. Experientia 48: 439–448

Khayat M, Yang WJ, Aida K, Nagasawa H, Tietz A, Funkenstein B, Lubzens E (1998) Hyperglycaemic hormones inhibit protein and mRNA synthesis in *in vitro*-incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus*. Gen Comp Endocrinol 110: 307–318

Laufer H, Biggers WJ, Ahl JS (1998) Stimulation of ovarian maturation in the crayfish *Procambarus clarkii* by methyl farnesoate. Gen Comp Endocrinol 111: 113–118

Lin CY, Chen SH, Kou GH, Kuo CM (1998) Identification and characterization of a hyperglycemic hormone from freshwater giant prawn, *Macrobrachium rosenbergii*. Comp Biochem Physiol A 121: 315–321

Liu L, Laufer H (1996) Isolation and characterization of sinus gland neuropeptides with both mandibular organ inhibiting and hyperglycemic effects from the spider crab *Libinia emarginata*. Arch Insect Biochem Physiol 32: 375–385

Marco HG, Brandt W, Stoeva S, Voelter W, Gade G (2000) Primary structures of a second hyperglycemic peptide and of two truncated forms in the spiny lobster, *Jasus lalandii*. Peptides 21: 19–27

Martin G, Sorokine O, Van Dorsselaer A (1993) Isolation and molecular characterization of a hyperglycemic neuropeptide from the sinus gland of the terrestrial isopod *Armadillidium vulgare* (Crustacea). Eur J Biochem 211: 601–607

Nagasawa H, Yang WJ, Aida K, Sonobe H (1999) Chemical and biological characterization of neuropeptides in the sinus glands of the kuruma prawn, *Penaeus japonicus*. In "Peptide Sience-Present and Future" Ed by Shimonishi Y, Kluwer Academic Publishers, London, pp 453–454

Ohira T, Katayama H, Aida K, Nagasawa H (2003) Expression of a recombinant crustacean hyperglycemic hormone of the kuruma prawn *Penaeus japonicus* in methylotrophic yeast *Pichia pastoris*. Fish Sci 69: 95–100

Ohira T, Katayama H, Tominaga S, Takasuka T, Nakatsuji T, Sonobe H, Aida K, Nagasawa H (2005) Cloning and characterization of a molt-inhibiting hormone-like peptide from the prawn *Marsupenaeus japonicus*. Peptides 26: 259–268

Sithigorngul W, Jaideechoey S, Saraithongkum W, Longyant S, Sithigorngul P (1999a) Purification and characterization of an isoform of crustacean hyperglycemic hormone from the eyestalk of *Macrobrachium rosenbergii*. J Exp Zool 284: 217–224

Sithigorngul P, Panchan N, Vilaivan T, Sithigorngul W, Petsom A (1999b) Immunochemical analysis and immunocytochemical localization of crustacean hyperglycemic hormone from the eyestalk of *Macrobrachium rosenbergii*. Comp Biochem Physiol B 124: 73–80

Tamone SL, Chang ES (1993) Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs *in vitro*. Gen Comp Endocrinol 89: 425–432

Yang WJ, Aida K, Nagasawa H (1995) Amino acid sequences of a

hyperglycaemic hormone and its related peptides from the Kuruma prawn, *Penaeus japonicus*. Aquaculture 135: 205–212

Yang WJ, Aida K, Nagasawa H (1997) Amino acid sequences and activities of multiple hyperglycemic hormones from the kuruma prawn, *Penaeus japonicus*. Peptides 18: 479–485

Yasuda A, Yasuda Y, Fujita T, Naya Y (1994) Characterization of crustacean hyperglycemic hormone from the crayfish (*Procambarus clarkii*): Multiplicity of molecular forms by stereoinversion and diverse functions. Gen Comp Endocrinol 95: 387–398

(Received November 15, 2005 / Accepted December 29, 2005)