ABSM

Crustacean Peptide Hormones: Structure, Gene Expression and Function

Hidekazu Katayama[1†], Tsuyoshi Ohira[2†] and Hiromichi Nagasawa[3*]

[1]*Department of Applied Biochemistry*
  School of Engineering
  Tokai University
 Hiratsuka, Kanagawa 259-1292, Japan
[2]*Department of Biological Sciences*
  Faculty of Science
  Kanagawa University
  Hiratsuka, Kanagawa 259-1293, Japan
[3]*Department of Applied Biological Chemistry*
  Graduate School of Agricultural and Life Sciences
  The University of Tokyo
  Bunkyo-ku, Tokyo 113-8657, Japan
*e-mail: anagahi@mail.ecc.u-tokyo.ac.jp

Abstract

Homeostasis, growth and reproduction in crustaceans are under endocrine control as is established in other animals. The identification of crustacean hormones and elucidation of mechanisms of hormonal regulation have been pursued extensively. Mechanisms are found to be partly similar to, but also considerably different from those in insects, although both groups of animals are arthropods. Molting in insects, for example, is positively regulated by prothoracicotropic hormone (PTTH), which is secreted by the brain/corpora allata complex and stimulates the prothoracic glands to promote the synthesis and secretion of ecdysteroids. On the other hand, molting in crustaceans is negatively regulated by molt-inhibiting hormone (MIH), which is secreted by the X-organ/sinus gland complex and suppresses the Y-organs from synthesizing ecdysteroids during the intermolt period. Therefore, it is rather difficult to fully elucidate physiological mechanisms in crustaceans based only on the results obtained in insects. Using experimental evidence, we have thus far looked into the roles of crustacean eyestalk hormones and androgenic gland hormone (AGH). Various eyestalk hormones have been isolated from the sinus glands and characterized: e.g., crustacean hyperglycemic hormone (CHH), MIH, vitellogenesis-inhibiting hormone (VIH), red pigment concentrating hormone (RPCH) and pigment dispersing hormone (PDH). CHH, MIH and VIH are structurally similar, forming the CHH-family. Insects also possess molecules similar to CHH, RPCH and PDH, but their functions are different. In addition, AGH has been studied mainly using terrestrial isopods. Here, we describe the present state of our understanding of the structure and function of these peptide hormones, and discuss future perspectives.

1. Introduction

Approximately 25,000 species of crustaceans have so far been identified and recorded on Earth, and most of these species live in freshwater or seawater, while a small number of species dwell on land. Some crustacean species including shrimps and crabs are of commercial importance in fisheries and aquaculture, while others play a key role as intermediates in the food chain, linking phytoplanktons and fishes within the aquatic ecosystem. Modern shrimp culture was first established in Japan over 50 years ago using the kuruma prawn *Marsupenaeus* (formerly *Penaeus*) *japonicus*, which is one of the most important commercial species in Japan, and the aquaculture technique has now spread to many countries, especially in tropical and subtropical

---

†These authors contributed equally to this work.

regions. At present, Japan is one of the largest shrimp-importing countries in the world, totaling over 200,000 tons per year. Considerable quantities of shrimp, mainly the tiger prawn, *Penaeus monodon*, had formerly been imported from Taiwan about 20 years ago, but Taiwan was later replaced by various southeast Asian countries due to a sudden decrease in shrimp production in Taiwan in relation to a serious viral disease outbreak. Despite the worldwide importance of shrimp culture as an industry, insufficient knowledge exists concerning basic mechanisms of growth and reproduction in crustaceans. Since crustaceans belong to the arthropods, it may appear that they are similar to insects in all aspects. Crustaceans do resemble insects in many ways, but still differ from them considerably, for example, in terms of control of body color, molting systems, neurosecretory systems, calcification of the exoskeleton, sexual differentiation, control of blood carbohydrate levels and so forth. Thus, it is necessary to understand many peculiar phenomena at the molecular level, and one way to do so is by characterizing hormones related to those phenomena.

Blood carbohydrates in crustaceans consist principally of glucose, while trehalose is found in insects. The existence of a hyperglycemic factor in crustacean eyestalks has been known since Abramowitz and his colleagues first demonstrated that the injection of extracts of eyestalks into crabs caused an increase in hemolymph glucose levels (Abramowitz *et al.* 1944). Mechanisms of carbohydrate homeostasis in crustaceans are thus quite different from those in insects. The physiological and biochemical properties of the hyperglycemic factor have been extensively investigated (reviewed by Keller 1992). Crustacean hyperglycemic hormone (CHH) was first characterized in the shore crab *Carcinus maenas* (Kegel *et al.* 1989). CHHs are always the major neuropeptides in the crustacean sinus gland and therefore have been characterized in many decapod and isopod species (Santos and Keller 1993). Recent studies have revealed that CHHs are also involved in various processes such as ion transport at the gills and water uptake for the purpose of enlarging body size after molting (Chung *et al.* 1999; Spanings-Pierrot *et al.* 2000).

Crustaceans as well as many other arthropod species characteristically shed the exoskeleton during growth, because the calcified hard exoskeleton interferes with growth. An eyestalk hormone responsible for molting was discovered as early as 1905 (Zeleny 1905), but its chemical identification was first accomplished using the American lobster *Homarus americanus* only about 20 years ago (Chang *et al.* 1990). In the 1960's, the structure of the molting hormone in insects was determined after many years of effort by a German research group; it was found to be a highly hydroxylated steroid (Hoppe and Huber 1965), which is synthesized from cholesterol. Soon after this

structural determination, crustaceans were also found to use the same compound in molting (Hampshire and Horn 1966). Thus, insects and crustaceans utilize a common steroid, referred to as ecdysteroid, for the purpose of molting. However, the regulation of molting hormone production by the prothoracic glands in insects is different from that by the Y-organs in crustaceans; in insects, the prothoracic glands are positively regulated by prothoracicotropic hormone (PTTH) secreted by the brain/corpora allata complex (Kawakami *et al.* 1990), while in crustaceans, the Y-organs are negatively regulated by molt-inhibiting hormone (MIH) secreted from the X-organ/sinus gland complex (Keller 1992). Therefore, there seems to be some diversity in regulatory systems of molting in arthropods. This difference is not yet well understood, and differences in signal transduction systems from hormone reception to biosynthesis of molting hormone should be clarified in the future. Recently, some biosynthetic enzymes involved in the biosynthetic pathway from cholesterol to ecdysone have been characterized at the cDNA level in both insects (Gilbert 2004; Truman 2005; Rewitz and Gilbert 2008) and crustaceans (Rewitz and Gilbert 2008; Asazuma *et al.* 2009).

Vitellogenesis is an essential physiological event in the reproduction of oviparous animals. Various nutrients including carbohydrates, proteins, lipids, minerals, and vitamins necessary for embryonic development are accumulated in the oocytes during this process. In crustaceans, vitellogenesis is negatively regulated by a neuropeptide, vitellogenesis-inhibiting hormone (VIH), which is synthesized in and secreted from the X-organ/sinus gland complex in the eyestalks. When we started to study on *M. japonicus* VIHs, peptides designated as VIHs had been purified and characterized from only two crustacean species, the American lobster *H. americanus* (Soyez *et al.* 1991) and the terrestrial isopod *Armadillidium vulgare* (Gréve *et al.* 1999). In *H. americanus*, two VIH isoforms (Hoa-VIH-I and -II) were isolated and had an identical sequence of 77 amino acid residues (Soyez *et al.* 1991). They may occur via the transformation of an L-amino acid residue to a D-amino acid residue in either sequence through post-translational modifications (Soyez *et al.* 1994). Hoa-VIH-I and *A. vulgare* VIH (Arv-VIH) were shown to inhibit the onset of vitellogenesis in an *in vivo* bioassay, and therefore are generally accepted to be VIHs. In *Penaeus semisulcatus*, vitellogenesis was inhibited by some CHHs purified from the sinus glands of *M. japonicus*, and therefore the CHHs of these shrimps may have dual functions of hyperglycemic and vitellogenesis-inhibiting activities (Khayat *et al.* 1998).

It was found that CHH is structurally similar to MIH, forming a peptide family designated as the CHH-family (Keller 1992). Many peptides belonging to the CHH-family have been characterized from various crustacean species and also from insects (Audsley *et*

al. 1992; Endo *et al.* 2000) and a spider (Gasparini *et al.* 1994). VIH and mandibular organ-inhibiting hormone (MOIH), which inhibits the synthesis of methyl farnesoate by the mandibular organs (Wainwright *et al.* 1996), are also produced in the eyestalk of crustaceans, and are included as members of the CHH-family. Ion transport peptide (ITP) was characterized in a locust (Audsley *et al.* 1992), and low molecular weight protein (LMWP) was found in spider venom (Gasparini *et al.* 1994). Therefore, CHH-family peptides are likely to play various roles in arthropods. CHH-family peptides have not yet been discovered in any animals other than arthropods, and are therefore considered to be molecules unique to the arthropods.

Many crustaceans have the ability to alter body color in order to adapt to their surrounding background, and this alteration is accomplished by specialized cells called chromatophores. Body color changes result from dispersion or concentration of movable pigment granules in the chromatophores, which in crustaceans include four types: erythrophores, xanthophores, leucophores and melanophores. To date, red pigment concentrating hormone (RPCH) and pigment dispersing hormone (PDH), which are produced by and released from the X-organ/sinus gland complex in the eyestalk, are well known as chromatophore-regulating neuropeptides (Keller 1992). RPCH was first isolated and sequenced from the pink shrimp *Pandalus borealis* (Fernlund and Josefsson 1972). This molecule is an octapeptide with an amidated C-terminus, and was the first neuropeptide to be characterized not only in crustaceans but also in invertebrates. Later, adipokinetic hormones (AKHs) in insects were found to be structurally related to RPCH (Mordue and Stone 1976; Gäde 1991). Thus, a large family of AKH/RPCH peptides was established. PDH was originally identified as distal retinal pigment hormone (DRPH), and was isolated from *P. borealis* and sequenced (Fernlund 1976). PDH consists of 18 amino acid residues with an amidated C-terminus. Thus far, PDHs have been shown to exist widely not only in crustaceans but also in insects (Rao and Riehm 1993). Homologous peptides to PDH in insects are known as pigment dispersing factors (PDFs), and some reports suggest that PDFs may play an important role in the regulation of circadian rhythms in insects (Park and Hall 1998; Park *et al.* 2000).

Sex determination and sexual differentiation in vertebrates have been studied extensively, and have been clarified to be under both genetic and hormonal regulations. In contrast, in invertebrates little is known about sex determination and sexual differentiation with the exception of the fruit fly, in which there is no hormonal regulation (Schütt and Nöthiger 2000). The mechanism of sexual differentiation in crustaceans seems to be considerably different from that in insects, and crustaceans seem to be more similar to vertebrates than to insects with respect to being under endocrine regulation (Charniaux-Cotton 1967). Androgenic gland hormone (AGH), which is produced by the androgenic glands (AGs) and is responsible for sexual differentiation in crustaceans, was discovered in 1954 (Charniaux-Cotton 1954). Since then, many efforts have been made to isolate and characterize AGH. In 1999, AGH was successfully isolated from the terrestrial isopod *A. vulgare*, and its structure was determined to be an insulin-like heterodimeric peptide having an *N*-linked glycan (Martin *et al.* 1999; Okuno *et al.* 1999).

In this monograph, we will present our findings on purification, structural determination, cDNA cloning, gene expression and intrinsic functioning of these crustacean peptide hormones, and discuss future perspectives of crustacean molecular endocrinology.

2. Eyestalk peptide hormones I: CHH-family peptides

It has been shown that various types of neuropeptides are produced in the crustacean eyestalks (Keller 1992). Most of these are synthesized in the X-organ and transferred to the sinus gland, from which they are released into the hemolymph. Among these, the amino acid sequences of four neuropeptides, CHH, MIH, VIH and MOIH, are similar, forming a peptide family referred to as the CHH-family (Keller 1992). CHH-family peptides are mostly 72 to 78 amino acid residues long having six conserved cysteine residues that form three intramolecular disulfide bonds. It is believed that they have evolved from a common ancestral molecule. CHH-family peptides have been isolated not only from decapod and isopod crustaceans, but also from insects (Audsley *et al.* 1992) and a spider (Gasparini *et al.* 1994).
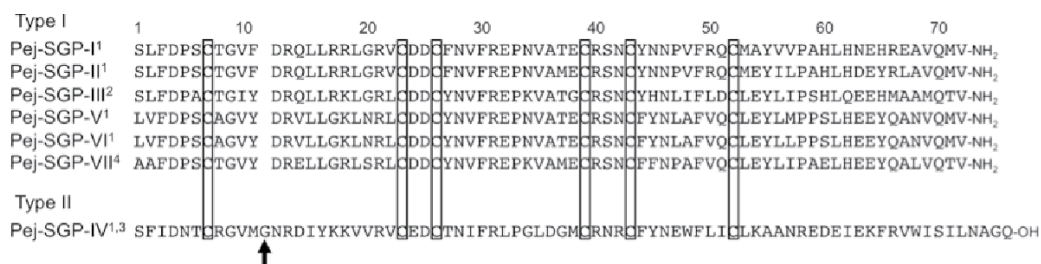
2-1. CHH

2-1A. Bioassay and purification

A CHH was first isolated and sequenced from the shore crab *C. maenas* (Kegel *et al.* 1989). Thereafter, the amino acid sequences of CHHs in various decapod species have been determined (reviewed by Santos and Keller 1993). However, no information concerning the structure of CHH in shrimps or prawns was available when we started to study crustacean eyestalk hormones. Therefore, we began purifying CHH molecules from the sinus glands of the kuruma prawn *M. japonicus* and determining their structures (Yang *et al.* 1995, 1996, 1997; Yang 1997; Nagasawa *et al.* 1999).

In our research, an *in vivo* injection bioassay using *M. japonicus* was performed essentially according to methods described previously (Leuven *et al.* 1982), but slightly modified as described below (Yang *et al.* 1995, 1996, 1997). Glucose levels in the hemolymph of normal prawns were 220 ± 30 μg/mL. They dramatically decreased to 35 ± 10 μg/mL 2 d (day) after bilateral

Fig. 1. Reversed-phase HPLC elution profile of extracts from 300 sinus glands of the kuruma prawn *Marsupenaeus japonicus*. Column: Asahi-Pak ODP-50 (4.6 × 150 mm). Solvent: linear gradient of 0–55% acetonitrile in 0.05% trifluoroacetic acid (TFA). Flow rate: 1 mL/min. Detection: absorbance at 225 nm. Temperature: 40°C. The concentration of acetonitrile is indicated by dashed line. Reprinted from Yang, Structures and functions of peptide hormones in the sinus gland of the kuruma prawn *Penaeus japonicus*, PhD Thesis, The University of Tokyo, © 1997, with permission from Wei-Jun Yang.

Table 1. Biological activities of Pej-SGP-I to -VII.

	Activity	
	CHH	MIH
Pej-SGP-I	++	–
Pej-SGP-II	+	–
Pej-SGP-III	+++	–
Pej-SGP-IV	–	+++
Pej-SGP-V	+++	+
Pej-SGP-VI	+++	+
Pej-SGP-VII	+++	nd

CHH, crustacean hyperglycemic hormone; MIH, molt-inhibiting hormone; nd, not determined.

Fig. 2. Amino acid sequencing analyses of Pej-SGP-III. Wavy line, residues identified by N-terminal sequencing; T3-1, -2, -3 and -4, tryptic peptides connected together by three disulfide bonds; E1 and E2, endoproteinase Glu-C peptides. Reprinted from *Aquaculture*, **135**, Yang *et al.*, Amino acid sequences of a hyperglycaemic hormone and its related peptides from the Kuruma prawn, *Penaeus japonicus*, 205–212, © 1995, with permission from Elsevier.

eyestalk ablation. To determine the appropriate time for hemolymph sampling following injection, a time-course experiment was conducted. After the injection of each CHH into eyestalk-ablated prawns, glucose levels were measured. The levels increased with time, reached a maximum within 2 h (hour) after injection, and decreased gradually thereafter. Thus, we drew up the conditions of a bioassay for hyperglycemic activity as follows.

Live kuruma prawns were obtained at a fish market in Tokyo, Japan and were kept in an aquarium at 20°C during the experiment. Samples dissolved in 100 μL of saline solution were injected into prawns (average body weight, 20 g) whose eyestalks had been ablated 2 d prior to the injection experiments. The hemolymph (200 μL each) was taken just prior to injection and 2 h after injection. Hemolymph glucose levels were determined using the glucose oxidase peroxidase method (Papadopoulos and Hess 1960). Hyperglycemic activity was assessed by the increase of hemolymph glucose levels following injection.

In order to purify *M. japonicus* CHH molecules, peptides were extracted from the sinus glands and fractionated by a single run of reversed-phase HPLC (Yang *et al.* 1995, 1996, 1997; Yang 1997; Nagasawa *et al.* 1999). A typical chromatogram from 300 sinus glands is shown in **Fig. 1**. Seven peak materials were designated as Pej-SGP-I to -VII in the order of elution. Hyperglycemic activities of the seven peaks were assessed by the methods described above, revealing that Pej-SGP-I, II, III, V, VI and VII showed considerable hyperglycemic activity. In contrast, Pej-SGP-IV exhibited almost no hyperglycemic activity, whereas it showed strong molt-inhibiting activity (**Table 1**), as described in Subsection 2-2A. Therefore, in *M. japonicus*, Pej-SGP-I, II, III, V, VI and VII are considered to be CHHs, whereas Pej-SGP-IV is considered to be an MIH.

2-1B. Structural determination

Next, we focused on the structural determination of the seven Pej-SGPs. The seven peak materials were reduced, carboxymethylated, and subsequently subjected to N-terminal amino acid sequence analysis (Yang *et al.* 1995, 1996, 1997; Yang 1997; Nagasawa *et al.* 1999). As a result, more than 43 amino acid residues for each peptide were identified. The N-terminal amino acid sequences of Pej-SGP-I, II, III, V,

```
Type I                1        10        20        30        40        50        60        70
Pej-SGP-I¹   SLFDPS C TGVF  DRQLLRRLGRV C DD C FNVFREPNVATE C RSN C YNNPVFRQ C MAYVVPAHLHNEHREAVQMV-NH₂
Pej-SGP-II¹  SLFDPS C TGVF  DRQLLRRLGRV C DD C FNVFREPNVAME C RSN C YNNPVFRQ C MEYILPAHLHDEYRLAVQMV-NH₂
Pej-SGP-III² SLFDPA C TGIY  DRQLLRKLGRL C DD C YNVFREPKVATG C RSN C YHNLIFLD C LEYLIPSHLQEEHMAAMQTV-NH₂
Pej-SGP-V¹   LVFDPS C AGVY  DRVLLGKLNRL C DD C YNVFREPNVATE C RSN C FYNLAFVQ C LEYLMPPSLHEEYQANVQMV-NH₂
Pej-SGP-VI¹  LVFDPS C AGVY  DRVLLGKLNRL C DD C YNVFREPNVATE C RSN C FYNLAFVQ C LEYLLPPSLHEEYQANVQMV-NH₂
Pej-SGP-VII⁴ AAFDPS C TGVY  DRELLGRLSRL C DD C YNVFREPKVAME C RSN C FFNPAFVQ C LEYLIPAELHEEYQALVQTV-NH₂

Type II
Pej-SGP-IV¹,³ SFIDNT C RGVMGNRDIYKKVVRV C ED C TNIFRLPGLDGM C RNR C FYNEWFLI C LKAANREDEIEKFRVWISILNAGQ-OH
                   ↑
```

Fig. 3. Comparisons between amino acid sequences of CHH-family peptides purified from the sinus glands of *Marsupenaeus japonicus*. Open boxes represent as conserved six cystein residues. An arrow indicates an insertion of a Gly residue at position 12. [1]Yang *et al*., 1997; [2]Yang *et al*., 1995; [3]Yang *et al*., 1996; [4]Nagasawa *et al*., 1999.

VI and VII were highly similar to each other and slightly less similar to the known CHHs from other crustaceans. In contrast, Pej-SGP-IV was more similar to *C. maenas* MIH and *H. americanus* VIH than to Pej-SGP-I, II, III, V, VI, VII and the known CHHs. These results showed good agreement with those of the bioassays as described above.

Since Pej-SGP-III is an abundant CHH molecule in the sinus glands of *M. japonicus*, further structural analysis of this peptide was first performed (Yang *et al*. 1995). Trypsin digestion of the intact Pej-SGP-III afforded a peptide fragment named T3 (**Fig. 2**). This fragment consisted of four peptide chains, among which three sequences (T3-1, -2 and -3) were easily assigned based on the N-terminal sequence. The fourth peptide (T3-4) starting from Ser[41] was assigned by subtracting the above three sequences from the total. T3-4 was considered to be a C-terminal peptide, but the residues could be identified up to the 70th residue and, judging from the mass spectral data, two more unidentified residues probably remained at the C-terminus.

In order to determine the C-terminal sequence, the intact Pej-SGP-III was digested with endoproteinase Glu-C. Four major fragment peptides named E1–E4 were obtained. E1 was found by sequence analysis to be a C-terminal fragment, and one more residue, Thr[71], could be identified. Thus, there remained only a C-terminal unidentified residue. The high resolution FAB mass spectrum of E1 showed a protonated molecular ion peak at m/z 887.43. The only possibility for the C-terminal residue which could satisfy this value was Val with an amidated structure (calculated value, 887.42). Thus the complete amino acid sequence of Pej-SGP-III was unambiguously determined (**Fig. 2**).

Subsequently, the complete amino acid sequences of the remaining five CHHs, Pej-SGP-I, II, V, VI and VII (Yang *et al*. 1997; Yang 1997; Nagasawa *et al*. 1999), and the MIH, Pej-SGP-IV (see Subsection 2-2B), were also determined similarly to the structural characterization of Pej-SGP-III. Amino acid sequences of the seven peptides are shown in **Fig. 3**. These peptides showed considerable sequence similarity to the CHH-family peptides characterized previously, and especially were found to have the conserved six cysteine residues which are involved in the formation of three disulfide bridges. Therefore, the seven peptides are thought to be members of the CHH-family peptide in *M. japonicus*. 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. According to this grouping, the six CHHs (Pej-SGP-I, II, III, V, VI and VII) can be classified into type I and the MIH (Pej-SGP-IV) belongs to type II. This classification is consistent with previous studies in that all of the known CHH molecules are classified as type I, and MIH, MOIH and VIH molecules are classified as type II (Lacombe *et al*. 1999).

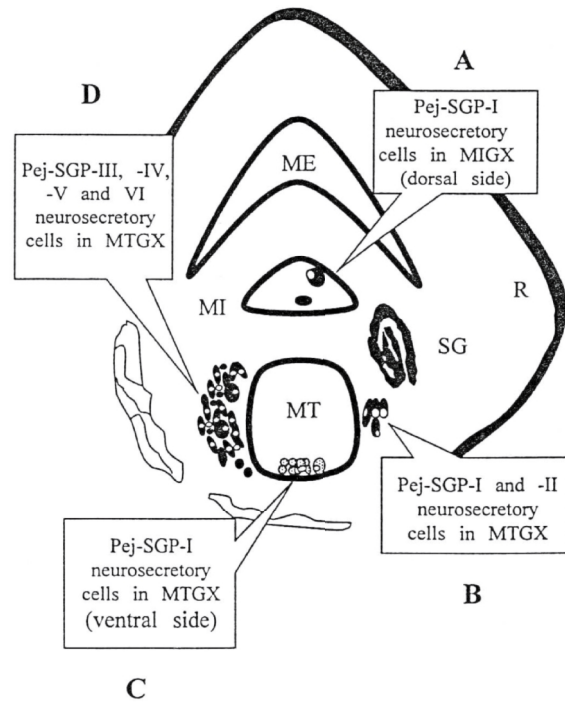### 2-1C. Immunohistochemical localization

In order to elucidate the localization of cells producing CHH-family peptides in *M. japonicus*, we developed five specific antibodies against Pej-SGP-I, -II, -III, -IV and -V (Shih *et al*. 1997, 1998). Immunohistochemical studies using these antibodies showed that neurosecretory cells of Pej-SGP-III and IV were located in the same cluster of the medulla terminalis ganglionic X-organ (MTGX), and that three kinds of neurosecretory cells stained with an anti-Pej-SGP-III antiserum and/or an anti-Pej-SGP-IV antiserum were present (**Figs. 4a, b**). The number of neurosecretory cells which were stained with both antisera was much smaller than that of neurosecretory cells which were stained with one antiserum only. Co-localization of two CHH-family peptides has been reported in previous studies on CHH- and VIH-producing cells using immunohistochemistry and *in situ* hybridization in *Homarus gammarus* (Rotllant *et al*. 1993) and *H. americanus* (De Kleijn *et al*. 1992). In contrast, no co-localization of MIH and CHH was observed at either the mRNA or peptide level in *C. maenas* (Klein *et al*. 1993).

The diagrammatic localization of neurosecretory cells stained with the five antibodies is shown in **Fig. 5**. Pej-SGP-I cells were found in the three clusters. The first cluster was in the medulla interna ganglionic X-
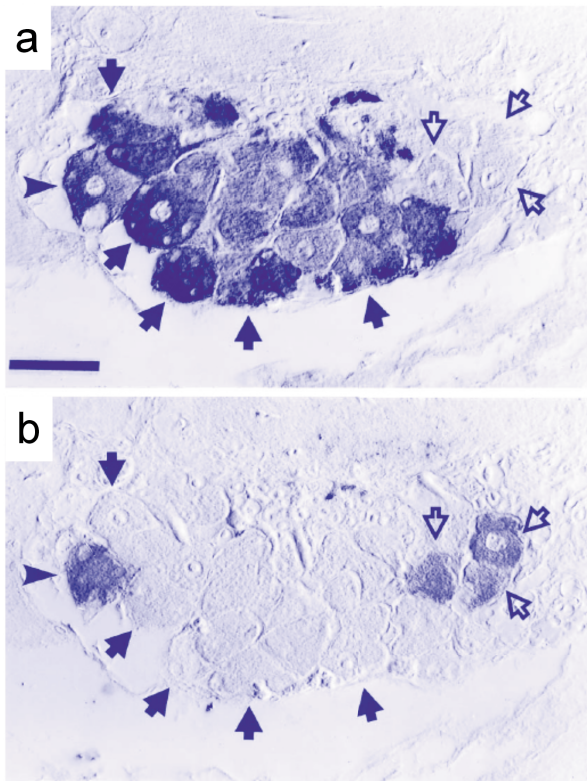
Fig. 4. Pej-SGP-III- and Pej-SGP-IV-immunopositive neurosecretory cells. (a) Pej-SGP-III-immunopositive neurosecretory cells in the medulla terminalis ganglionic X-organ (MTGX). (b) Pej-SGP-IV-immunopositive neurosecretory cells in the MTGX. Arrows indicate Pej-SGP-III (➡), Pej-SGP-IV (⇨) and Pej-SGP-III and -IV (➤) neurosecretory cells. Scale bar represents 50 μm. Reprinted with permission from *Zool. Sci.*, **15**, Shih *et al.*, Immunohistochemical identification of hyperglycemic hormone- and molt-inhibiting hormone-producing cells in the eyestalk of the kuruma prawn, *Penaeus japonicus*, 389–397, Fig. 4, © 1998, Zoological Society of Japan.

Fig. 5. Schematic representation of the right eyestalk in the kuruma prawn *Marsupenaeus japonicus*, showing the localization of neurosecretory cells (horizontal section). ME, medulla externa; MI, medulla interna; MT, medulla terminalis; SG, sinus gland; MIGX, medulla interna ganglionic X-organ; MTGX, medulla terminalis ganglionic X-organ; R, rerinula. Reprinted from Kawashima and Kikuyama (eds)., *Proceedings of the XIII International Congress of Comparative Endocrinology*, Shih *et al.*, Immunohistochemical localization of crustacean hyperglycemic hormones (CHHs) and a molt-inhibiting hormone (MIH) in the eyestalk of *Penaeus japonicus*, 87–91, © 1997, with permission from Monduzzi Editore, Bologna.

organ (MIGX) (Position A in **Fig. 5**). The second cluster was in the MTGX near to the sinus gland (Position B in **Fig. 5**). The third cluster was also in the MTGX, but located at the ventral side of the medulla terminalis (MT) near to the root of the eyestalk (Position C in **Fig. 5**). Pej-SGP-II cells were found in the MTGX near to the sinus gland (Position B in **Fig. 5**). Pej-SGP-V cells existed in the MTGX opposite to the sinus gland, being of the same cluster as those of Pej-SGP-III and IV (Position D in **Fig. 5**). As far as we know, the neurosecretory cells of CHH-family peptides in crustaceans are known to exist in the same cluster of the MTGX located at the opposite side of the sinus gland (Jaros and Keller 1979). Therefore, the results of our study are the first to show that not all of the CHH-family peptides are produced in the same cell cluster. Different patterns of localization of these neurosecretory cells for *M. japonicus* CHH-family peptides may imply that they have different physiological and regulatory roles.

2-1D. cDNA cloning

When we started our studies on CHHs of the kuruma prawn *M. japonicus*, cDNAs encoding CHHs had been isolated from the shore crab *C. maenas* (Weidemann *et al.* 1989), the American lobster *H. americanus* (De Kleijn *et al.* 1995), and the crayfish *Orconectes limosus* (De Kleijn *et al.* 1994a). A common structure for these CHH precursors was demonstrated by cDNA sequence analysis. The CHH precursors consisted of a signal peptide, a CHH precursor-related peptide (CPRP), and a CHH. The CPRPs were 33–38 residues long and structurally conserved. The CPRPs had been purified from sinus glands of the above three crustacean species (Tensen *et al.* 1991), but their biological function had not yet been clarified. Moreover, there was no information concerning the structure of CHH precursor

```
                                                          -24
                                                         Met Val
5'---TGAACTGCATTGCAACTCAATATTTCTAAGTCTCGTGTCATTTGCA ATG GTG  52

      -20                      Signal Peptide
Thr Pro Arg Met Leu Ser Ala Leu Ser Ala Val Leu Leu Leu Val Leu
ACT CCT CGC ATG TTG TCA GCG CTG TCA GCA GTC TTG CTG TTG GTT CTT 100

                    -1   1          CHH Precursor-Related Peptide
Thr Ala Ser Ser Ser Ala Arg Ser Phe Asp Ala Ser Pro Ser Ala Thr
ACT GCC TCG TCC TCG GCG CGG TCT TTC GAC GCC TCT CCG TCT GCG ACT 148

                                            20
Ser Gly Asn His Ser Leu Asn Lys Arg Ser Leu Phe Asp Pro Ala Cys
TCG GGA AAC CAC AGC CTC AAT AAG CGG TCT CTC TTC GAC GCT GCG TGC 196

              Pej-SGP-III              40
Thr Gly Ile Tyr Asp Arg Gln Leu Leu Arg Lys Leu Gly Arg Leu Cys
ACC GGC ATC TAC GAC CGG CAG CTG CTG AGG AAG TTG GGT CGC CTG TGC 244

Asp Asp Cys Tyr Asn Val Phe Arg Glu Pro Lys Val Ala Thr Gly Cys
GAC GAC TGC TAC AAC GTG TTC CGG GAA CCC AAA GTA GCC ACT GGA TGC 292

    60
Arg Ser Asn Cys Tyr His Asn Leu Ile Phe Leu Asp Cys Leu Glu Tyr
AGG AGT AAT TGT TAC CAC AAC CTC ATT TTC CTC GAT TGC CTG GAG TAC 340

              80
Leu Ile Pro Ser His Leu Gln Glu Glu His Met Ala Ala Met Gln Thr
CTC ATC CCG AGC CAC CTG CAG GAG GAG CAT ATG GCC GCC ATG CAG ACA 388

        93
Val Gly Lys End
GTC GGG AAG TAA AAGTCCCCCTCCGAGAAATTCTCTTCGTTGATGGCATCTGCGAACGA 444

CCTGGATTCACGAACGTTTTCTTATGTAAATCTCGAACTTAAGTGCAGTCGGTATTCTCGGTT 507

TTCTTTTTCGTTGTTAGTGATTTCTGATCTAATGTTTGTTGGAAGAGAAAGAGTTCATGTATTT 570

GTTTGTGTTTACGTTTGCGTCAATGCCTTTGAGATATATGTGAACTCAGCTGTGATCTACATC 633

TTAAATAAACTCATAATACGGCTGTACGCATAAAACAACCTGTAAAGACCTTTTTAATTTTCC 696

ATTTTGTCGTCAGGGTCTACTTCGAGAAGGAATTGTATGAAATAAGATATAAATTTAAATCGT 759

CGTATTTTCAGTTGG---3'                                          774
```

Fig. 6. Nucleotide sequence of a cDNA encoding a Pej-SGP-III precursor in the kuruma prawn *Marsupenaeus japonicus* and its deduced amino acid sequence. Amino acid numbering starts at the N-terminal residue of the propeptide of Pej-SGP-III with the presumptive signal peptide indicated by negative numbers. The black box indicates the putative dibasic cleavage site between Pej-SGP-III and CHH precursor-related peptide (CPRP). The light gray box indicates the putative amidation signal. The putative polyadenylation signals are underlined. The sequence has been deposited in the DNA Data Bank of Japan (accession No. D87864). Reprinted with permission of John Wiley & Sons, Inc. from *Mol. Mar. Biol. Biotechnol.*, **6**, Ohira *et al.*, Cloning and sequence analysis of a cDNA encoding a crustacean hyperglycemic hormone from the Kuruma prawn *Penaeus japonicus*, 59–63, Fig. 1, © 1997, Wiley-Liss, Inc., a Wiley Company.

in shrimps or prawns at that time. Therefore, we attempted to isolate a cDNA encoding a precursor of Pej-SGP-III, which was the first CHH to be characterized among the six CHHs in *M. japonicus* (Ohira *et al.* 1997a).

Total RNA isolated from the eyestalk of *M. japonicus* was subjected to RT-PCR to obtain a cDNA fragment encoding Pej-SGP-III. The reaction product was used as a probe to screen an eyestalk cDNA library, and subsequently three clones encoding a Pej-SGP-III precursor were isolated. The nucleotide sequence of one of the cDNA clones is shown in **Fig. 6**. An open reading frame (ORF) of 354 bp was found, and this ORF was conceptually translated into a putative protein of 117 amino acid residues. As expected, the sequence of Ser^{20}–Val^{91} of this protein was identical to that of Pej-
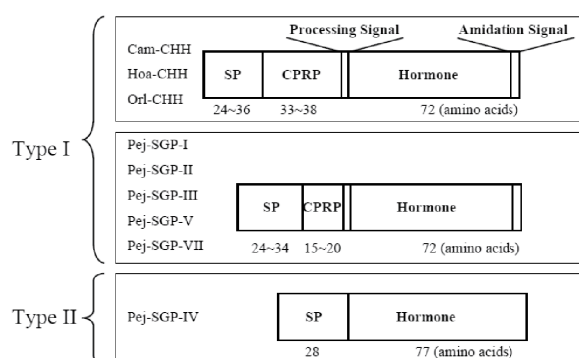
Fig. 7. Schematic representation of the organization of CHH-family peptide precursors. Cam-CHH, *Carcinus maenas* CHH; Hoa-CHH, *Homarus americanus* CHH; Orl-CHH, *Orconectes limosus* CHH; Pej-SGP-I, II, III, V and VII, *Marsupenaeus japonicus* CHHs; Pej-SGP-IV, *M. japonicus* MIH; SP, signal peptide; CPRP, CHH precursor-related peptide.

SGP-III. This segment Ser^{20}–Val^{91} was flanked C-terminally by Gly^{92}–Lys^{93} and followed by a stop codon. Val^{91} of the mature Pej-SGP-III is amidated (Yang *et al.* 1995); Lys^{93}, therefore, is expected to be removed from the precursor, and Gly^{92} might be the amide donor to Val^{91}. Segment Met^{-24}–Ala^{-1} had a highly hydrophobic core (Met^{-19}–Leu^{-7}) and therefore was likely to constitute a signal peptide (Von Heijne 1986). The remaining sequences of Arg^1–Asn^{17} and Lys^{18}–Arg^{19} were thought to be a putative CPRP and a putative dibasic cleavage site, respectively. This cDNA also included a 5′ flanking sequence (46 bp) and a 3′ untranslated region (374 bp) containing a consensus polyadenylation signal site and a variant one (AATTTAAA) 132 bp and 16 bp upstream from poly (A) tail, respectively.

Following the cDNA cloning of the Pej-SGP-III, nucleotide sequences of cDNAs encoding other CHHs in *M. japonicus* were determined. cDNA clones of Pej-SGP-I, V, and VII were isolated by the same methods as those for Pej-SGP-III (Ohira *et al.* 1997b). A Pej-SGP-II cDNA was amplified by RT-PCR coupled with 5′- and 3′-RACE (Ohira 2000). Although we looked for a Pej-SGP-VI cDNA, unfortunately it could not be cloned. Nucleotide sequences of those cDNAs encoding Pej-SGP-I, II, V, and VII are presently accessible through public DNA databases (their accession Nos. AB007507, AB035724, AB007508, and AB007509).

The schematic organization of CHH precursors in *M. japonicus* and other species are shown in **Fig. 7**. The manner of organization of the five precursors was identical with that of *C. maenas*, *H. americanus*, and *O. limosus*, but differed from that of a Pej-SGP-IV precursor, which is an MIH in *M. japonicus* (see Subsection 2-2B). The CHH-family peptides can therefore be grouped into two types as previously mentioned. Also,

the precursors of the two types have a distinct organization: type I precursors such as CHHs in *C. maenas*, *H. americanus*, and *O. limosus* consist of a signal peptide, a CPRP and a mature hormone, whereas type II precursors lack a CPRP (reviewed by De Kleijn and Van Herp 1995). It has already been mentioned that Pej-SGP-I, II, III, V, VI and VII are grouped into type I, whereas only Pej-SGP-IV is classified into type II based on amino acid sequence of mature hormones (see Subsection 2-2B). Therefore, the results revealed by cDNA cloning of the five CHHs are consistent with the previous results, because CPRP were found in the five precursors (**Fig. 7**).

The five CPRPs in *M. japonicus* (15–20 residues) were significantly shorter than those in *C. maenas*, *H. americanus*, and *O. limosus* (33–38 residues). The primary structures of the CPRPs were conserved among *C. maenas*, *H. americanus* and *O. limosus*, suggesting a common biological function. The CPRPs in Pej-SGP-I, II, III, V and VII, however, showed low sequence similarity to those in other crustacean species. These observations would argue against the possibility that the CPRPs in *M. japonicus* have the same function as the CPRPs in other crustacean species.

2-1E. Preparation of recombinant CHHs

In general, recombinant proteins produced using bacterial expression systems are very useful tools for studies on the determination of their tertiary structures and the production of antibodies. The *Escherichia coli* expression system enables the production of larger amounts of recombinant proteins at lower cost than those of other expression systems, such as the baculovirus and yeast expression systems. Using the *E. coli* expression system, we have succeeded in producing a recombinant Pej-SGP-IV, an MIH in *M. japonicus*, showing molt-inhibiting activity comparable to that of the native Pej-SGP-IV (see Subsection 2-2C). Therefore, we first attempted to express a recombinant Pej-SGP-III, one of the six CHHs in *M. japonicus*, using the same *E. coli* expression system. However, the initial trial failed. Thereafter, the utility of the baculovirus expression system was also examined, but Pej-SGP-III could not be successfully produced. Since the methylotrophic yeast *Pichia pastoris* expression system has been used in the production of several peptide hormones, such as mammalian gonadotrophic hormones and human insulin (Cereghino and Cregg 2000), we examined the use of *P. pastoris* as a host organism in the expression of recombinant Pej-SGP-III (Ohira *et al.* 2003a).

A cDNA encoding Pej-SGP-III was processed by PCR and the resulting product was ligated into an expression vector. *P. pastoris* was transformed with this vector, and a recombinant Pej-SGP-III having an additional glycine residue at the C-terminus (rPej-SGP-III-Gly), a form considered to be a putative precursor
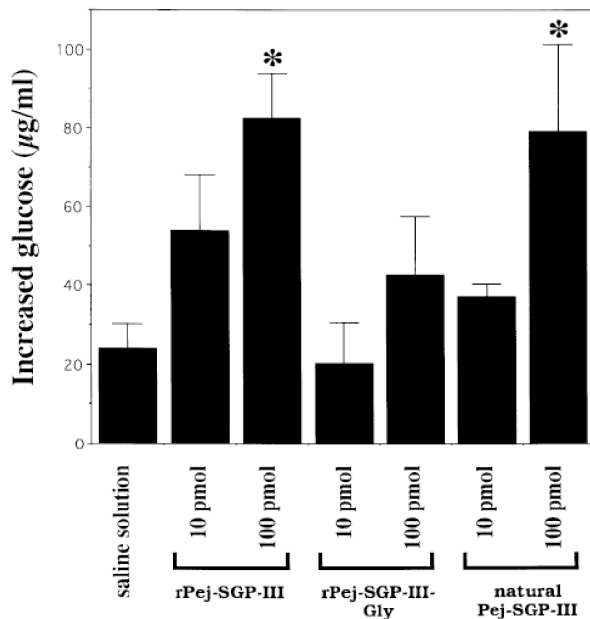
Fig. 8. Comparison of hyperglycemic activities of rPej-SGP-III, rPej-SGP-III-Gly and natural Pej-SGP-III purified from the sinus glands. The saline solution column represents the injection of saline solution only as a negative control. Results are expressed as the means ± SE of five independent bioassays. Points with asterisks (∗) indicate values significantly different from that of saline solution ($P < 0.05$, Dunnett's multiple comparison test). Reprinted with permission from *Fish. Sci.*, **69**, Ohira *et al.*, Expression of a recombinant crustacean hyperglycemic hormone of the kuruma prawn *Penaeus japonicus* in methylotrophic yeast *Pichia pastoris*, 95–100, Fig. 4, © 2003, The Japanese Society of Fisheries Science.

of this hormone, was expressed. rPej-SGP-III-Gly secreted into the culture medium was purified by reversed-phase HPLC and its yield was about 0.4 mg from 1 liter of culture. rPej-SGP-III-Gly was subjected to an amidating reaction using a peptidylglycine α-amidating enzyme, and the amidated recombinant Pej-SGP-III (rPej-SGP-III) was purified by reversed-phase HPLC. The rPej-SGP-III showed hyperglycemic activity in an *in vivo* bioassay almost comparable to that of the native Pej-SGP-III (**Fig. 8**).

We also tried to produce other recombinant *M. japonicus* CHHs using different methods of making the recombinant Pej-SGP-III. In the case of the production of a recombinant Pej-SGP-I, the *E. coli* expression system was used (Katayama *et al.* 2002). Although biologically active recombinant Pej-SGP-I was obtained, the yield of the final product was poor (0.3 mg from 1 liter culture). This was because, the expressed recombinant Pej-SGP-I aggregated in an insoluble form, consequently yielding an inefficient refolding reaction. In order to overcome this problem, we recently introduced a new system using a Nus-tag for
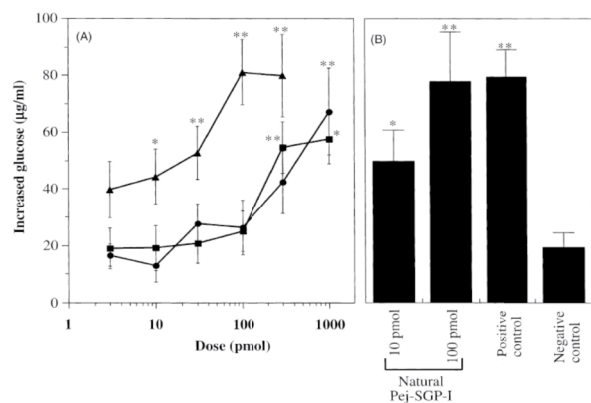
Fig. 9. Comparison of the hyperglycemic activities of recombinant and natural peptides, and sinus gland extract. (A) Dose-response curves of rPej-SGP-I-OH (●), rPej-SGP-I-Gly (■) and rPej-SGP-I-amide (▲) in terms of hyperglycemic activity. (B) Hyperglycemic activity of the natural Pej-SGP-I; negative control, injection of phosphate buffered saline (PBS); positive control, injection of the sinus gland extract (one sinus gland equivalent) of the kuruma prawn *Marsupenaeus japonicus*. Results are expressed as the means ± SE (n = 5–21). Asterisks indicate values significantly different from the negative control. *$P < 0.05$; **$P < 0.01$ using the program of Fisher's PLSD. Reprinted from *Peptides*, **23**, Katayama *et al*., Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone, 1537–1546, © 2002, with permission from Elsevier.

Table 2. Secondary structure composition in recombinant and native peptides of Pej-SGP-I predicted from CD spectra. Reprinted from *Peptides*, **23**, Katayama *et al*., Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone, 1537–1546, © 2002, with permission from Elsevier.

Molecule	α-Helix	β-Sheet	Turn	Random
rPej-SGP-I-Gly	29.6	0	32.7	37.7
rPej-SGP-I-OH	29.1	0	34.6	36.3
rPej-SGP-I-amide	35.3	0	41.4	23.3
Native Pej-SGP-I	42.7	0	19.7	37.6

Values indicate type of secondary structure present in terms of percentage (%).

solubilization to produce a recombinant Pej-SGP-VII (Nagai *et al*. 2009). Therefore, soluble and biologically active recombinant Pej-SGP-VII could successfully be obtained using a simpler and more efficient procedure than that previously used for the recombinant Pej-SGP-I. The yield of soluble recombinant Pej-SGP-VII attached with the Nus-tag at the N-terminal side and a Gly residue at the C-terminal end was about 160 mg from 1 liter of culture.

Recombinant CHHs of crustacean species except for *M. japonicus* were also produced by other workers using *E. coli* (Gu *et al*. 2000; Mettulio *et al*. 2004) and yeast expression systems (Treerattrakool *et al*. 2003). Injection of those recombinant CHHs elevated hemolymph glucose levels; however, it has not been determined whether their biological activities are comparable to those of native ones. Such recombinant CHHs seem to have shown lower hyperglycemic activity compared with that of recombinant *M. japonicus* CHHs, probably due to the lack of amidation at the C-terminus. In addition, their structural characteristics, including secondary structure and disulfide bond arrangement, have not yet been examined.

2-1F. Structure-activity relationship

CHH-family peptides are divided into two subtypes, types I and II, based on the structure of the mature hormone as described above. All CHH molecules are classified into type I, and MIH, MOIH and VIH molecules are classified into type II except for *Procambarus bouvieri* MIH belonging to type I (Aguilar *et al*. 1996). Most type I peptides are amidated at the C-terminus, but a few exceptions are observed. In *C. maenas*, CHH-like molecule isolated from the pericardial organ (PO-CHH) and the CHH isolated from the sinus gland (SG-CHH) share an identical N-terminal sequence, positions 1–40, but the remaining C-terminal sequences differ considerably (Dircksen *et al*. 2001). SG-CHH exhibits a remarkable hyperglycemic activity, but PO-CHH which lacks an amide moiety at the C-terminus shows no hormonal activity. In the South African spiny lobster *Jasus lalandii*, two CHHs with a C-terminal amide moiety have been isolated from the sinus gland. In addition, it was found that C-terminally truncated forms of two CHHs, lacking seven C-terminal amino acid residues and having a free C-terminus, exist concurrently in the sinus glands (Marco *et al*. 2000). The former two CHHs showed hyperglycemic activity, whereas the latter two truncated CHHs did not. Therefore, it is highly possible that the C-terminal sequence of CHH, especially the C-terminal amide moiety, is significant in conferring the hormonal activity, although no direct evidence has been obtained.

In order to examine the above hypothesis, we produced recombinant Pej-SGP-I molecules, one of the six CHHs in *M. japonicus*, with or without the C-terminal amide moiety using a bacterial expression system (Katayama *et al*. 2002). As expected, increases in hemolymph glucose levels after injection of the recombinant Pej-SGP-I with a free carboxyl-terminus (rPej-SGP-I-OH) were observed at high dosages only, whereas the recombinant Pej-SGP-I having an amidated C-terminus (rPej-SGP-I-amide) showed remarkable hyperglycemic activity, which was comparable to that of the native Pej-SGP-I (**Fig. 9**). These
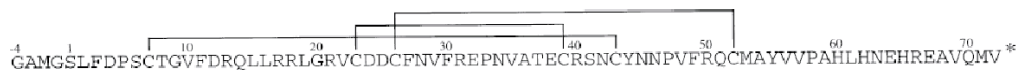
Fig. 10. Complete primary structure of rPej-SGP-I. Four amino acid residues at the N-terminus, Gly-Ala-Met-Gly, are derived from the nucleotide sequence of an expression vector. An additional glycine residue was attached at the C-terminus (∗) in rPej-SGP-I-Gly, and the C-terminus was amidated in rPej-SGP-I-amide. Reprinted from *Peptides*, **23**, Katayama *et al.*, Significance of a carboxyl-terminal amide moiety in the folding and biological activity of crustacean hyperglycemic hormone, 1537–1546, © 2002, with permission from Elsevier.

results indicate that the C-terminal amide moiety is significant in conferring hyperglycemic activity. This was the first report that describes direct evidence for the importance of C-terminal amidation in CHH. The C-terminal amidation caused a change in the C-terminal charge from a negatively charged C-terminus to a neutral one and, therefore, it is likely that the difference between rPej-SGP-I-OH and rPej-SGP-I-amide in the C-terminal charge may cause binding affinity to the CHH receptor.

The secondary structure of rPej-SGP-I-amide as estimated by circular dichroism (CD) spectrum differed from that of rPej-SGP-I-OH (**Table 2**), indicating that the C-terminal amide moiety of CHH is also significant in the folding of the molecule to its natural conformation. The difference in hyperglycemic activity between rPej-SGP-I-OH and rPej-SGP-I-amide may be partly due to differences in secondary structure and/or C-terminal charge. The arrangements of three disulfide bonds in rPej-SGP-I-OH and rPej-SGP-I-Gly, a precursor of rPej-SGP-I-amide, 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} (**Fig. 10**). This arrangement is identical to those of other CHH-family peptides determined thus far (Kegel *et al.* 1989; Huberman *et al.* 1993; Martin *et al.* 1993; Yasuda *et al.* 1994; Aguilar *et al.* 1995, 1996; Nagasawa *et al.* 1999; Kawakami *et al.* 2000; Katayama *et al.* 2001) and, therefore, it is likely that this arrangement of disulfide bonds is also the same as in the native Pej-SGP-I. These results indicate that the difference in conformation between rPej-SGP-I-OH and rPej-SGP-I-amide is caused not by the variation of disulfide bond arrangement, but by the conformational change of the C-terminal region.

Although the secondary structure of rPej-SGP-I-amide predicted by the CD spectrum was different from that of the native Pej-SGP-I (**Table 2**), the hyperglycemic activity of rPej-SGP-I-amide was comparable to that of the natural peptide (**Fig. 9**). Since the rPej-SGP-I-amide possessed four extra amino acid residues at the N-terminus of native Pej-SGP-I (**Fig. 10**), it is likely that the N-terminal extension of four residues causes a change in secondary structure but does not cause a significant change in biological activity. In our previous studies, recombinant Pej-SGP-IV expressed in *E. coli* exhibited an identical amino acid sequence to the native one except that the recombinant Pej-SGP-IV had an additional alanine residue at the N-terminus of the native peptide (Ohira *et al.* 1999; Katayama *et al.* 2001). It was reported that the CD spectrum of the recombinant Pej-SGP-IV was close to that of the native Pej-SGP-IV, and that the MIH activity of recombinant Pej-SGP-IV was as high as that of the native one in an *in vitro* bioassay (Ohira *et al.* 1999; Katayama *et al.* 2001). All of these results suggest that short extensions at the N-terminus of CHH-family peptides may cause slight changes in whole conformation, whereas they do not cause to affect biological activity.

All type II peptides, such as MIH, VIH and MOIH, unexceptionally have an additional glycine residue at position 12, which is lacked in all CHHs. Pej-SGP-IV carries five α-helices, and Gly^{12} is located in the short N-terminal helix (Katayama *et al.* 2003). The tertiary structure of Pej-SGP-III, one of the six CHHs in *M. japonicus*, was modeled based on the structure of Pej-SGP-IV. By comparison of tertiary and surface structures of Pej-SGP-III with those of Pej-SGP-IV, the N-terminal helix of Pej-SGP-IV was presumed to be one of the significant regions for conferring MIH activity (Katayama *et al.* 2003). In order to examine the significance of the absence of a glycine residue at position 12 in natural CHHs, we prepared a mutant rPej-SGP-I-amide (rPej-SGP-I-amide-Gly^{12}) with an additional glycine residue inserted between positions 11 and 12 (Katayama and Nagasawa 2004). rPej-SGP-I-amide-Gly^{12} had the same disulfide bond arrangement as rPej-SGP-I-amide, and exhibited a similar CD spectrum to that of rPej-SGP-I-amide, indicating that the two recombinant peptides possessed similar conformations.

The dose-response relationship of rPej-SGP-I-amide-Gly^{12} is shown in **Fig. 11**. Injection of 100 pmol of rPej-SGP-I-amide-Gly^{12} increased hemolymph glucose levels slightly, and its hyperglycemic activity reached a maximum at 300 pmol. It was demonstrated that the rPej-SGP-I-amide showed low hyperglycemic activity at 10 pmol and high activity at 100 pmol (**Fig. 9**). Therefore, the hyperglycemic activity of rPej-SGP-I-amide-Gly^{12} was lower than that of the rPej-SGP-I-amide by about one order of magnitude. Thus, these
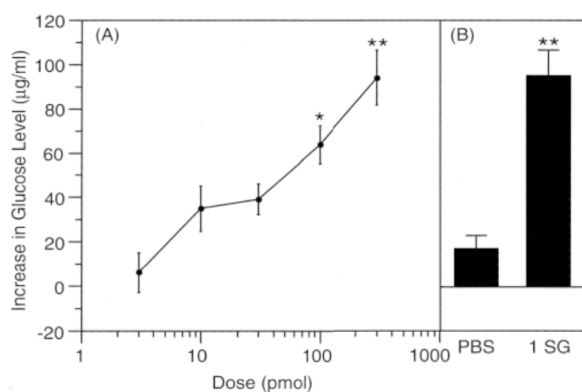
Fig. 11. (A) Dose-response relationship of Pej-SGP-I-amide-Gly[12]. (B) Negative control, injection of phosphate buffered saline (PBS); positive control, injection of sinus gland extract (one sinus gland equivalent) of *Marsupenaeus japonicus*. Results are expressed as the means ± SE (*n* = 7–14). Asterisks indicate values significantly different from the negative control. Symbols, (*) and (**), indicate *P* < 0.01 and *P* < 0.001, respectively, which were calculated with the program of Fisher's PLSD. Reprinted with permission from *Zool. Sci.*, **21**, Katayama *et al.*, Effect of a glycine residue insertion into crustacean hyperglycemic hormone on hormonal activity, 1121–1124, Fig. 2, © 2004, Zoological Society of Japan.

Fig. 12. Dose-response relationship of MIHs. Triangles, circles and squares show the native MIH, recombinant MIH and recombinant MIH-B, respectively. The results are expressed as the means ± SE (*n* = 3–8).

results suggest that the insertion of a glycine residue is one of the indices for structural and functional divergence of the CHH-family peptides.

2-2. MIH

2-2A. Bioassay and purification

In 1905, Zeleny demonstrated that eyestalk ablation shortened the molt interval of the crab *Uca pugilator* (Zeleny 1905). A molt-inhibiting factor, the so-called "MIH" was first isolated and characterized in the sinus gland located in the eyestalk of green shore crab, *C. maenas* (Webster and Keller 1986; Webster 1991). After that, MIHs were characterized from several decapod species (Webster 1991; Chung *et al.* 1996).

From the sinus gland extract of *M. japonicus*, a peptide exhibiting molt-inhibiting activity was purified by a single-step reversed-phase HPLC (Yang *et al.* 1996). Molt-inhibiting activity was measured using an *in vitro* culture of Y-organs of the crayfish, *Procambarus clarkii*, and an ecdysteroid radioimmunoassay (RIA) (Sonobe *et al.* 1991). In this work, a pair of Y-organs was dissected out of an eyestalk-ablated animal. One gland was incubated in a medium containing 1% bovine serum albmin (BSA), and the other was done in the same medium containing each peptide separated by the reversed-phase HPLC. After 6 h incubation at 25°C, ecdysteroid levels in the culture media were measured by RIA, and the inhibition ratio was calcu-

lated from the differences between the BSA and tested peptide treatments. By this method, one fraction on an HPLC chromatogram containing Pej-SGP-IV showed strong MIH activity, and therefore it was likely that this peptide might be an intrinsic MIH (**Table 1**).

The dose-response relationship of purified MIH for the inhibition of ecdysteroid secretion in *in vitro* crayfish Y-organ culture is shown in **Fig. 12** (Yang *et al.* 1996). The lowest inhibition (*ca.* 10%) was observed at a dosage level of 0.125 sinus gland equivalents in 500 μL (1.08 nM), and the inhibition was enhanced in a dose-dependent manner. Maximal inhibition (*ca.* 45%) was observed at a concentration of 1–5 sinus gland equivalents in 500 μL. This value is comparable to the effects of *C. maenas* MIH on the Y-organ culture of the same species (Webster and Keller 1986), suggesting that this purified peptide is functional in *M. japonicus*.

2-2B. Chemical structure and cDNA cloning

To determine the amino acid sequence of MIH, we firstly analyzed the N-terminal sequence using an automated protein sequencer, and the first 73 residues except for 8 residues at positions 7, 24, 27, 40, 44, 53, 69 and 71 were identified. Next, to determine these unidentified residues and the C-terminal sequence, MIH was digested with lysyl endopeptidase, and the digests were separated by reversed-phase HPLC. One of the digests was reduced by dithiothreitol and was *S*-carboxymethylated, yielding two peptides on reversed-phase HPLC. All of these fragments were analyzed on the automated protein sequencer, and the complete amino acid sequence of MIH was determined to consist of 77 amino acid residues (Yang *et al.* 1996). The C-terminal structure was confirmed by FAB mass spectral analysis of the C-terminal fragment after enzymatic digestion to be a free C-terminus. Six Cys residues exist at the positions conserved among CHH-family

```
                    TCCTTCGACCCCTACTCCTCGGCTTCCCTCCGTCCCTACGCTTTTACACTC  51
     ATG TAT CGG CTA GCA ATG AGG ACA TGG CTG GCG ATA GTG ATT GTA GTA  99
-28  Met Tyr Arg Leu Ala Met Arg Thr Trp Leu Ala Ile Val Ile Val Val

     GTT GGG ACA AGC CTC TTG TTC GAC ACC GCT TCG GCG AGC TTC ATA GAC  147
-12  Val Gly Thr Ser Leu Leu Phe Asp Thr Ala Ser Ala Ser Phe Ile Asp

     AAC ACC TGT AGG GGC GTG ATG GGT AAT CGT GAC ATT TAC AAG AAA GTA  195
  5  Asn Thr Cys Arg Gly Val Met Gly Asn Arg Asp Ile Tyr Lys Lys Val

     GTG CGA GTG TGT GAG GAT TGC ACT AAT ATC TTC CGA TTG CCA GGA CTG  243
 21  Val Arg Val Cys Glu Asp Cys Thr Asn Ile Phe Arg Leu Pro Gly Leu

     GAC GGC ATG TGC AGA AAT CGG TGC TTC TAC AAC GAA TGG TTC CTG ATT  291
 37  Asp Gly Met Cys Arg Asn Arg Cys Phe Tyr Asn Glu Trp Phe Leu Ile

     TGC TTG AAG GCC GCC AAC AGG GAG GAC GAG ATC GAG AAG TTC AGA GTG  339
 53  Cys Leu Lys Ala Ala Asn Arg Glu Asp Glu Ile Glu Lys Phe Arg Val

     TGG ATC AGC ATC CTG AAC GCC GGA CAG TGA GGCTGAATGTACCAGGACCCGAT  392
 69  Trp Ile Ser Ile Leu Asn Ala Gly Gln  *

     CCCCTCCCCTCCCCAGGCCTCGCTCATATAGACCAACATTCTTGACACTTGACCAACGATTAT  455
     CATCATTAATATTCTTTATGATTTTAAACTTATTTCGTCTCGTTATCTCTGTTAGTTTACTG  518
     GTACCAGTAAATGATATGCCAGTCTGAGCCTACAAGGCTGGCGGTCATGTAAAGTATGTATAC  581
     GAAAAATTACGTGTGAAATATTCCCACTAATCTGCTGTCATTTGCTGTAATGGACATCCCTCA  644
     TCCTCTTTATTATGTACTTTATTTAATATATTGATTTGAATCTGGAAATCAAACTAAAAGAAA  707
     TATCTATTTTGTATTTTGATCTCATATGTATATTCATCGAGCGTATCTTTTCATGTGATTTGA  770
     TTGCTTCCTCATTGTTATGATGGACACGAATAAAAGTTTCTCTG(A)n                814
```

Fig. 13. Nucleotide and deduced amino acid sequences of *Marsupenaeus japonicus* MIH (Pej-SGP-IV). The numbers at the left and right sides indicate the amino acid and nucleotide numbers, respectively. Signal sequence is indicated by an underline, and the putative poly-adenylation signal is shown by a shaded box. The sequence has been deposited in the DNA Data Bank of Japan (accession No. AB004652).

Fig. 14. Reversed-phase HPLC elution profile of the recombinant MIH after the refolding reaction. Column, TSKgel Octadecyl-4PW (4.6 × 150 mm); flow, 1.0 mL/min. A hatched peak indicates the correctly folded recombinant MIH. SDS/polyacrylamide gel electrophoresis of the hatched peak material is shown as in-set. Reprinted with permission from *Biosci. Biotechnol. Biochem.*, **63**, Ohira *et al.*, Expression of a recombinant molt-inhibiting hormone of the kuruma prawn *Penaeus japonicus* in *Escherichia coli*, 1576–1581, Fig. 4, © 1999, Japan Society for Bioscience, Biotechnology, and Agrochemistry.

peptides. *M. japonicus* MIH showed 44% sequence identity with *C. maenas* MIH (Webster 1991), and this value is higher than that between *M. japonicus* MIH and CHHs in the same species (~30%). *M. japonicus* MIH also showed high sequence identity with *P. clarkii* MIH (49%), which is consistent with the evidence that *M. japonicus* MIH possessed the molt-inhibiting activity in our bioassay system using *P. clarkii* Y-organs.

In order to clone a cDNA encoding MIH, total RNA was isolated from the eyestalk, and subjected to RT-PCR. Using a set of degenerate primers designed based on the amino acid sequence of MIH, a partial cDNA encoding MIH was obtained. This RT-PCR product was used as a probe to screen an eyestalk cDNA library, and 20 positive clones were identified. Sequence analysis of ten positive clones demonstrated that two of these clones encoded a MIH precursor. The nucleotide sequence of one of them and its deduced amino acid sequence are shown in **Fig. 13** (Ohira *et al*. 1997c). An ORF of 318 bp was found and this ORF encoded putative 105 residues protein. The sequence of the C-terminal 77 residues of the protein was identical to that of MIH, and N-terminal 28 residues were likely to constitute a signal peptide. Unlike CHH precursors, no CPRP-like sequence was found between the signal and mature peptides in the precursor of MIH.

The MIH gene was specifically expressed in the eyestalk, and no expression was observed in the hepatopancreas, abdominal muscle, brain, thoracic ganglia and abdominal ganglia by Northern blot analysis (Ohira *et al*. 1997c). We next investigated changes in levels of MIH transcripts in the eyestalk during the molt cycle. Unexpectedly, no decrease in the MIH expression level was observed at the early and late premolt stages, suggesting that the synthesis, modification and/or secretion of MIH was regulated post-transcriptionally.

Until now, MIHs have been isolated and characterized from many crustacean species (Nakatsuji *et al*. 2009). Different from the case of CHHs whose C-terminus was amidated without any exceptions, the C-terminal structures of MIHs were varied; *P. clarkii* MIH possesses shorter sequence (75 amino acid residues long) than that of *M. japonicus* and amidated terminus (Nagasawa *et al*. 1996), and *C. maenas* MIH possessed elongated C-terminus (78 residues long) (Webster 1991). These observations suggest that the C-terminal structure of MIH is not important for conferring MIH activity.

2-2C. Preparation of the recombinant MIH

In order to achieve further functional and structural analyses such as measurement of *in vivo* biological activity and tertiary structure determination, a large amount of MIH would be required. Therefore, we attempted to establish a bacterial expression system for recombinant MIH (rMIH) (Ohira *et al*. 1999).

The cDNA encoding *M. japonicus* MIH was inserted into an expression plasmid, and *E. coli* competent cells were transformed with it. Positive clones were selected on LB plates containing antibiotics, and the bacterial cells from a single colony were cultured in LB me-

dium. After 2 h induction of the recombinant protein expression by addition of isopropyl-β-D-thiogalactoside (IPTG), the bacterial cells were harvested. After cell breakage by sonication, the soluble and insoluble fractions were obtained. In SDS-polyacrylamide gel electrophoresis (PAGE) analysis of these fractions, a heavily stained band corresponding to rMIH was observed only in the insoluble fraction, indicating that rMIH was expressed not as a soluble protein but in the form of inclusion bodies.

After solubilization of the inclusion bodies by 6 M guanidine-HCl, the resultant solution was applied to reversed-phase HPLC, in which four major peaks were observed. All these peak materials gave the same value in the mass spectral analysis, which coincided well with the theoretical value of rMIH. In sequence analysis, these four materials gave the same sequence, which were identical with the MIH except that these four had an additional alanine residue at the N-terminus derived from the nucleotide sequence of the expression plasmid. These results indicated that these four materials were the disulfide isomers of rMIH. These four peak materials were combined, and subjected to the *in vitro* refolding reaction.

The refolding reaction was carried out in a redox buffer containing 1.3 M urea/1 mM glutathione (reduced form, GSH)/1 mM glutathione (oxidized form, GSSG) at 4°C for overnight. After the reaction, the four peak materials were converged into one peak on the reversed-phase HPLC chromatogram (**Fig. 14**). This peak material gave the protonated ion peak at *m/z* 9135 (calculated value, 9136 for $(M+H)^+$) in mass spectral analysis and also gave the N-terminal sequence ASFID- in sequence analysis, suggesting that this peak material is rMIH which is expected to have a proper folding and correct disulfide arrangement. The yield of this peak material was about 1 mg for 1 liter of culture.

The *in vitro* molt-inhibiting activity of rMIH was examined using a *P. clarkii* Y-organ culture assay. The lowest inhibition (*ca.* 12%) was observed at a concentration of 0.2–1.0 nM. The inhibition was enhanced with increased dosage, and reached its maximum (*ca.* 41%) at the concentration of 10–20 nM (**Fig. 12**). These values are almost comparable to those of the native MIH. Although rMIH possesses an extra Ala residue at the N-terminus, this extension did not cause a significant change in biological activity.

In order to confirm the folding of rMIH, the CD spectrum of rMIH was measured. A CD spectrum of the native MIH was also measured at the same time under the same conditions. The spectral analysis indicated that rMIH possesses α-helical structure, and the helix content was calculated to be 46% (Katayama *et al*. 2001). This spectral pattern was almost the same as that of the native peptide, suggesting that rMIH and the native MIH share the same conformation. It was also similar to the CD spectra of other CHH-family

peptides reported previously (Huberman *et al*. 1989; Gasparini *et al*. 1994).

Next, we examined the nature of disulfide pairing in rMIH. MIH was first digested by thermolysin, and the digests were separated by reversed-phase HPLC. The structure of one of the fragments was determined to consist of four peptide chains connected by three disulfide bonds based on mass spectral and amino acid sequence analyses. To obtain the peptide fragments containing only one disulfide bridge, this fragment was further digested sequentially with TPCK-treated trypsin and endoproteinase Asp-N, giving three double-chain fragments containing one disulfide bond. Each fragment was analyzed by MALDI-TOF mass spectrometer and a protein sequencer, establishing that the disulfide pairing occurred between Cys^7–Cys^{44}, Cys^{24}–Cys^{40} and Cys^{27}–Cys^{53}. The disulfide bond arrangement in the native MIH was also determined in the same manner, and the same results as those in the case of rMIH were obtained. This disulfide pairing is essentially the same as those in other CHH-family peptides whose disulfide bond arrangements were determined previously (Kegel *et al*. 1989; Huberman *et al*. 1993; Martin *et al*. 1993; Yasuda *et al*. 1994; Gasparini *et al*. 1994; Aguilar *et al*. 1995, 1996; Nagasawa *et al*. 1999; Kawakami *et al*. 2000), suggesting that the type II peptide share a similar conformation with those of type I CHH-family peptides.

All these results including biological assays, CD spectrum measurement and disulfide bond arrangement determination indicated that rMIH is not only biologically but also chemically identical with the native peptide except that an extra Ala residue was attached at the N-terminus in rMIH. Using this recombinant peptide, it became possible for us to conduct tertiary structure determination, *in vivo* bioassays, and screening of MIH receptor.

2-2D. Tertiary structure

We attempted to determine the tertiary structure of MIH using nuclear magnetic resonance (NMR) methods (Katayama *et al*. 2003). To measure NMR spectra, a sample solution of high concentration is required, but the solubility of rMIH in water or phosphate buffered saline (PBS) at neutral pH is poor. On the other hand, acidic solutions or aqueous organic solvents can dissolve rMIH at high concentrations above 1 mM. In particular, 30% aqueous acetonitrile dissolved rMIH in concentrations over 2 mM, and rMIH in this solution showed essentially the same CD spectrum as in PBS. Thus, we used 30% aqueous acetonitrile as the solvent for the NMR experiments.

All non-labile ^1H, ^{13}C and ^{15}N atoms of rMIH were assigned based on standard triple resonance NMR experiments. A total of 2152 restraints including 2109 distance and 43 torsion angle restraints was obtained from the NMR data and used in the structural calcula-
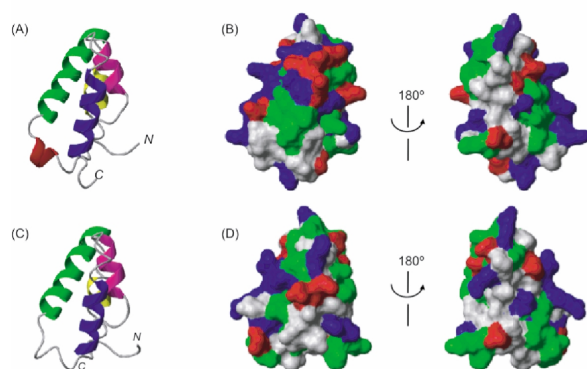
Fig. 15. (A) Ribbon model of the energy-minimized average structure of MIH. (B) Surface structure of MIH. The view angle of the left figure is the same as that of panel A, and that of the right figure is the reverse. The acidic, basic and hydrophobic residues are colored red, blue and green, respectively. (C) Ribbon model of the homology-modeled structure of Pej-SGP-III. (D) Surface structure of Pej-SGP-III. The view angle of the left figure is the same as that of panel C, and that of the right figure is the reverse. Reprinted with permission from *J. Biol. Chem.*, **278**, Katayama *et al.*, The solution structure of molt-inhibiting hormone from the Kuruma prawn *Marsupenaeus japonicus*, 9620–9623, Fig. 3, © 2003, The American Society for Biochemistry and Molecular Biology.

tions of rMIH. A set of 10 structures was selected out of 50 calculated structures based on agreement with the experimental data. In these selected structures, there were no violations greater than 0.4 Å and 2° in interproton distances and torsion angles, respectively. The pairwise RMS deviations among the 10 structures were 0.51 ± 0.09 Å for the backbone heavy atoms (N and C) and 1.24 ± 0.10 Å for all non-hydrogen atoms in the well-defined region (Asn[5]–Ala[75]). The conformation of the N-terminal (Ser[1]–Asp[4]) and C-terminal (Gly[76]–Gln[77]) regions was much less defined, because only a few restraints were obtained for these regions. In the Ramachandran ϕ–ψ plot for the well-defined region of the 10 selected structures, 99.8% of ϕ–ψ pairs were located in the favored and allowed regions.

Figure 15A shows the best-fit superposition of the backbone atoms of rMIH. The rMIH molecule consists of five α-helices (α1: Val[10]–Asn[13], α2: Ile[16]–Phe[31], α3: Pro[34]–Cys[40], α4: Glu[49]–Lys[55] and α5: Glu[62]–Ile[72]) and four loops between the α-helices (L1-2, L2-3, L3-4 and L4-5). The disulfide bonds linked N-terminal region and L3-4, α2 and α3, and α2 and α4. This α-helical structure is consistent with the results of CD spectral analysis.

The conformation of rMIH was stabilized by a number of hydrophobic interactions in the cluster of nine hydrophobic residues (Leu[16], Val[20], Val[23], Phe[46], Phe[50], Leu[54], Phe[66], Ile[70] and Leu[73]) and the three

disulfide bonds. These hydrophobic and cysteine residues are conserved in most CHH-family peptides, suggesting that the CHH-family peptides harbor similar foldings. We searched for proteins structurally similar to MIH using the DALI server (Holm and Sander 1993), but no similar structure was found, indicating that CHH-family peptides form a novel class of folds.

The tertiary structure of Pej-SGP-III (Yang *et al.* 1995), one of the *M. japonicus* CHHs which has 32% sequence identity and 66% sequence similarity to *M. japonicus* MIH, was homology-modeled using the SWISS-MODEL server (Guex and Peitsch 1997). **Figure 15C** shows the modeled structure of the CHH. This structure is very similar to the solution structure of rMIH except that CHH lacks α1. The solution structure of rMIH shows that the Gly residue insertion typical for type II CHH-family peptides is located at α1, and the absence of α1 in CHH may be due to the lack of the Gly residue. Since the C-terminal sequence of CHH is shorter than that of MIH by a few amino acid residues, the CHH lacked the C-terminal tail region present in MIH. The C-terminal tail of MIH was located sterically close to α1, and the surface properties including electrostatic potential and hydrophobicity of these peptides were different in this terminal region. Therefore, it was presumed that the region containing α1 and the C-terminal tail was important for molt-inhibiting activity.

As described in the previous section, it was demonstrated that the C-terminal amide moiety of CHH is significant in conferring biological activity. In addition, the C-terminally truncated CHH from the South African spiny lobster *J. lalandii*, which lacked six C-terminal amino acid residues, showed no hyperglycemic activity (Marco *et al.* 2000). These observations suggest that the functional site of CHH may be located at the C-terminal region. On the basis of our results from the NMR experiments, we proposed the hypothesis that the functionally important sites of CHH-family peptides may be located at the region containing α1 and the C-terminal region.

2-2E. Structure-activity relationship

In order to confirm the hypothesis that the functional site of MIH is located in the region encompassing the α1 and the C-terminus, various mutant molecules of MIH were prepared (Katayama *et al.* 2004). The amino acid residues to be mutated were selected according to the following two criteria: first, residues must reside in the region containing the α1 helix and C terminal tail; second, residues must have a side chain exposed at the molecular surface. We selected residues exposed to the molecular surface for mutation, partly because the surface residues would directly interact with the receptor, and partly because conformational changes due to the mutations would be minimized. Thus, seven mutants, N13A (a substitution mutant at position 13
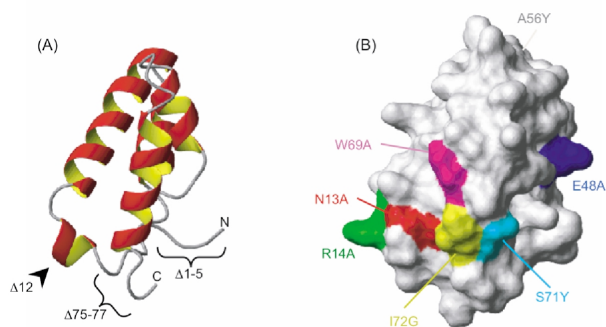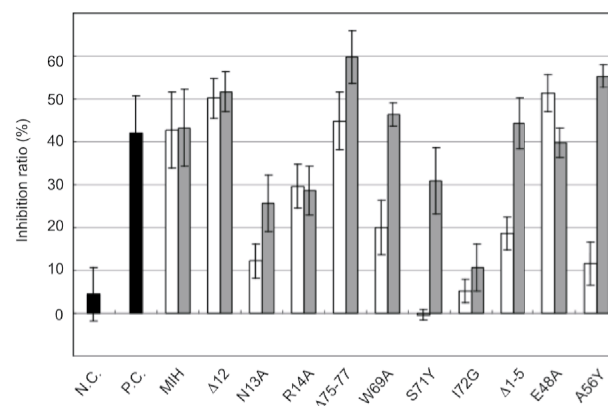
Fig. 16. (A) Ribbon model of MIH. Mutated sites of deletion mutants are indicated. (B) Surface structure of the MIH. Colors indicate the sites of various mutations. Reprinted with permission from *Biochemistry*, **43**, Katayama *et al.*, Structure-activity relationship of molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*, 9629–9635, Fig. 1, © 2004, American Chemical Society.

Fig. 17. Molt-inhibiting activity of the various mutants, controls and the recombinant MIH. N.C., no sample was added as the negative control; P.C., sinus gland extract of the crayfish *P. clarkii* (0.5 sinus gland equivalents) as the positive control. Mutants and the recombinant MIH were tested at 20 nM (open columns) and 200 nM (shaded columns). Results are expressed as the means ± SE (*n* = 3–9). Reprinted with permission from *Biochemistry*, **43**, Katayama *et al.*, Structure-activity relationship of molt-inhibiting hormone from the kuruma prawn *Marsupenaeus japonicus*, 9629–9635, Fig. 4, © 2004, American Chemical Society.

from Asn to Ala), R14A, W69A, S71Y, I72G, Δ12 (a deletion mutant of Gly^{12}), and Δ75–77 (a mutant truncated three residues at the C-terminus) were designed. In addition, E48A, A56Y and Δ1–5 (a mutant truncated five residues at the N-terminus) were also designed in order to examine the influence of the putative non-functional position (**Fig. 16**).

These mutant peptides were expressed in *E. coli* in essentially the same manner as for rMIH. The bacterial cells were then harvested, suspended in PBS, and sonicated. Soluble and insoluble fractions obtained by centrifugation were subjected to SDS-PAGE, and heavily stained bands corresponding to recombinant mutant peptide candidates were detected only in the insoluble fractions in all mutants. These results indicated that all of the mutants were expressed in *E. coli* cells as inclusion bodies. Therefore, refolding was considered to be necessary to obtain mutant peptides with the proper conformation.

All mutants were obtained as a single peak on reversed-phase HPLC after refolding. MALDI-TOF mass spectral and N-terminal amino acid sequence analyses of each peak material showed the expected single molecular ion peak and N-terminal sequence, respectively, except for Δ1–5. The N-terminal sequence analysis of Δ1–5 gave the sequence, M/T-T/X-X/R-R/G-G/V-, indicating that the N-terminal methionine residue had only been partially removed by methionine aminopeptidase in *E. coli*. Since these two peptides could not be separated by HPLC, we used a mixture of the two Δ1–5 peptides in the following experiments. The N-terminal sequence of the other mutants, ASFIDNTXRG-, was identical to the native peptide except for the N-terminal alanine residue that was derived from the expression vector.

In order to confirm that the mutant peptides possessed the same conformation as the native peptide, the CD spectra of the mutants in PBS were recorded at room temperature. Since the solubility of S71Y in PBS was not enough for measuring CD spectrum, we could not record the CD spectrum of this mutant under the same conditions as for the other peptides. As expected, the CD spectra of the other mutants in PBS showed a pattern typical for α-helical proteins and were quite similar to the spectra of the native and recombinant molecules of MIH. Therefore, we concluded that the mutant peptides retained proper conformation regardless of the mutations.

In the dose-response relationship of the recombinant MIH, inhibition of ecdysteroid synthesis *in vitro* was observed at a concentration of 2 nM with maximum inhibition observed at 20 nM or higher (**Fig. 12**). Consequently, we assessed the molt-inhibiting activity of each mutant peptide at two concentrations, 20 and 200 nM. The results of the bioassays are shown in **Fig. 17**. Three mutants, Δ12, Δ75–77 and E48A, exhibited high molt-inhibiting activity comparable to that of rMIH at 20 and 200 nM. R14A, W69A, A56Y, and Δ1–5 showed high activity at 200 nM, but significantly lower activity than rMIH at 20 nM, indicating that the mutations in these four peptides brought about a decrease in activity by approximately one order of magnitude. Neither N13A nor S71Y exhibited activity at 20 nM with low activity at 200 nM, indicating that N13A and S71Y are less active than rMIH by more than one order of magnitude. I72G showed no activity at the two con-

centrations tested, indicating that the activity of I72G is lower than that of rMIH by at least two orders of magnitude. The disulfide bond arrangement in I72G was determined to be the same as that of rMIH, indicating that the loss of activity in I72G was not due to the failure in the refolding reaction.

N13A and R14A, mutated at the α1 helix, exhibited lower activities than the recombinant MIH, although Δ12, expected to lack the helical structure of the α1 helix due to the shortened peptide chain, retained activity comparable to that of rMIH. These results suggest that the amino acid residues located at the C-terminal end of the α1 helix, but not the helical structure itself, are significant for conferring molt-inhibiting activity, which is not inconceivable because some type I CHH-family peptides show molt-inhibiting activity.

W69A, S71Y and I72G, mutated at the C-terminus, showed lower activities than rMIH, whereas Δ75–77, lacking the three C-terminal amino acid residues, was as active as rMIH. These results suggest that the residues located at the C-terminal side of the C-terminal helix (α5 helix), not including the terminal three residues, are significant for conferring molt-inhibiting activity. The C-termini of MIHs from many crustaceans show various structures; some peptides have a shortened, amidated C-terminus (Nagasawa *et al.* 1996; Marco *et al.* 2000), while others possess an extended C-terminal sequence (Webster 1991; Chung *et al.* 1996). Taken together, it is likely that a few residues at the C terminus of MIH are not recognized by its receptor.

Among the many mutants, a notable decrease in molt-inhibiting activity was observed in I72G. Since the disulfide bond arrangement in I72G was the same as that of the native MIH, it is likely that the Ile[72] mutation caused a decrease in molt-inhibiting activity by means of reduced binding ability to its receptor but not through a conformational change in the peptide. The bulky, hydrophobic side chain of Ile[72] may be essential for receptor binding. N13A and S71Y also exhibited very low activity. The changes in hydrophobicity in N13A and in the bulkiness and aromaticity in S71Y could affect their activities. All these results suggest that Ile[72] is the most significant residue of the residues mutated in this study for conferring biological activity, and that Asn[13] and Ser[71], both of which are sterically close to Ile[72], are also very important.

On the other hand, it was unexpected that the A56Y mutation within the putative non-functional region of the peptide would exhibit lower molt-inhibiting activity than that of the recombinant MIH by approximately one order of magnitude. Two reasons are considered for this contradiction. First, a functionally important site may also be located around the Ala[56] residue, and the decrease in activity may be because the aromatic ring of Tyr directly inhibits receptor-hormone binding; second, a slight conformational change which was not detectable in the CD spectrum may have been caused by this mutation, and as a result the binding affinity for the hormone receptor may have been reduced. In the NOESY NMR spectrum of the recombinant MIH, nuclear Overhauser effects (NOEs) were detected between the side chain of the Ala[56] residue and those of the Val[23] and the Ile[30] residues. Therefore, it is likely that Tyr[56] in the A56Y mutation could not fit into the space due to its bulky side chain, causing weaker hydrophobic interactions.

The hypothesis was likewise contradicted with the decreased molt-inhibiting activity of the Δ1–5 mutation. In the crayfish *P. clarkii*, two CHHs, CHH-I and CHH-II, have been isolated from the sinus gland (Yasuda *et al.* 1994). These two CHHs have identical amino acid sequences except that CHH-II has a D-Phe, which is L-Phe in CHH-I, at the third position from the N-terminus. These peptides have both CHH and MIH activities. CHH-I exhibits hyperglycemic activity as high as CHH-II, although it displays lower molt-inhibiting activity than CHH-II by approximately one order of magnitude. These results, combined with our observations, suggest that the considerable structural change in the N-terminal region of CHH-family peptides may not affect hyperglycemic activity, but does cause a reduction in molt-inhibiting activity.

Comparing the sequence of *M. japonicus* MIH with that of *P. clarkii* MIH, the positions mutated in this study are well conserved except for Ser[71], which is Gly[71] in *P. clarkii* MIH. Mutations at the conserved position among them tend to decrease MIH activity, strongly suggesting that the functional site of MIHs is located at the same position of the molecules each other. Since we used hetero-species bioassay system using *P. clarkii* Y-organs in this structure-activity relationship study, it is expected that the similar results might be obtained in structure activity relationship study of *P. clarkii* MIH.

2-2F. *In vivo* effects

In order to determine the function of MIH *in vivo*, an injection experiment using rMIH was performed (Okumura *et al.* 2005). When rMIH was injected into juvenile prawns once a day on the 2nd, 3rd, 4th, 5th, 6th and 7th days after ecdysis at the dose of 250 or 2500 ng/g-body weight (BW), the molt interval was elongated from 9.0 ± 0.4 d (means $\pm$ s.d.) in the control group to 9.2 ± 0.6 d and 9.5 ± 0.5 d in 250 and 2500 ng/g-BW groups, respectively. The molt interval differed significantly between the control and the 2500 ng/g-BW groups ($P < 0.05$). To examine the effects of MIH on the ecdysteroid level, 3 μg/g-BW of rMIH was injected to juvenile prawns at premolt stage D_1. The ecdysteroid level in the control group was increased 1.94 ± 1.09-fold, whereas the increment was signifi-
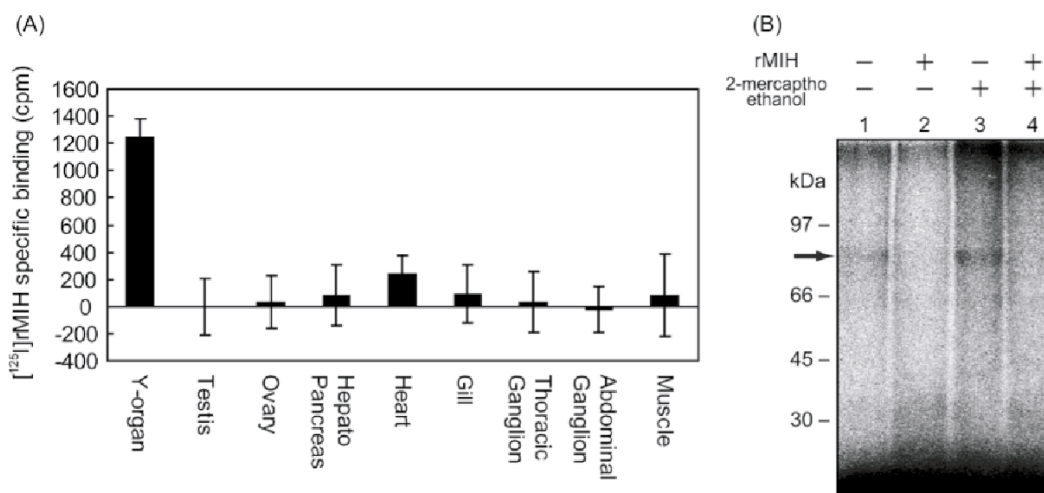
Fig. 18. (A) [125I]-Recombinant MIH binding to the membrane fractions of various tissues. The results are expressed as the means ± SD. (B) Chemical cross-linking of [125I]-recombinant MIH to the membrane fraction of the Y-organ. An Arrow indicates the band derived from the protein specifically binds to the recombinant MIH. Reprinted with permission of John Wiley & Sons, Inc. from *Ann. N.Y. Acad. Sci.*, **1040**, Asazuma *et al.*, Characterization of a molt-inhibiting hormone (MIH) receptor in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*, 215–218, Figs. 1 and 3, © 2005, Wiley-Liss, Inc., a Wiley Company.

cantly suppressed to 1.28 ± 0.39-fold in the test group ($P < 0.05$). To measure the biological activity of *M. japonicus* MIH *in vitro*, a hetero-species Y-organ incubation assay was used. In contrast, an *in vivo* assay was performed using the same species, and the results indicated that the MIH has an inhibitory effect on molting in this species as well.

MIH titers in the hemolymph are in the range of ~10^{-11-12} M in several species (Nakatsuji and Sonobe 2003; Chung and Webster 2005). In our injection experiments, although hormone titers may temporally increase to ~10^{-6} M, the effects on molt interval and hemolymph ecdysteroid level were not great. It has been reported that a 20-fold injection of 250 pmol/animal is required to inhibit molting in the case of the crayfish *P. clarkii* (Nakatsuji and Sonobe 2004). Considering the very short half-life of MIH in the hemolymph (Chung and Webster 2003; Nakatsuji and Sonobe 2004), it is likely that the low activity *in vivo* is due to the limited exposure of MIH to the Y-organ.

2-2G. Mode of action

In order to clarify the molecular mechanism of MIH signaling, we attempted to characterize the MIH receptor by performing a binding assay and chemical cross-linking experiments using radiolabeled rMIH (Asazuma *et al.* 2005).

The rMIH was radiolabeled with [125I], and the labeled peptide was purified by reversed-phase HPLC. To survey MIH receptor distribution in *M. japonicus*, the binding capacities of [125I]rMIH to the membrane fractions prepared from various tissues were compared.

Non-specific binding of each membrane fraction was measured in the presence of 1000-fold amounts of non-labeled rMIH. Specific binding was calculated by subtracting non-specific binding from total binding. Significant specific binding of [125I]rMIH was observed only in the membrane fraction of Y-organs, whereas no specific binding was observed in other tissues tested (**Fig. 18A**). These results strongly suggest that the MIH receptor exists specifically on the Y-organ.

To elucidate the kinetics of the MIH receptor, a saturable binding assay was carried out with various amounts of [125I]rMIH ranging from 0 to 500 pM. Non-specific binding of each measuring point was determined by the count in the presence of a 10,000-fold amount of non-labeled rMIH. Scatchard analysis revealed a single population of high-affinity MIH receptors on the Y-organ membrane ($K_d = 4.76 \times 10^{-10}$ M, $B_{max} = 5.51 \times 10^{-12}$ M).

To characterize the MIH receptor, the membrane fraction from the Y-organs was incubated with [125I]rMIH, and the mixture was cross-linked with a bifunctional cross-linking reagent, bis(sulfosuccinimidyl)suberate. The covalently cross-linked mixture was then analyzed by SDS-PAGE and autoradiography. A major band with the size of ~80 kDa was observed (**Fig. 18B**). On the other hand, this band disappeared when the binding reaction was carried out in the presence of an excess amount of non-labeled rMIH. These data strongly suggested that the band of 80 kDa was derived from the conjugate of [125I]rMIH with the MIH receptor. In addition, this band did not change the mobility on SDS-PAGE under reducing conditions, indicating that the
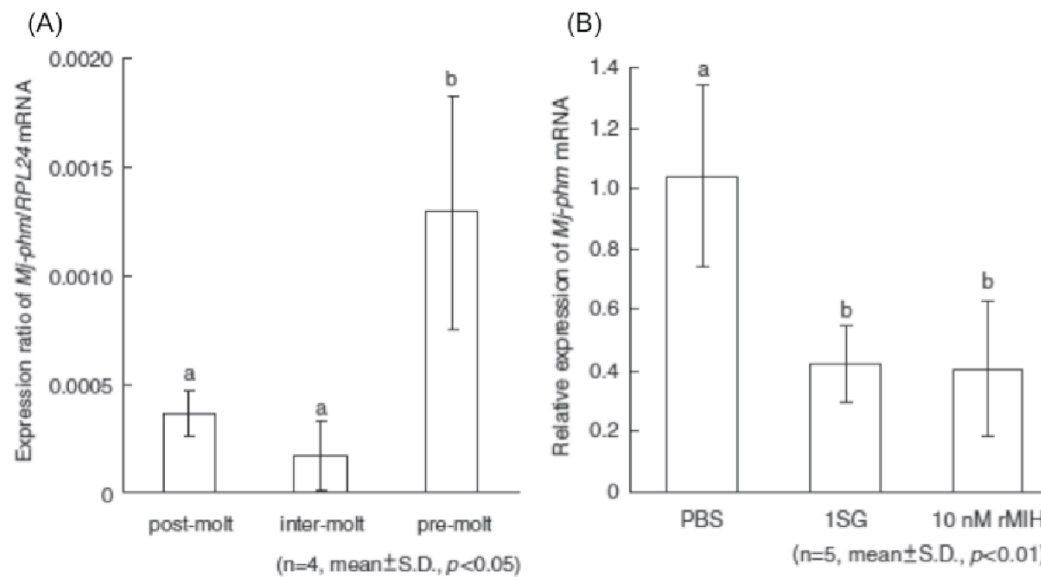
Fig. 19. (A) Expression levels of *phantom* mRNA through molting periods. Transcription levels of *phantom* and *RPL24* (a gene for 60s ribosomal protein) in the Y-organ of prawns at three different molting stages (inter-molt, pre-molt, post-molt) were analyzed using quantitative PCR. The expression levels were represented as ratios of *phantom* to *RPL24*. Different letters above the error bars indicate significant differences. Values are represented as the means ± SD ($n = 4$). (B) The expression of *phantom* in the Y-organ incubated *in vitro* with the recombinant MIH (rMIH). A pair of the Y-organs of the eyestalk-ablated prawn was incubated in a separated well with or without (control) phosphate buffered saline (PBS), sinus gland extract (SG), and 10 nM rMIH. The expression levels of *phantom* were determined by quantitative PCR. The relative expression level was represented as a percentage, control as 100%. Values are represented as the means ± SD ($n = 5$). Statistical analyses were performed using one-way ANOVA ($P < 0.05$). Reprinted with permission of John Wiley & Sons, Inc. from *Arch. Insect Biochem. Physiol.*, **72**, Asazuma *et al.*, Inhibitory effect of molt-inhibiting hormone on Phantom gene expression in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus*, 220–233, Figs. 5 and 6, © 2009, Wiley-Liss, Inc., a Wiley Company.

MIH receptor on the Y-organ has a molecular mass of approximately 70 kDa and possibly is not composed of subunits linked by disulfide bonds.

It has been demonstrated that the cyclic nucleotide analogs and phosphodiesterase inhibitors mimick MIH action in the suppression of Y-organ activity in several crustacean species (Saidi *et al.* 1994; Nakatsuji *et al.* 2006, 2009). In addition, MIH treatment increased cGMP levels in Y-organ *in vitro*. Given the fact that cGMP is involved in ecdysteroidogenesis in the Y-organ, a membrane guanylate cyclase (MGC) was suggested as a candidate MIH receptor in the Y-organ (Zheng *et al.* 2006). Indeed, cDNA for MGC was recently isolated from Y-organ of the blue crab *Callinectes sapidus* which exhibits a molecular weight of 137 kDa on a Western blot analysis, thus implicating that it may be a putative receptor for MIH (Zheng *et al.* 2008). In contrast, the putative receptor of MIH in the Y-organ that was chemically cross-linked with [125I]MIH revealed approximately 70 and 51 kDa proteins in *M. japonicus* and *C. sapidus*, respectively (Asazuma *et al.* 2005; Zmora *et al.* 2009). Overall, further studies are required for characterizing receptor(s) and elucidating the specific mode of actions of MIH on the inhibition of ecdysteroidogenesis in the Y-organ.

Recently, it was found that the Halloween genes encoding cytochrome P450 monooxygenases from insects were characterized as the steroidogenic enzymes catalyzing the final four steps of the ecdysteroid synthetic pathway from 5β-ketodiol to 20-hydroxyecdysone (Gilbert 2004; Truman 2005). To elucidate whether ecdysteroidogenesis in the Y-organs is regulated by MIH, the expression levels of an orthologue of a member of the Halloween genes, *phantom*, in the Y-organ of *M. japonicus* was analyzed (Asazuma *et al.* 2009). As expected, *phantom* gene expression significantly increased at the pre-molt stage, and decreased after ecdysis (**Fig. 19**). In addition, the exposure of the Y-organs to MIH significantly decreased *phantom* expression levels *in vitro*. These observations strongly suggest that the transcription of the *phantom* gene in the Y-organ is negatively regulated by MIH, although the question of how MIH regulates gene expression levels still remains unclear. In order to clarify the mechanism of MIH action on ecdysteroidogenesis, further studies are required.

2-2H. Other MIH-like peptides

Gu *et al.* cloned a fragment of a cDNA encoding an

```
                        AGGTGCAGCGGCACCTGCTAGATACTTCAAGCAAATCCGCAAGCA  45
ATG AGG GCA TGG CTG CTG TTA GCT ATC GTG GCG GCG GGA TCG TGC CTA  93
-23 Met Arg Ala Trp Leu Leu Leu Ala Ile Val Ala Ala Gly Ser Cys Leu

    TTT CCC GAA CTC TCT TCA GCC AAT ATC CTG TAC AGT AGC TGC CGA GGT  141
-7  Phe Pro Glu Leu Ser Ser Ala Asn Ile Leu Tyr Ser Ser Cys Arg Gly

    GTG ATG GGT AAC CGC GAC ATC TAC AGC AAG GTA GAG CGT GTT TGC AAC  189
10  Val Met Gly Asn Arg Asp Ile Tyr Ser Lys Val Glu Arg Val Cys Asn

    GAC TGT ACC AAT CTC TAC CGG TTG CCG CAA CTT GAC GGC TTG TGC AGA  237
26  Asp Cys Thr Asn Leu Tyr Arg Leu Pro Gln Leu Asp Gly Leu Cys Arg

    AAC CGA TGC TTC AAT AAT CAG TGG TTC CTG CTG TGC CTG AAT TCG GCG  285
42  Asn Arg Cys Phe Asn Ans Gln Trp Phe Leu Leu Cys Leu Asn Ser Ala

    AAG AGA GAA GAC GAA CTT AAT AAT TTC AGA CTA TGG ATC AGC ATC CTC  333
58  Lys Arg Glu Asp Glu Leu Asn Asn Phe Arg Leu Trp Ile Ser Ile Leu

    AAT GCC GGT CGC GAA TGG TAA ACTCTCCTCAAGGCATCCTACACATCCCCAGCCTC  389
74  Asn Ala Gly Arg Glu Trp  *

    CCGCTTCATCAAGGACACCTGACAGGACGTTGACACGGCCCCGAGGCTCCTTCCGCGCTATTA  452
    AAATTGATCATCGAAATAGACATCTCCGATCCTGATGCTTTGACGCCTGAGTTCCTTTGTGAA  515
    TACCTCTTGGTTTGGTATTTTTGTGCTCGAGGAAGACTAGGAGTGTTCGGAAACATCGTATTA  578
    AATCATGATGTTTTCCTCTTGTGTCTAACTTTATTGACGCATTACTCCTCATATATAAGTCCT  641
    GTTTTCCAAAGGTATTGGATGGAAGAGCAATTCCTTTAGATTCCTGCCGGTGTGATTTCCAGT  704
    ATTATAGATTTATTGCATGTTTATCATTCACAAATTAATTGGATTCCTAATTATCCGAGTTCA  767
    ATAAGTCTTAATTTTTCGGTCGAAATAAAATTGTCTT(A)n              804
```
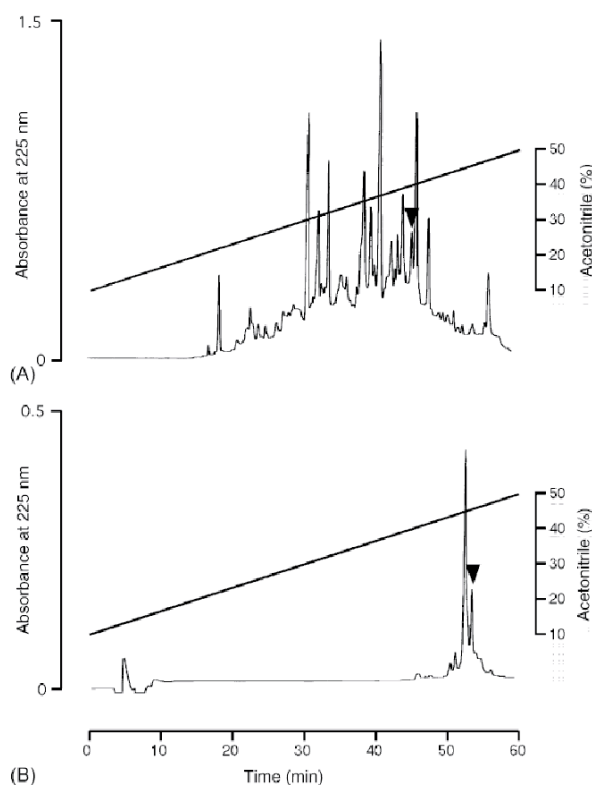
Fig. 20. Nucleotide and deduced amino acid sequences of *Marsupenaeus japonicus* MIH-B. The numbers at the left and right sides indicate the amino acid and nucleotide numbers, respectively. Signal sequence is indicated by underlines, and the poly-adenylation signal is shown by a shaded box. The sequence has been deposited in the DNA Data Bank of Japan (accession No. AB162448).

Fig. 21. Purification of the native MIH-B from the sinus gland. (A) Reverse-phase HPLC elution profile of the sinus gland extract. Separation was carried out on an Asahi-Pak ODP-50 column (4.6 × 250 mm) with a 60-min linear gradient of 10–50% acetonitrile containing 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.8 mL/min. (B) Reverse-phase HPLC elution profile of the MIH-B fraction from the first HPLC. Separation was performed in the same manner as in the first HPLC except for the use of 0.05% heptafluorobutyric acid (HFBA) instead of 0.05% TFA. Arrowheads indicate the MIH-B fractions. Reprinted from *Peptides*, **26**, Ohira *et al.*, Cloning and characterization of a molt-inhibiting hormone-like peptide from the prawn *Marsupenaeus japonicus*, 259–268, © 2005, with permission from Elsevier.

MIH-like peptide named MIH-B from *M. japonicus*, but the full-length sequence of this cDNA had not yet been reported (Gu *et al.* 2002). In order to determine its nucleotide and amino acid sequences, and to analyze its biological activity, we cloned it using RT-PCR methods (Ohira *et al.* 2005).

A cDNA fragment encoding MIH-B was amplified by RT-PCR using degenerate oligonucleotide primers designed based on the partial amino acid sequence of MIH-B. Subsequently, the 5′- and 3′-regions of the cDNA were amplified by 5′- and 3′-RACE, respectively, using specific primers. In order to confirm the nucleotide sequence of the cDNA, PCR was performed to amplify the cDNA fragments including the full-length ORF. The MIH-B precursor cDNA consisted of 804 bp comprising a 5′-untranslated region (45 bp), an ORF (309 bp), a stop codon (TAA), and a 3′-untranslated region (450 bp). The 3′-untranslated region contained a consensus polyadenylation signal (AATAAA) 9 bp upstream from the poly (A) tail. The ORF of MIH-B was conceptually translated into a putative 102-residue peptide consisting of a signal peptide (23 residues) and a putative hormone portion (**Fig. 20**).

The putative hormone consisted of 79 amino acid residues containing six Cys residues which were conserved in all CHH-family peptides. The amino acid sequence of MIH-B was identical to that of the partial sequence from positions 13 to 68 previously determined by Gu *et al*. This peptide had 73% identity with MIH, and was highly homologous to MIHs character-

ized from other crustacean species.

The MIH-B gene was strongly expressed in the thoracic ganglia and abdominal ganglia, and weakly in the eyestalk. No expression was observed in the other tissues tested by RT-PCR analysis. This expression pattern was very similar to that of Mee-MIH-B, which is another MIH molecule in the shrimp, *Metapenaeus ensis*, having high amino acid sequence identity (66%) with *M. japonicus* MIH-B (Gu *et al.* 2002). MIH-B shares some other characteristics with Mee-MIH-B: the precursor has a shorter signal peptide (23 amino acid residues) than the other MIH precursors; the mature peptide consists of 79 amino acid residues; and the recombinant peptide shows low molt-inhibiting activity as described below. Therefore, MIH-B seems to be closely related to Mee-MIH-B in terms of not only

molecular evolution but also biological function.

In order to confirm the existence of MIH-B as a peptide molecule in live prawns, we attempted to purify the peptide separately from thoracic ganglia and sinus glands. The extract of thoracic ganglia, however, included a great many proteins and peptides, making it impossible to purify MIH-B. On the other hand, MIH-B could be purified from the sinus glands by two steps of reversed-phase HPLC (**Fig. 21**). In MALDI-TOF mass spectral analysis, the purified peptide gave a protonated molecular ion peak at *m/z* 9399.0, which is coincident with the calculated mass number of MIH-B (9401.7 for (M+H)$^+$). Amino acid sequence analysis of this peptide yielded the N-terminal sequence, NILYSSXRGV-, which was identical to the putative N-terminal sequence of MIH-B. The yield of this peptide was 0.5 μg from 510 sinus glands.

In order to obtain a large quantity of MIH-B, recombinant MIH-B (rMIH-B) was expressed using an *E. coli* expression system essentially according to the methods that were used for rMIH. As in the case of rMIH, rMIH-B was expressed in the form of inclusion bodies, and it was, therefore, likely that a refolding reaction was necessary in order to obtain the peptide in its native conformation. Firstly, we attempted to conduct a refolding reaction for rMIH-B in the same redox buffer solution as was used in the refolding reaction for producing rMIH in its native conformation, but the entire product became insoluble. Next, we added acetonitrile to the reaction buffer to a final concentration of 10% in order to increase the solubility of rMIH-B. The reaction then yielded rMIH-B as a single peak on RP-HPLC. Five amino acid residues, ANILY-, could be determined by N-terminal amino acid sequence analysis of the purified peptide. This sequence was identical to that of the native peptide except for the first Ala residue, which arose from the nucleotide sequence of the expression vector. In the MALDI-TOF mass spectrum of the purified rMIH-B, a protonated molecular ion peak was observed at *m/z* 9471.5, which coincided well with the calculated value of 9472.7 for (M+H)$^+$. The yield of the refolded rMIH-B was about 2 mg from 1 liter of culture.

In order to examine the conformation of the rMIH-B, the arrangement of disulfide bonds in rMIH-B was analyzed. The recombinant MIH-B could be successively digested with trypsin and chymotrypsin, and the digests were separated by reversed-phase HPLC. Each fragment peptide was then analyzed by TOF-MS and amino acid sequence analyses. The results showed that rMIH-B had three disulfide bonds connected between Cys[7] and Cys[44], Cys[24] and Cys[40], and Cys[27] and Cys[53]. This arrangement was identical with that of other CHH-family peptides reported previously (Kegel *et al.* 1989; Huberman *et al.* 1993; Martin *et al.* 1993; Yasuda *et al.* 1994; Gasparini *et al.* 1994; Aguilar *et al.* 1995, 1996; Nagasawa *et al.* 1999; Kawakami *et al.* 2000).

The CD spectrum of rMIH-B showed a pattern similar to that of other CHH-family peptides reported previously (Huberman *et al.* 1989; Gasparini *et al.* 1994), which was typical for α-helix-rich proteins. All these results indicated that rMIH-B possessed the native conformation.
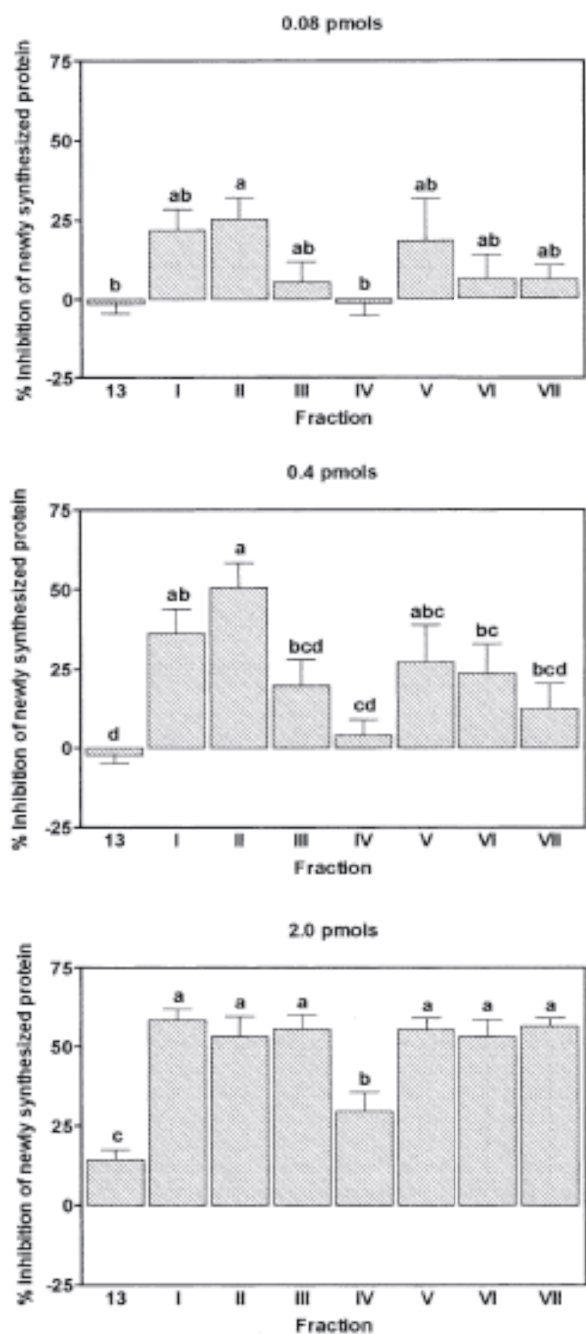
The dose-response relationship of rMIH-B in terms of molt-inhibiting activity was examined. rMIH-B weakly inhibited ecdysteroid synthesis by the Y-organ *in vitro* at a concentration of 2 nM (**Fig. 12**). The inhibition was enhanced with increasing peptide concentrations, and reached a maximum at a concentration of 100 nM (**Fig. 12**). These results indicated that rMIH-B was less active than rMIH by approximately one order of magnitude.

Next, we examined the hyperglycemic activity of rMIH-B. However, no elevation of hemolymph glucose level was observed with the injection of 10 or 100 pmol of rMIH-B, indicating that rMIH-B possessed no hyperglycemic activity.
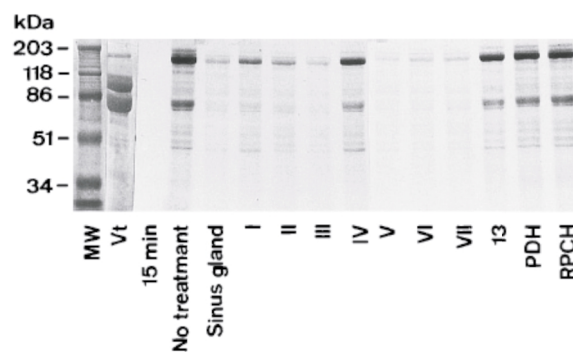
As described above, the residues, Ile[16], Val[20], Val[23], Phe[45], Phe[50], Leu[54], Phe[66], Ile[70] and Leu[73], in MIH form the hydrophobic core in the center of the molecule, and they are completely conserved in MIH-B. In addition, the surface residues around α1 helix (Asn[13] and Arg[14]) and the C-terminal part (Trp[69], Ser[71] and Ile[72]), which may contribute to direct binding to the MIH receptor, are also conserved. Thus, MIH-B seemed to share MIH-like molecular characteristics with MIH. The arrangement of disulfide bonds of rMIH-B was identical to that of other CHH-family peptides reported previously, and rMIH-B showed a CD spectrum similar to other CHH-family peptides, suggesting that MIH-B has a conformation similar to that of other CHH-family peptides. However, rMIH-B showed lower activity than MIH by approximately one order of magnitude. It is likely that the low molt-inhibiting activity of rMIH-B was due not to unnatural conformation, but to slight differences in the local structure of the receptor recognition site of MIH-B.

It is generally presumed that MIH exists in the eyestalks, because bilateral eyestalk ablation causes shortening of the molt interval (Zeleny 1905). Levels of *M. japonicus* MIH-B peptide in the eyestalk were very low (about 1 ng per sinus gland), and were about 40-fold lower than that of MIH. The lower activity and content of MIH-B suggest a much lower contribution to Y-organ regulation compared to MIH, and it might not act as an intrinsic MIH. Since rMIH-B showed no hyperglycemic activity *in vivo* and no vitellogenesis-inhibiting activity *in vitro* (Tsutsui *et al.* 2005a), the physiological function(s) of MIH-B is still unclear.

In an expressed sequence tag experiment using a cDNA library from *M. japonicus* eyestalk, it was demonstrated that another MIH-like gene designated "putative" MIH-C was expressed in the eyestalk (Yamano and Unuma 2006). The deduced amino acid sequence

Fig. 23. Analysis of the effect of *Marsupenaeus japonicus* crude sinus gland extract (0.1 sinus gland equivalent), CHH-family peptides (Fractions I–VII) and non-CHH-family peptides (Fraction 13, PDH, and RPCH) at the highest concentration (2 pmol/mL) on *Penaeus semisulcatus* ovarian protein synthesis. Labeled proteins (30 μg) from ovarian fragment incubated *in vitro* with different peptide were separated by SDS-PAGE (10% acrylamide). Reprinted from *Gen. Comp. Endocrinol.*, **110**, Khayat *et al*., Hyperglycaemic hormones inhibit protein and mRNA synthesis in *in vitro*-incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus*, 307–318, © 1998, with permission from Elsevier.

Fig. 22. Influence of *Marsupenaeus japonicus* CHH-family peptides (Fractions I–VII) and a non-CHH-family peptide (Fraction 13) on *Penaeus semisulcatus* ovarian protein synthesis. The values represent the percentage of inhibition in fragments removed from six different ovaries (means ± SE; $n = 6$). Statistical analysis of the results indicates a significant difference ($P < 0.05$) in the highest concentration (2 pmol/mL) between the inhibition values of Fraction 13 and Fraction IV and the other CHH-family peptides. Means with the same letter are not significantly different ($P > 0.05$) by analysis of the variance. Reprinted from *Gen. Comp. Endocrinol.*, **110**, Khayat *et al*., Hyperglycaemic hormones inhibit protein and mRNA synthesis in *in vitro*-incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus*, 307–318, © 1998, with permission from Elsevier.

of MIH-C shared 73% and 84% identities with those of MIH and MIH-B, respectively, and it was structurally more related to MIH-B than to MIH. The function of putative MIH-C peptide has not yet been characterized. In the *M. japonicus* genome, it is presumed that more MIH-like genes exist; along these lines, further experiments are required for understanding the molecular and functional diversity of MIHs.

2-3. VIH

2-3A. Bioassay

An *in vivo* bioassay is the most appropriate means of accurately examining VIH action; however, it is difficult to conduct such assays throughout the isolation process, because large quantities of natural hormone and lengthy procedures are required. Therefore, we have established an *in vitro* bioassay for vitellogenesis-inhibiting activity (Khayat *et al.* 1998; Tsutsui *et al.* 2005a), as described below.

In *M. japonicus*, the eight CHH-family peptides, the six CHHs (Pej-SGP-I, II, III, V, VI, VII) belonging to type I and the two MIHs (Pej-SGP-IV and MIH-B) belonging to type II, were identified from the sinus glands (see Subsections 2-1B and 2-2H). Since the two VIHs from *H. americanus* and *A. vulgare* are members of the CHH-family, the eight peptides were considered to be candidates for VIH in *M. japonicus*. Therefore, vitellogenesis-inhibitory activities of the seven of eight CHH-family peptides (Pej-SGP-I to -VII) were exam-
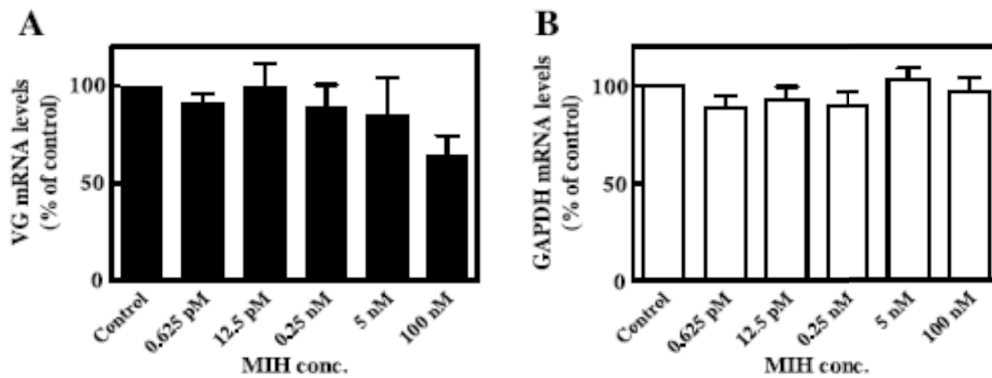
Fig. 24. The effects of MIH (Pej-SGP-IV) on vitellogenin (VG) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Relative VG mRNA levels are expressed as percentage changes relative to control values in (A). The relative GAPDH mRNA levels from the same samples are shown in (B). The results are presented as the means ± SE of five prawns each for 100, 5, and 0.25 nM, and four prawns each for 12.5 and 0.625 pM. Reprinted from *Gen. Comp. Endocrinol.*, **144**, Tsutsui *et al.*, The effects of crustacean hyperglycemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, *Marsupenaeus japonicus*, 232–239, © 2005, with permission from Elsevier.

ined using an *in vitro* incubation system with ovaries isolated from the green tiger prawn *P. semisulcatus* (Khayat *et al.* 1998). In this system, the levels of *de novo* protein synthesis in the incubated ovarian fragments were used as an index of vitellogenesis. As a result, MIH (Pej-SGP-IV) showed quite weak activity (**Fig. 22**), although this MIH is structurally more related to the known VIHs, both of which are classified into type II, than to CHHs (see Subsection 2-1B). In contrast, the six CHHs belonging to type I inhibited protein synthesis in the incubated ovarian fragments (**Fig. 22**), implying that these six CHHs are involved in the regulation of vitellogenesis. However, their effects were observed on overall protein synthesis in the ovary, and were not specific for vitellogenesis-related proteins (**Fig. 23**).

Vitellogenin (VG) is the dominant vitellogenesis-related protein. In crustaceans, VG is synthesized in the hepatopancreas and/or ovary (Chen *et al.* 1999; Yang *et al.* 2000; Tseng *et al.* 2001; Abdu *et al.* 2002; Avarre *et al.* 2003; Tsang *et al.* 2003; Tsutsui *et al.* 2004; Mak *et al.* 2005; Yang *et al.* 2005; Raviv *et al.* 2006), undergoes processing (Okuno *et al.* 2002a; Avarre *et al.* 2003), and is incorporated into developing oocytes as mature vitellin likely through receptor-mediated endocytosis (Warrier and Subramoniam 2002). In *M. japonicus*, it has been confirmed that both VG mRNA levels in the hepatopancreas and ovary, and VG protein levels in the hemolymph increase significantly during vitellogenesis (Jasmani *et al.* 2000; Tsutsui *et al.* 2000, 2005b). On the basis of this information, an *in vitro* bioassay for vitellogenesis-inhibiting activity using *M. japonicus* ovarian fragments has been established, in which VG mRNA levels in incubated ovarian fragments are used as an in-

dex of vitellogenesis (Tsutsui *et al.* 2005a). Using this assay system, it has been shown that *M. japonicus* MIH, type II peptide, had no significant effect on VG mRNA levels in the incubated ovary (**Fig. 24**), whereas Pej-SGP-III, one of the six CHHs belonging to type I, strongly reduced VG mRNA levels, but did not affect glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels (**Fig. 25**). Recently, the remaining five CHHs belonging to type I, in addition to Pej-SGP-III, were subjected to the same bioassay, and then all of them showed obvious VIH activities (Tsutsui *et al.* 2013). These results indicate that the six CHHs are probably involved not only in the control of carbohydrate metabolism but also in the regulation of vitellogenesis in *M. japonicus*. Recently, a recombinant *H. americanus* VIH was shown to have weak but significant vitellogenesis-inhibiting activity using the same assay (Ohira *et al.* 2006), as described in the next section. Moreover, VIH activities of CHH-family peptides from the whiteleg shrimp *Litopenaeus vannamei* were successfully examined by the same methods (Tsutsui *et al.* 2007). These suggest that the system is a very useful tool for the investigation of VIH not only in *M. japonicus* but also in other decapod crustaceans.

2-3B. Structure-activity relationship

H. americanus VIH was originally characterized as a type II peptide having a free C-terminus, based on the results of amino acid sequencing and mass spectrometry (Soyez *et al.* 1991). However, cDNA cloning revealed that the VIH precursor had an amidating signal sequence at the C-terminal end (De Kleijn *et al.* 1994b), and therefore it was later determined that mature *H. americanus* VIH possesses a C-terminal amide moiety (Soyez 1997). Another VIH
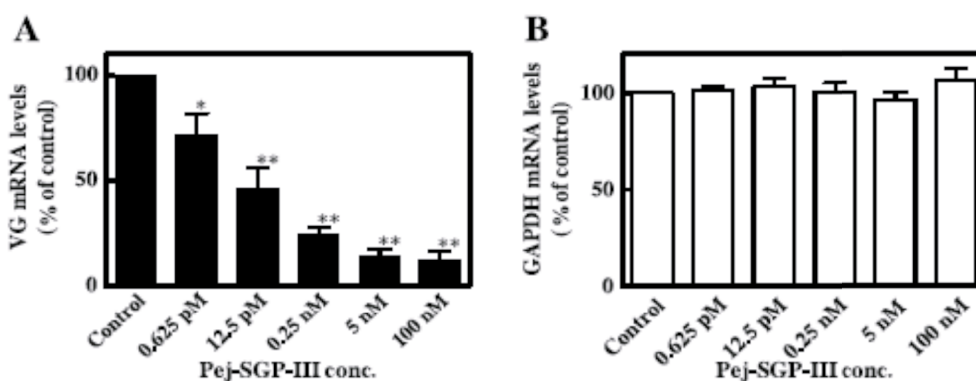
Fig. 25. The effects of Pej-SGP-III on vitellogenin (VG) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Relative VG mRNA levels are expressed as percentage changes relative to control values (means ± SE; $n = 5$). (A) Relative VG mRNA levels; (B) relative GAPDH mRNA levels from the same samples. Data were examined for significance: $*P < 0.05$; $**P < 0.01$. Reprinted from *Gen. Comp. Endocrinol.*, **144**, Tsutsui et al., The effects of crustacean hyperglycemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, *Marsupenaeus japonicus*, 232–239, © 2005, with permission from Elsevier.
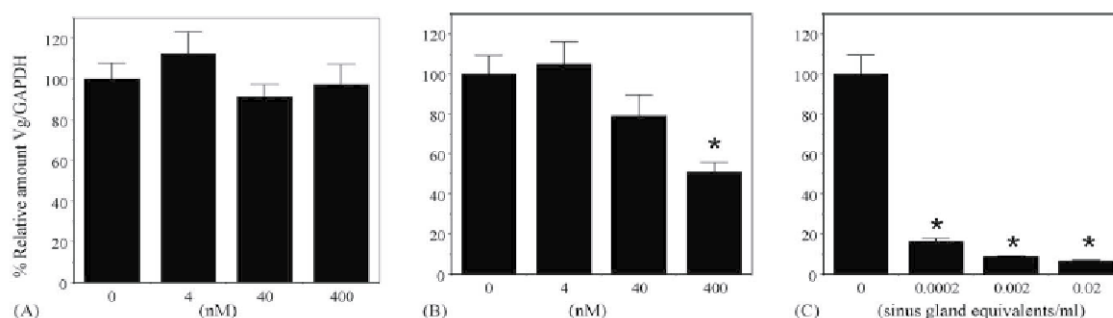
Fig. 26. The effects of rHoa-VIH-OH (A), rHoa-VIH-amide (B), and sinus gland extracts (C) on vitellogenin (VG) mRNA levels in the ovary of *Marsupenaeus japonicus*. Relative VG mRNA levels (VG/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA) were expressed as a percentage change (means ± SE; $n = 5$) relative to each non-treatment group (control value = 100). Asterisks indicate values significantly different from each non-treatment group, $P < 0.05$ (*). Reprinted from *Peptides*, **27**, Ohira et al., Production and characterization of recombinant vitellogenesis-inhibiting hormone from the American lobster *Homarus americanus*, 1251–1258, © 2006, with permission from Elsevier.

from *A. vulgare*, which also belongs to the type II group, has a free C-terminus in contrast to the case of *H. americanus* VIH (Gréve et al. 1999). In this way, whether the C-terminal amidation in VIH is necessary to confer biological activity remains a point of clarification.

In order to elucidate the above point, we produced two types of recombinant *H. americanus* VIHs (rHoa-VIHs), rHoa-VIH-amide and rHoa-VIH-OH, and assessed their vitellogenesis-inhibiting activities using a *M. japonicus* ovarian fragment incubation system (Ohira et al. 2006). The rHoa-VIH-amide significantly reduced vitellogenin mRNA levels in the ovary, while rHoa-VIH-OH had no effect in this assay system (**Fig. 26**). The arrangements of the three disulfide bonds in rHoa-VIH-Gly, a precursor of rHoa-VIH-amide, and rHoa-VIH-OH were identical to that of other CHH-

family peptides determined thus far (Kegel et al. 1989; Huberman et al. 1993; Martin et al. 1993; Yasuda et al. 1994; Aguilar et al. 1995, 1996; Nagasawa et al. 1999; Kawakami et al. 2000; Katayama et al. 2001), and therefore, it was likely that this arrangement of disulfide bonds is also the same as that in the native *H. americanus* VIH. These results indicated that the difference in conformation between rHoa-VIH-OH and rHoa-VIH-amide is caused not by the variation of disulfide bond arrangement, but by the presence or absence of the C-terminal amide. In *M. japonicus*, the C-terminal amide moiety of CHH was important for the folding of the molecule to its natural conformation and was consequently necessary for conferring hyperglycemic activity (see Subsection 2-1F). Therefore, the C-terminal amidation of *H. americanus* VIH may also have a role in maintaining its conformation.

rHoa-VIH-amide showed definite biological activity in the *in vitro* bioassay using *M. japonicus* ovary, although its efficacy was much lower than that of sinus gland extracts from *M. japonicus* (**Fig. 26**). As mentioned above, Pej-SGP-III, one of the six CHHs in *M. japonicus* belonging to the type I group with a C-terminal amide, reduced vitellogenin mRNA levels to 15% of that of the non-treatment control at only 5 nM using the same assay system (**Fig. 25**). On the other hand, Pej-SGP-IV, MIH in *M. japonicus* belonging to type II with a free C-terminus, was not effective even at 100 nM (**Fig. 24**). In another case, two C-terminally shortened CHHs with a free C-terminus from *J. lalandii*, both of which are type I peptides, showed much weaker VIH activities than those of the two CHHs having a C-terminal amide using the *P. semisulcatus* ovarian incubation system (Marco *et al.* 2002). These results suggest that the molecular characteristics required for vitellogenesis-inhibiting activity in penaeid shrimp species reside in the type I peptide with an amidated C-terminus. This postulation seems to be consistent with our present results, because rHoa-VIH-OH belonging to type II had no activity and even rHoa-VIH-amide showed very low vitellogenesis-inhibiting activity. The reason for the low activity of rHoa-VIH-amide might be due to the use of a heterologous bioassay or the presence of another VIH that is more potent. As the biological activity of native *H. americanus* VIH has also been characterized by *in vivo* studies in a different shrimp species, *Palaemonetes varians* (Soyez *et al.* 1987), it is necessary to establish a bioassay system using *H. americanus* for further examination of rHoa-VIH-amide activity.

3. Eyestalk peptide hormones II: Chromatophorotropic peptides

In 1972, Fernlund and Josefsson reported the isolation and complete structure of crustacean red pigment concentrating hormone (RPCH) from the pink shrimp *P. borealis* (Fernlund and Josefsson 1972). This shrimp RPCH was an octapeptide (pELNFSPGWamide) and showed activity of chromatophoral pigment concentration and dark-adaptation eye pigment migration. Thereafter, the primary structures of RPCH from several crustacean species were determined, revealing that all structures were identical (Gaus *et al.* 1990; Keller 1992), suggesting that only one RPCH molecule exists in crustaceans.

In 1976, crustacean pigment dispersing hormone (PDH) was purified from the sinus glands of *P. borealis* and its primary structure was determined (Fernlund 1976). This octadecapeptide with an amidated C-terminus had an effect on the light-adapting movement of pigment in the compound eye. Later, this molecule was also found to cause pigment dispersion in epidermal chromatophores and therefore has been referred
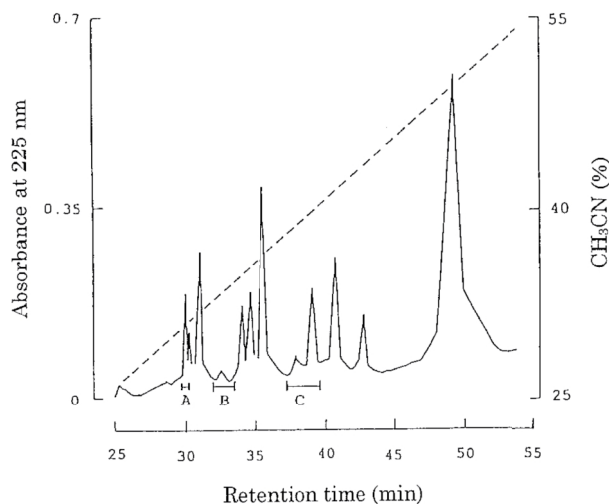
Fig. 27. Reversed-phase HPLC elution profile of extracts from 300 sinus glands of the kuruma prawn *Marsupenaeus japonicus*. Column: Asahi-Pak ODP-50 (4.6 × 150 mm). Solvent: linear gradient of 0–65% acetonitrile in 0.05% TFA for 65 min. Flow rate: 1 mL/min. Detection: absorbance at 225 nm. Temperature: 40°C. The concentration of acetonitrile is indicated by the dashed line. Fraction A showed pigment concentrating activity. Fractions B and C showed pigment dispersing activity. Reprinted from *Gen. Comp. Endocrinol.*, **114**, Yang *et al.*, Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus*, 415–424, © 1999, with permission from Elsevier.

to as α-PDH (Rao and Riehm 1993). Thereafter, PDHs from several crustacean species were isolated and sequenced. Interestingly, all of them were found to differ from α-PDH of *P. borealis* at more than 6 positions out of 18 amino acid residues and were thus designated as β-PDH (Rao and Riehm 1993). The most significant difference between α- and β-PDHs is at position 3, where Gly is found in α-PDH and Glu in β-PDHs, causing β-PDH to be more potent than α-PDH. Up until now, α-PDH has only been found in species of the *Pandalus* genus.

In order to obtain more information on the structure-activity relationship of chromatophorotropic peptides, we characterized three chromatophore-regulating peptides of the kuruma prawn *M. japonicus* (Yang *et al.* 1999), as described below.

3-1. RPCH

3-1A. Purification and structure determination

Peptides were extracted from the sinus glands and fractionated by reversed-phase HPLC. RPCH activity was observed at fraction A (**Fig. 27**). Fraction A was then applied to a second reversed-phase HPLC using a different column, and the peptide showing RPCH activity could be purified (Pej-RPCH). As expected, the N-terminus was confirmed to be blocked based on N-
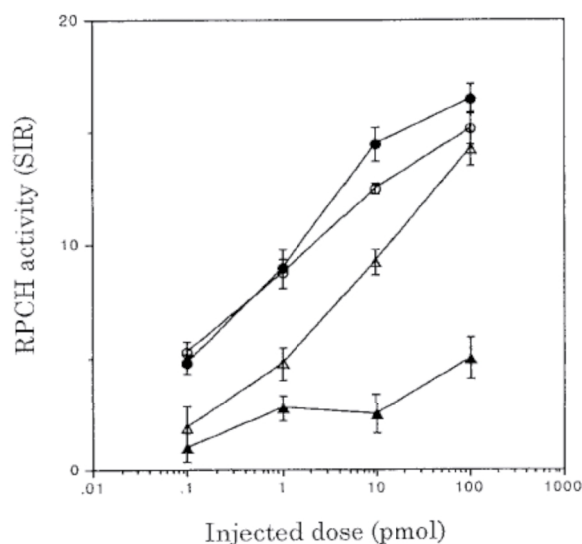
Fig. 28. Dose-response relationship of Pej-RPCH to four types of chromatophores. ○, Erythophores; ●, xanthophores; △, melanophores; ▲, leukophores. The activities are expressed as standard integrated response (SIR), and are presented as the means ± SE. Each point is derived from responses elicited by five prawns. Reprinted from *Gen. Comp. Endocrinol.*, **114**, Yang *et al.*, Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus*, 415–424, © 1999, with permission from Elsevier.

terminal amino acid sequence analysis. Digestion of Pej-RPCH with Pfu pyroglutamate aminopeptidase afforded a corresponding de-blocked peptide. Amino acid sequence analysis of the de-blocked peptide unambiguously revealed the sequence of residues 2–8, LNFSPGW. To determine whether the C-terminal residue was amidated, the intact Pej-RPCH was subjected to high resolution FAB mass spectral analysis. A protonated molecular ion peak was observed at *m/z* 930.44, which coincided well with the calculated value of the amidated peptide (930.44 for $(M+H)^+$). Thus, the amino acid sequence of Pej-RPCH was determined as pELNFSPGW-NH$_2$.

Pej-RPCH was identical with RPCH previously determined in several crustacean species. This is in contrast to the insect AKHs, other members of the AKH/RPCH family, in which both amino acid sequences and peptide lengths vary among species. This may be due to a strict ligand-receptor interaction in the case of RPCH, which does not allow any changes in residues and peptide lengths.

3-1B. Biological activity

To examine RPCH activity, the kuruma prawns whose eyestalks were ablated one day prior to the experiment were used for an *in vivo* assay, as all types of the chromatophores were highly dispersed at this time.
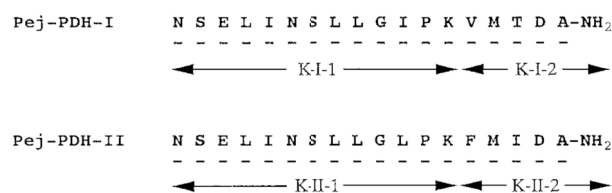
Fig. 29. Summary of sequence analyses of Pej-PDH-I and Pej-PDH-II. (- - -), residues identified by amino-terminal sequence analysis. Fragments (K-I-1, K-I-2, K-II-1, and K-II-2) were generated by lysyl endopeptidase digestion of Pej-PDH-I and -II, respectively. Reprinted from *Gen. Comp. Endocrinol.*, **114**, Yang *et al.*, Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus*, 415–424, © 1999, with permission from Elsevier.

After the injection of peptide, the extent of concentration or dispersion was estimated using the index of Hogben and Slome (1931); maximal pigment concentration was recorded as score 1, and maximal dispersion as score 5; scores 2, 3, and 4 represented intermediate states. The observed activity was expressed in terms of Standard Integrated Response (SIR) values, as defined previously (Fingerman *et al.* 1967).

The dose-response relationships of Pej-RPCH to the four types of chromatophores (erythrophores, xanthophores, leucophores and melanophores) were shown in **Fig. 28**. The sensitivities of the four types of chromatophores to Pej-RPCH were found to be considerably different. The most sensitive response was observed in erythrophores and xanthophores, and the least sensitive response was seen in leucophores. Melanophores showed a medium-level response.

3-2.　PDH

3-2A. Purification and structure determination

Pigment dispersing activity was found in fractions B and C (**Fig. 27**). These fractions were further purified separately on reversed-phase HPLC, and consequently two peptides, Pej-PDH-I and -II, were obtained from fractions B and C, respectively. Eighteen residues each were identified by the N-terminal amino acid sequence analyses of intact Pej-PDH-I and -II (**Fig. 29**). To confirm the amino acid sequences and to determine the C-terminal residues, intact Pej-PDH-I and -II were separately digested with lysyl endopeptidase and the digests were separated by reversed-phase HPLC. Amino acid sequences of the fragment peptides, K-I-1 and -2, and K-II-1 and -2, were confirmed on an automated protein sequencer (**Fig. 29**). High-resolution FAB mass spectra of both C-terminal fragments (K-I-2 and K-II-2) were analyzed to determine their C-terminal structures. Protonated molecular ion peaks for K-I-2 and K-II-2 were observed at *m/z* 535.25 and *m/z*
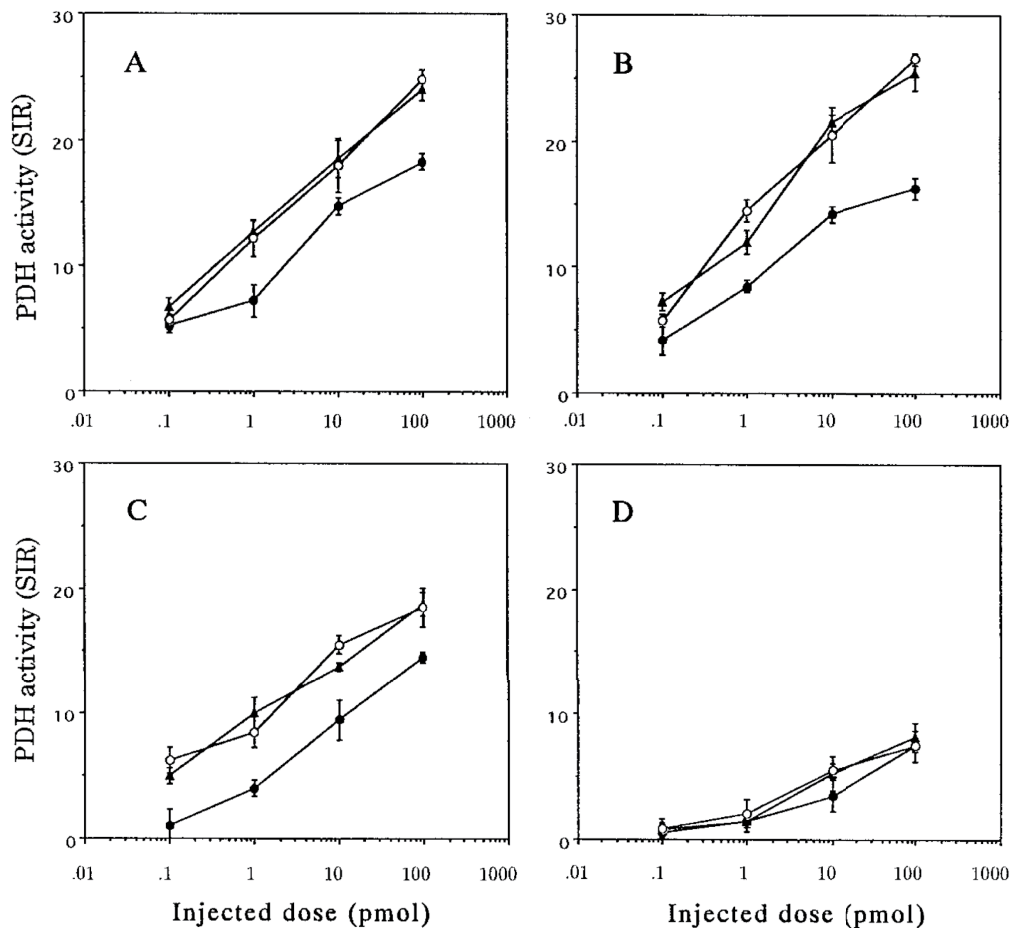
Fig. 30. Dose-response relationships of Pej-PDH-I and -II to four types of chromatophores. (A) Erythophores; (B) xanthophores; (C) melanophores; (D) leukophores. Activities are expressed as standard integrated response (SIR), elicited by Pej-PDH-I (●), Pej-PDH-II (▲), and synthetic [Ile^{16}]-Pej-PDH-I (○). The results are presented as the means ± SE. Each point is derived from responses elicited by five prawns. Reprinted from *Gen. Comp. Endocrinol.*, **114**, Yang *et al.*, Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus*, 415–424, © 1999, with permission from Elsevier.

595.30, respectively, and these values coincided well with the calculated values for the amidation forms of K-I-2 and K-II-2 (535.25 and 595.28, respectively), establishing the amidated C-terminus of both K-I-2 and K-II-2. Thus, the complete amino acid sequences of Pej-PDH-I and -II were determined (**Fig. 29**).

The two PDH showed considerable sequence similarity to other PDHs characterized from several crustacean species previously. Pej-PDH-I was most closely related to the PDHs of *Penaeus aztecus* (Phillips *et al.* 1988) and *Pandalus jordani* (Rao *et al.* 1989) with one residue substitution, while Pej-PDH-II was most similar to the PDHs of *C. sapidus* (Klein *et al.* 1994) and *L. vannamei* (PDH-II) (Desmoucelles-Carette *et al.* 1996) with replacement of two residues. In Pej-PDH-II, this was the first occasion in which Phe and Ile were found at positions 14 and 16 of a crustacean PDH; up until now, only Val or Leu and Asn or Thr had been found at these positions in all of the known PDHs.

3-2B. Biological activity

Prawns whose eyestalks were ablated seven days previously were injected with peptide samples to test PDH activity, as all types of the chromatophores were most concentrated. The dose-response relationships of the two PDHs to the four types of chromatophores are shown in **Fig. 30**. Pej-PDH-II was 5-, 7-, and 10-fold more potent than Pej-PDH-I for erythrophores, xanthophores, and melanophores, respectively, on the basis of the quantity of peptides required to elicit an SIR value of 10. Like RPCH, the sensitivities of four types of chromatophores to Pej-PDH-I and -II were found to differ, and the order of sensitivity was the same as that of Pej-RPCH: erythrophores = xanthophores > melanophores > leucophores.

Pej-PDH-II was more potent than Pej-PDH-I in all types of chromatophores except for leucophores. The reason for the differences in activity between the two PDHs was thought to be due to one or more differ-

(A)

```
                    -22        -20              Signal peptide
             Met Arg Ser Ile Ala Val Val Val Val Val Met Ala
             TCTAGG ATG CGC AGT ATC GCA GTG GTC GTT CTC CTG GTG GTG ATG GCT   48
                                                          -1   1
             Leu Ser Leu Gln Gly Thr Val Ala Asp Ser Ser Leu Lys Tyr Phe Glu
             TTG AGT CTT CAG GGA ACA GTG GCC GAT TCG AGC CTT AAG TAT TTC GAG   96
             PDH precursor-related peptide        20
             Arg Glu Val Val Ser Glu Leu Ala Ala Gln Ile Leu Arg Val Ala Gln
             CGT GAG GTG GTG TCC GAG TTG GCG GCG CAG ATC CTG CGT GTG GCT CAG  144

             Gly Pro Ser Ala Phe Val Ala Gly Pro His│Lys Arg│Asn Ser Glu Leu  40
             GGA CCC TCG GCC TTC GTC GCC GGA CCT CAC│AAG CGG│AAC TCG GAG CTG  192
             Pej-PDH-I
             Ile Asn Ser Leu Leu Gly Ile Pro Lys Val Met Thr Asp Ala│Gly Arg│
             ATC AAC TCC CTC CTG GGG ATC CCC AAG GTC ATG ACC GAC GCC│GGA AGG│ 240
             57
             Arg│End
             AGA│TAA ATCATGTAGATCTATTGTCCTTAGACGAGGACCACGGACTTTGTTTAGTCTCCCA  301

             CTACACCTTCGCCTACCTTTCTTTGGTAAAAATTCCTGCTGCAGCATTAGTCCTTATATTTGT  364

             TTTCTGGGATATTGAATAAAATGCATTGAAT(A)n                                395
```

(B)

```
                      -22      -20              Signal peptide
               Met Cys Arg Ala Ala Val Leu Leu Phe Leu Met
               CACCTCACTCTGCAAG ATG TGC CGC GCT GCA GTG CTG TTG TTT CTT ATG   49
                                                       -1   1
               Leu Ala Val Ala Ala Val Met Val Thr Glu Ala Gln Ile Arg Glu Pro Thr
               TTG GCG GTG GCA GCT GTC ATG GTG ACA GAA GCG CAA AGA GAG CCT ACA   97
               PDH precursor-related peptide        20
               Ala Ser Lys Cys Gln Ala Ala Thr Glu Leu Ala Ile Gln Ile Leu Gln
               GCT TCA AAG TGT CAG GCT GCA ACA GAA CTG GCA ATA CAG ATC TTG CAG  145

               Ala Val Lys Gly Ala His Thr Gly Val Ala Ala Gly Pro His│Lys Arg│
               GCA GTG AAG GGT GCT CAC ACT GGG GTA GCC GCA GGA CCC CAC│AAA AGG│ 193
               40
               Asn Ser Glu Leu Ile Asn Ser Leu Leu Gly Leu Pro Lys Phe Met Ile
               AAC TCA GAG CTC ATC AAT TCC CTC TTG GGC CTC CCC AAG TTC ATG ATC  241
               Pej-PDH-II
                                 58
               Asp Ala│Gly Arg Arg│End
               GAC GCT│GGC AGG AGG│TGA TGAGATGCCTCTTTCAGGATAATTAGAACACTGAGCCCA  298

               AATTCTCATGAACTAGCCATAAGTTTACAAGTTCATTTTACTAGGACTGTATTAGAGGATATA  361

               CTCCTGGAATTTCTGTTACTTTGATGTACAAACTCTGAGCATTACTGCATGACTTTATCGAGT  424

               TGCAACAATAAAAAGCAAATGT(A)n                                        446
```

Fig. 31. Nucleotide and deduced amino acid sequences of the cDNAs for Pej-PDH-I (A) and -II (B) precursors in the kuruma prawn *Marsupenaeus japonicus*. Amino acid numbering starts at the N-terminal residue of each propeptide of Pej-PDH-I and -II, and the putative signal peptide is indicated by negative numbers. The shaded boxes indicate the putative dibasic cleavage site. The open boxes indicate the putative amidation signal. Each polyadenylation signal sequence is underlined. The nucleotide sequences of the cDNAs for Pej-PDH-I and -II have been deposited in the DDBJ/EMBL/GenBank databases (accession Nos. AB073367 and AB073368, respectively). With kind permission from Springer Science + Business Media: <*Mar. Biotechnol.*, Molecular cloning of cDNAs encoding two pigment-dispersing hormones and two corresponding genes from the kuruma prawn (*Penaeus japonicus*), 4, 2002, 463–470, Ohira *et al.*, Fig. 2>.

ences in amino acid residues at position 11, 14, or 16. Previous studies on the structure-activity relationship of PDH showed that PDHs with Ile[11] and Leu[11] were equally potent (Rao and Riehm 1989). Moreover, the difference of residue at position 14, Val in Pej-PDH-I and Phe in Pej-PDH-II, was not thought to be significant enough to cause differences in activity, because these two residues have similar hydrophobic properties. We thus considered that the difference at position 16, Thr in Pej-PDH-I and Ile in Pej-PDH-II, had the greatest potential to influence activity and therefore decided to synthesize [Ile[16]]-Pej-PDH-I. As expected,

this synthetic peptide showed PDH activity comparable to that of Pej-PDH-II (**Fig. 30**), even though [Ile[16]]-Pej-PDH-I had different residues at positions 11 and 14 from Pej-PDH-II. Until now, only Asn and Thr, have been found at position 16, and replacement of Asn by Thr at this position did not cause considerable change in activity (Rao and Riehm 1989). Thus, Ile[16] in Pej-PDH-II provided new information regarding the structure-activity relationship of PDH. Similar results have been reported by *P. jordani* (Rao and Riehm 1993), where α-PDH substituted by Ala at position 16 was 2-fold more potent than α-PDH with Thr, suggesting that hydrophobic residues at this position increase the activity.

3-2C. Cloning of cDNAs and corresponding genes

Until now, cDNAs encoding PDH/PDF family peptide precursors have been cloned from 4 crustacean species: the shore crab *C. maenas* (Klein *et al.* 1992), the crayfish *O. limosus* (De Kleijn *et al.* 1993), the blue crab *C. sapidus* (Klein *et al.* 1994) and the whiteleg shrimp *L. vannamei* (Desmoucelles-Carette *et al.* 1996), and from 2 insect species: the lubber grasshopper *Romalea microptera* (Davis *et al.* GenBank accession No. U42472) and the fruit fly *Drosophila melanogaster* (Park and Hall 1998). A common structure of the PDH/PDF family peptide precursors consisting of a signal peptide, a PDH/PDF precursor-related peptide (PPRP) and a PDH/PDF was demonstrated by sequence analyses of the cDNAs.

A genomic DNA for the fruit fly *D. melanogaster* PDF has been reported (Park and Hall 1998). This PDF gene has no intron and is present as a single copy per haploid genome. Until now, this has been the only instance in which gene structure of a PDH/PDF family peptide has been elucidated. Therefore, we attempted to isolate cDNAs encoding the two PDHs of *M. japonicus* and two corresponding genes (Ohira *et al.* 2002).

Two cDNAs encoding Pej-PDH-I and -II were amplified by a combination of 5′- and 3′-RACE (**Fig. 31**). The amino acid sequences of the two PDH precursors deduced from the nucleotide sequences of the cDNAs revealed that they possess a structure common to the PDH/PDF family peptide precursors. The two PDH precursors consisted of a signal peptide, a PPRP, a dibasic cleavage site, a PDH and an amidation signal. Amino acid sequences of mature peptides were highly conserved among all PDHs and PDFs, whereas PPRP exhibits lower sequence similarity among crustacean species, and sequence homology is not observed between crustaceans and insects. This observation suggests that PPRP may not have a biological function. Similar structural divergence in peptides where regions other than that corresponding to the mature peptide is observed in those of the AKH/RPCH family (Bogerd *et al.* 1995) and in those of the CHH-family (see Sub-
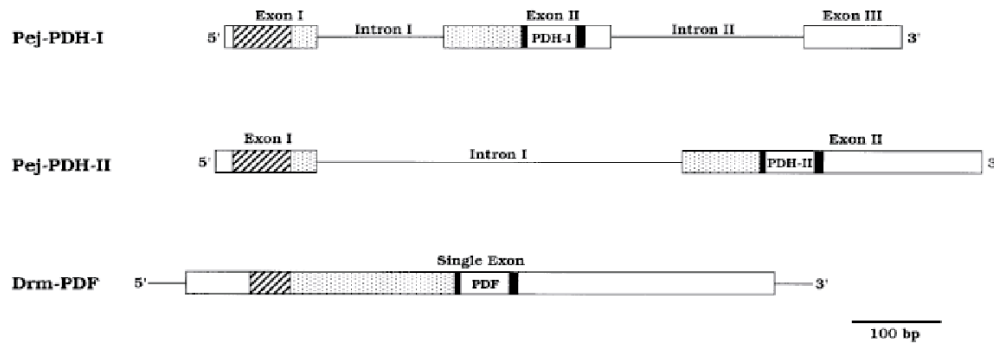
Fig. 32. Schematic representation of the organization of genes for Pej-PDH-I, -II, and *Drosophila melanogaster* PDF (Drm-PDF). Open boxes indicate the 5′- and 3′-untranslated regions. Hatched and shaded boxes indicate the signal peptide and the PDH/PDF precursor-related peptide (PPRP), respectively. Black boxes located at both sides of the mature PDH/PDF indicate the dibasic cleavage site (the N-terminal side) and the amidation signal (the C-terminal side), respectively. The nucleotide sequences of the genes for Pej-PDH-I and -II have been deposited in the DDBJ/EMBL/GenBank databases (accession Nos. AB073369 and AB073370, respectively). With kind permission from Springer Science + Business Media: <*Mar. Biotechnol.*, Molecular cloning of cDNAs encoding two pigment-dispersing hormones and two corresponding genes from the kuruma prawn (*Penaeus japonicus*), **4**, 2002, 463–470, Ohira *et al.*, Fig. 4>.

section 2-1D).

We also cloned two corresponding genes by PCR using genomic DNA derived from a single prawn as a template (**Fig. 32**). The exon/intron organization of Pej-PDH-I gene differed from that of Pej-PDH-II. The Pej-PDH-I gene consisted of three exons and two introns, while Pej-PDH-II gene consisted of two exons and a single intron. Such organization differed from that of the *D. melanogaster* PDF gene, which lacks an intron (Park and Hall 1998). These differences in exon/intron organization among the genes for the PDH/PDF family peptides are also seen in genes for CHH-family (Udomkit *et al*. 2000; Krungkasem *et al*. 2002) and in that for insulin-related peptides in insects (Kromer-Metzger and Lagueux 1994). However, the above cases seem to be exceptional, as exon/intron organization of genes for the same family peptides are generally conserved. The differences in the structure of genes for PDH/PDF family peptides may reflect their evolutionary pathway. With more information on other crustacean and insect species, it may be possible to estimate the evolutionary pathway of the PDH/PDF gene.

4. AGH

In 1954, it was first discovered by Charniaux-Cotton that the androgenic gland (AG) plays a pivotal role in crustacean sex differentiation (Charniaux-Cotton 1954). In AG-ablated *A. vulgare* males, it was demonstrated that endopodites cease to elongate and a vitellogenin gene is expressed at the same levels as in the normal females (Suzuki *et al*. 1990). On the other hand, when AGs were implanted into *A. vulgare* females, body color changed from brown, a typical color for females, into the grey-black color of the male body, and the gonads were transformed into testes with fully developed seminal vesicles (Suzuki and Yamasaki 1998). These observations strongly indicated that the AGs secrete a sex differentiation factor, AGH. In order to purify AGH, an *in vivo* bioassay system was first established by Katakura *et al*. (1975). When AG extract was injected into young *A. vulgare* females, elongation of endopodites, an external male characteristic, was observed after the ensuing molt. Using this phenomenon as an index of the AGH activity, we attempted to purify AGH as described below.

4-1.   Trial for purification of AGH

At the early stage of study for purification of AGH of the terrestrial isopod, *A. vulgare* (**Fig. 33**), the whole reproductive organ was used as starting material, because it was difficult to collect only AGs due to their small size and sticky cell surface. However, since the whole reproductive organ contained various materials other than AGH, the purification could not be easily achieved. On one occasion, we thought that we had successfully isolated AGH, because the active fraction was recovered in a single UV-peak on reversed-phase HPLC after several steps of purification. This fraction was subjected to structural analysis, which showed that the fraction contained at least four peptides with closely related structures (Nagasawa *et al*. 1995). However, the activity of the purified fraction was not very high. Comparison of the specific activity of this fraction with that of later-purified AGH revealed that the activity of the former was much weaker than the latter by three orders of magnitude. Thus, it turned out that the fraction contained AGH at a ratio of only 0.1% and most

Fig. 33. The terrestrial isopod *Armadillidium vulgare*.

Fig. 34. Elution profile of reversed-phase HPLC at the final third step. Hatched peak material showed AGH activity. Solvent, aqueous acetonitrile containing 0.05% heptafluorobutyric acid (HFBA); column, Asahi-Pak ODP-50 (4.6 × 150 mm); flow, 1.0 mL/min. Reprinted with permission from *Zool. Sci.*, **14**, Okuno *et al.*, Purification and properties of androgenic gland hormone from the terrestrial isopod *Armadillidium vulgare*, 837–842, Fig. 4, © 1997, Zoological Society of Japan.

of the other materials were non-AGH peptides. Since the peptide thus characterized was found to be distributed only in the seminal vesicle using immunohistochemistry and an antiserum raised against a synthetic peptide fragment, we designated it as seminal vesicle-specific peptide (SVSP) (Nagasawa *et al*. 1995). Therefore, we later changed the starting material for purification of AGH from whole reproductive organs to androgenic glands only. Because of this change in strategy, AGH could thereafter be isolated successfully.

4-2. Isolation of AGH

AGH was purified from isolated androgenic glands of male *A. vulgare* by three steps of reversed-phase HPLC (Okuno *et al*. 1997). Only 160 ng of the material was finally obtained from 2,000 animals at about an 11% rate of recovery (**Fig. 34**). The elution of AGH activity by molecular sieve HPLC indicated that the molecular weight of AGH was 11,000–13,000. The purified AGH-active fraction showed masculinizing activity when 38 pg of this preparation was injected into a young female of the same species. Inactivation experiments using a crude preparation of AGH showed that AGH activity was lost by treatment with trypsin or by reductive alkylation. The AGH activity was not affected by heat treatment at 100°C for 3 min. These results indicated that AGH was a heat-stable protein with disulfide bond(s). Since sequence analysis of the final preparation thus obtained yielded two phenylthiohydantoin derivatives of amino acids at each cycle of Edman degradation, we judged that it was still impure. This was later found to be wrong, because it was clarified that AGH was really a heterodimeric peptide. It was necessary to wait for the next opportunity to analyze the N-terminal sequence of another batch of AGH, in which similar results were obtained. Thus, we concluded that AGH is a heterodimer.

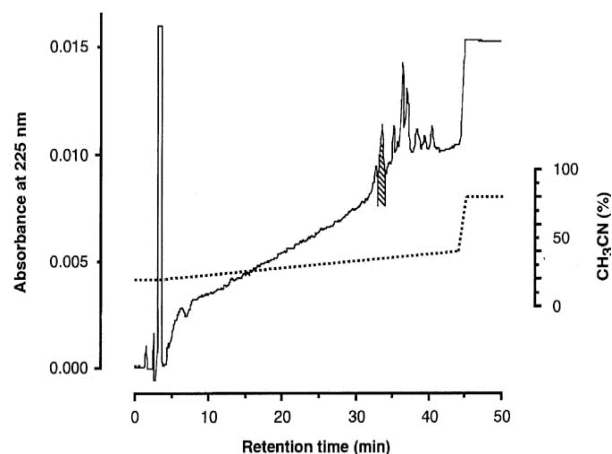At almost the same time, a French research group

obtained a similar result using the same species infected by *Wolbachia* (Martin *et al*. 1998), whose AGs became enlarged possibly due to the inhibition of AG activity and possibly AGH secretion, resulting in a decrease of male characteristics in this animal, the so-called "intersex" type (Martin *et al*. 1990). Because these workers were able to obtain a high concentration of AGH in such AGs, they succeeded in the isolation of AGH from a much smaller number of animals than we used. This group postulated an overall heterodimeric structure of an AGH molecule in terms of chain length of each A and B chain, the positions of Cys residues and an *N*-glycosylation site, and the structure of its precursor molecule including the presence of C peptide, but did not reveal any amino acid residues in detail.

4-3. Cloning of a cDNA encoding AGH

Since a pair of amino acid residues with almost equimolar amounts was identified at each cycle by the N-terminal sequence analysis of native AGH, we could not determine amino acid sequences of two peptide chains consisting of AGH. On the other hand, one of the two peptides could be recovered after reductive carboxymethylation, and its N-terminal 10-residue sequence was obtained. Then, by subtraction of each amino acid residue from the doubly-identified residues for the whole molecule, the amino acid sequence of the other peptide chain could be estimated (Okuno *et al*. 1999).

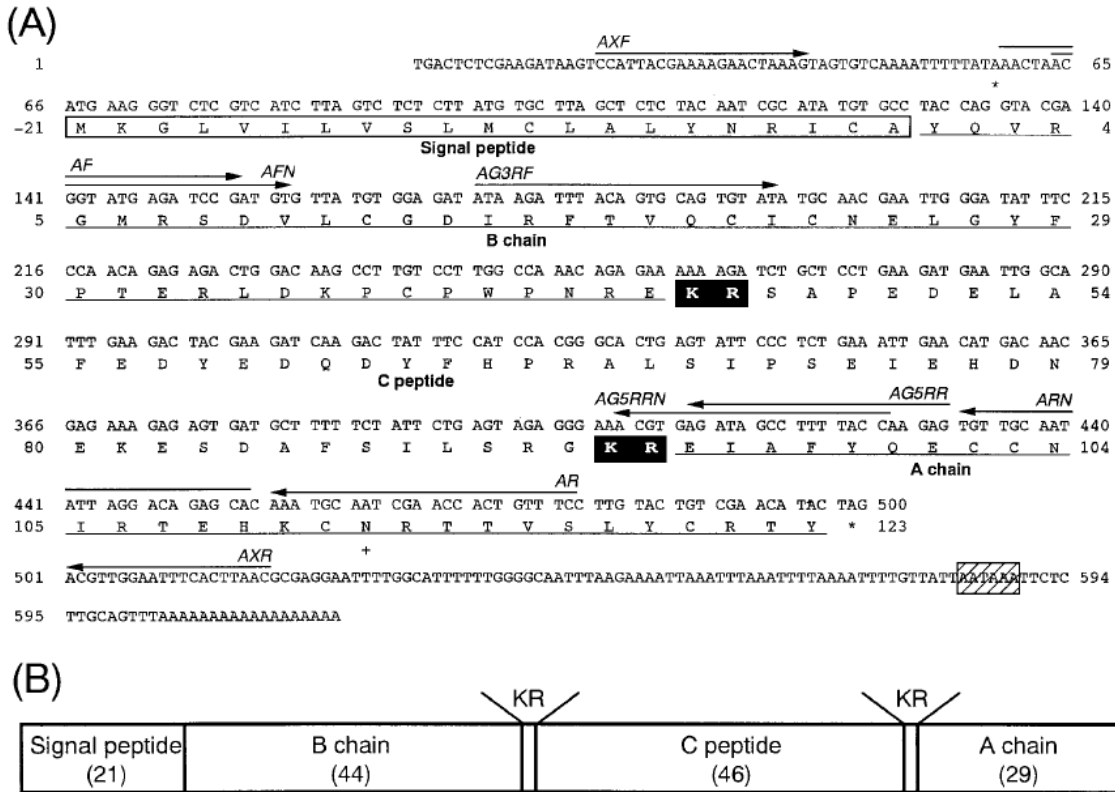A cDNA fragment encoding AGH was amplified by

(A)

```
                                                          AXF
  1                              TGACTCTCGAAGATAAGTCCATTACGAAAAGAACTAAAGTAGTGTCAAAATTTTTATAAACTAAC  65
                                                                                        *

 66  ATG AAG GGT CTC GTC ATC TTA GTC TCT CTT ATG TGC TTA GCT CTC TAC AAT CGC ATA TGT GCC TAC CAG GTA CGA 140
-21   M   K   G   L   V   I   L   V   S   L   M   C   L   A   L   Y   N   R   I   C   A | Y   Q   V   R    4
                                         Signal peptide
      AF                    AFN              AG3RF
141  GGT ATG AGA TCC GAT GTG TTA TGT GGA GAT ATA GTA TTT ACA GTG CAG TGT ATA TGC AAC GAA TTG GGA TAT TTC 215
  5   G   M   R   S   D   V   L   C   G   D   I   R   F   T   V   Q   C   I   C   N   E   L   G   Y   F   29
                                          B chain
216  CCA ACA GAG AGA CTG GAC AAG CCT TGT CCT TGG CCA AAC AGA GAA AAA AGA TCT GCT CCT GAA GAT GAA TTG GCA 290
 30   P   T   E   R   L   D   K   P   C   P   W   P   N   R   E   K   R | S   A   P   E   D   E   L   A   54
291  TTT GAA GAC TAC GAA GAT CAA GAC TAT TTC CAT CCA CGG GCA CTG AGT ATT CCC TCT GAA ATT GAA CAT GAC AAC 365
 55   F   E   D   Y   E   D   Q   D   Y   F   H   P   R   A   L   S   I   P   S   E   I   E   H   D   N   79
                         C peptide
                                              AG5RRN                        AG5RR
                                                                                            ARN
366  GAG AAA GAG AGT GAT GCT TTT TCT ATT CTG AGT AGA GGG AAA CGT GAG ATA GCC TTT TAC CAA GAG TGT TGC AAT 440
 80   E   K   E   S   D   A   F   S   I   L   S   R   G | K   R | E   I   A   F   Y   Q   E   C   C   N  104
                                                                                           A chain
                                                       AR
441  ATT AGG ACA GAG CAC AAA TGC AAT CGA ACC ACT GTT TCC TTG TAC TGT CGA ACA TAC TAG 500
105   I   R   T   E   H   K   C   N   R   T   T   V   S   L   Y   C   R   T   Y   *  123
      AXR                      +
501  ACGTTGGAATTTCACTTAACGCGAGGAATTTTGGCATTTTTTGGGGCAATTTAAGAAAATTAAATTTAAATTTTAAAATTTTGTTAT[ATAAA]TTCTC 594

595  TTGCAGTTTAAAAAAAAAAAAAAAAAAAA
```

(B)

Signal peptide (21)	B chain (44)	C peptide (46)	A chain (29)

KR (between B chain and C peptide), KR (between C peptide and A chain)

Fig. 35. The nucleotide sequence of a cDNA encoding the *A. vulgare* AGH precursor and its deduced amino acid sequence. (A) Arrows indicate the regions used as the primers for PCR. The stop codon and the putative *N*-glycosylation site are indicated by an asterisk and a symbol (+), respectively. The putative signal peptide is boxed. The A and B chains are underlined. The hatched box indicates the consensus sequence for polyadenylation signal. The filled box indicates the possible dibasic cleavage sites between B chain and C peptide, and between C peptide and A chain. The nucleotide sequence has been deposited in DDBJ, EMBL and GenBank databases under accession No. AB029615. (B) Schematic representation of the organizations of preproAGH. The numbers in parentheses indicate the number of amino acid residues in each domain of AGH. The possible cleavage sites (Lys-Arg) are indicated by KR. Reprinted from *Biochem. Biophys. Res. Commun.*, **264**, Okuno *et al.*, Characterization and cDNA cloning of androgenic gland hormone of the terrestrial isopod *Armadillidium vulgare*, 419–423, © 1999, with permission from Elsevier.

PCR using a set of primers designed based on the amino acid sequence analyzed. By combining the results obtained by 5′- and 3′ RACE, a full length cDNA covering an open reading frame was obtained (**Fig. 35**) (Okuno *et al.* 1999). An open reading frame of 432 bp encoded a preproAGH consisting of a signal peptide (21 residues), B chain (44 residues), C peptide (46 residues), and A chain (29 residues). Two dibasic processing sequences were present between the B chain and the C peptide, and between the C peptide and the A chain. The A chain possessed a putative *N*-linked glycosylation site. It was postulated that through processing including the removal of a signal peptide, disulfide bond formation, the removal of C peptide, and the addition of carbohydrate moiety, the A and B chains might form a heterodimeric glycopeptide of a mature hormone. Disulfide bond formation should proceed the removal of the C peptide. The characteristic

organization of preproAGH was similar to that of insulin, although both peptide chain length and amino acid sequence differed.

4-4. Verification of the cDNA as that encoding AGH using anti-AGH antisera

In order to prove that this cloned cDNA actually encodes AGH, recombinant single-chain precursor molecules consisting of B chain, C peptide and A chain were produced using both baculovirus and bacterial expression systems (Okuno *et al.* 2002b). The former system can produce an *N*-glycosylated peptide, while the latter can yield a non-glycosylated peptide. Neither recombinant precursors containing C peptide showed AGH activity as described below. Three kinds of antisera were raised in rabbits against three antigens; a recombinant putative AGH precursor produced by the *E. coli* expression system, a synthetic peptide
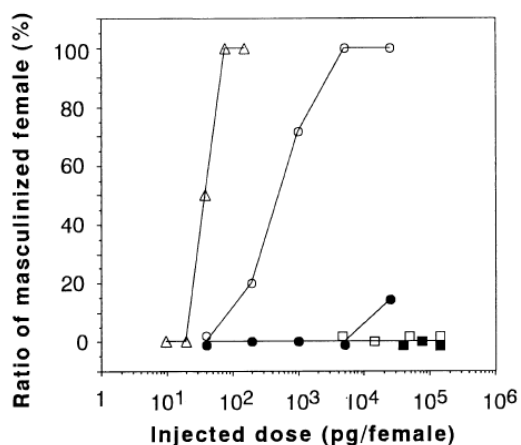
Fig. 36. Dose-response relationship of the recombinant and native AGHs. Triangles show the native AGH. Open and filled circles indicate the recombinant AGH expressed by baculovirus expression system with and without lysyl endopeptidase treatment, respectively. Open and filled squares show the recombinant AGH expressed by *E. coli* expression system with and without lysyl endopeptidase treatment, respectively. Reprinted from *Peptides*, *23*, Okuno *et al.*, Preparation of an active recombinant peptide of crustacean androgenic gland hormone, 567–572, © 2002, with permission from Elsevier.

corresponding to the N-terminal eight residues of the A chain, and a synthetic peptide corresponding to the N-terminal nine residues of the B chain. The first one was used directly for immunization, while the synthetic peptides were used as antigens after conjugation with BSA. After incubation of partially purified preparation of AGH with each of the three antisera, AGH activity decreased significantly in all cases (Okuno *et al.* 2001), strongly indicating that the cDNA encoded AGH, although it constituted indirect evidence.

In order to examine the distribution of AGH peptide, an immunohistochemical study was performed. *A. vulgare* AG showed strong immunoreactivity to all three antisera, while the testis, the seminal vesicle and the vas deferens did not (Hasegawa *et al.* 2002). These results indicated that AGH peptides are specifically localized in AG.

4-5. Preparation of biologically active recombinant AGH

The simplest method for confirming that this heterodimeric glycopeptide is the biologically active AGH is measurement of the biological activity using recombinant peptide. Along these lines, we prepared the recombinant AGHs using both *E. coli* and baculovirus expression systems (Okuno *et al.* 2002b). The recombinant AGHs were expressed as a single-chain precursor protein consisting of B chain, C peptide and A chain, and only the recombinant AGH produced by the baculovirus system possessed a carbohy-

drate moiety at the *N*-glycosylation site in the A chain. Both recombinant precursor proteins showed no biological activity *in vivo*, indicating that the precursor protein is inactive regardless of the *N*-linked glycan. In order to examine whether the removal of C peptide by proteolytic cleavage is essential for conferring the activity, we tried to remove the C peptide from the precursor proteins with proteases. When treated with lysyl endopeptidase at 37°C for 2 h, peptide bonds at Lys[45]–Arg[46] and Lys[81]–Glu[82] were cleaved and the peptide was partially removed, although a part of the C peptide, Glu[82]–Arg[94], was still attached to the recombinant proteins. Using these double-chain recombinant proteins, the biological activity was assessed (**Fig. 36**). As expected, the digested recombinant AGH prepared by the baculovirus system showed biological activity at a dose of 0.25 ng/individual, but was less active than the native AGH by about approximately one order of magnitude. This difference may be due to the partly retained C peptide in the recombinant AGH. On the other hand, the recombinant AGH prepared by the *E. coli* system and subsequently digested by the same way showed no activity even at a dose of 440 ng/individual, indicating that the carbohydrate moiety at the A chain is essential for conferring AGH activity.

The arrangement of four disulfide bonds in the recombinant AGH prepared by the baculovirus system was determined by analyzing the structure of protease digests. The results clearly indicated that two intrachain disulfide bridges between Cys[21]–Cys[38] and Cys[103]–Cys[111] and two interchain disulfide bridges between Cys[12]–Cys[102] and Cys[23]–Cys[120] were connected. The carbohydrate structure of the recombinant AGH was also estimated to be Man$_3$-GlcNAc$_2$(Fuc)- by mass spectral analysis. This carbohydrate structure is typically found in glycoproteins produced by insect cells, and was different from that of the native AGH reported previously, which has a larger glycan with a sulfate group at its terminal (Martin *et al.* 1999). The weak activity of the recombinant AGH may be partly due to the difference in carbohydrate structure.

4-6. Structure-activity relationship of AGH

In order to investigate the structure-activity relationship of AGH, we synthesized the hormone by chemical means. First, we attempted to synthesize AGH by an expressed protein ligation strategy (Muir *et al.* 1998), in which 110 residues N-terminal peptide segment is expressed in an *E. coli* expression system, and the C-terminal peptide having a carbohydrate moiety is chemoselectively condensed by a native chemical ligation method (Dawson *et al.* 1994). This scheme is shown in **Fig. 37**. After the condensation reaction, disulfide bonds were formed by *in vitro* refolding reaction in a redox buffer containing reduced/oxidized forms of glutathione. The C peptide portion was cleaved off by treatment with metalloendopeptidase,
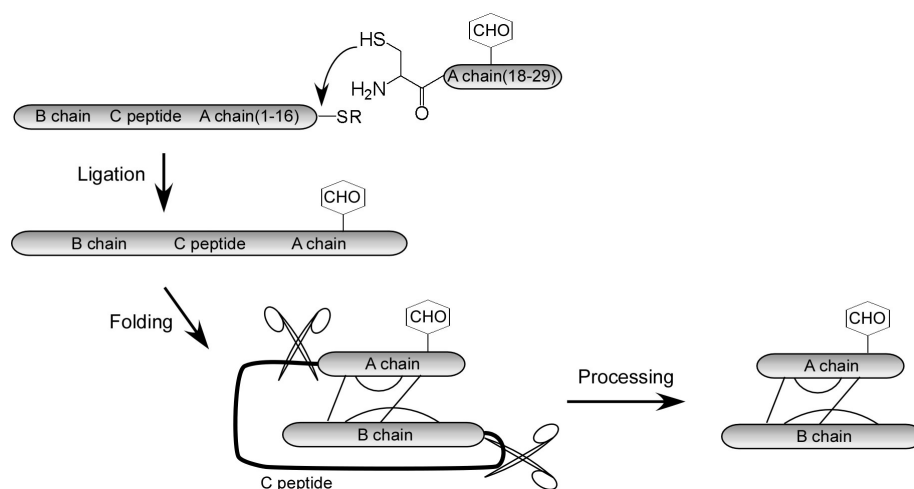
Fig. 37. Scheme for preparing the semi-synthetic AGH by the expressed protein ligation method. Reprinted with permission from *Biochemistry*, **49**, Katayama *et al.*, Correct disulfide pairing is required for the biological activity of crustacean androgenic gland hormone (AGH): Synthetic studies of AGH, 1798–1807, Graphical Abstract, © 2010, American Chemical Society.

yielding the heterodimeric glycopeptide. Unexpectedly, the semi-synthetic AGH showed no biological activity *in vivo* regardless of the carbohydrate structure (Katayama *et al.* 2010). In order to confirm the conformation of the semisynthetic AGH, the disulfide bond arrangement was determined by analyzing protease digests, establishing that four disulfides are connected between Cys^{12}–Cys^{103}, Cys^{21}–Cys^{38}, Cys^{23}–Cys^{120} and Cys^{102}–Cys^{111}. The results clearly indicated that the disulfide bond arrangement in the semisynthetic peptide is different from that in the ecombinant AGH prepared by the baculovirus expression system. These results indicated that the AGH with correct disulfide bonds could not be obtained by an *in vitro* refolding reaction, and that the native conformation is not the thermodynamically most stable form at least *in vitro*.

To confirm that the loss of biological activity resulted from disulfide bond isomerization, total chemical synthesis with selective disulfide bond formation was carried out according to the scheme shown in **Fig. 38** (Katayama *et al.* 2010). The A and B chains were separately synthesized by a 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis strategy. To form disulfide bonds selectively, three protecting groups, trityl, 4-methoxybenzyl and acetamidomethyl, were used for Cys residues, and stepwise deprotection and oxidation reactions were performed. The synthetic AGH carrying the GlcNAc moiety at the *N*-glycosylation site in the A chain showed rather weak activity *in vivo*. On the other hand, the synthetic AGH without a carbohydrate chain showed no biological activity at any dosage levels tested. These results clearly indicated that carbohydrate chain attached to the A chain and the correct disulfide pairing are essential for conferring AGH activity. Weak activity of synthetic AGH may be due in part to that the presence of GlcNAc may not be sufficient for generating complete activity, and a larger glycan moiety may be required for achieving full AGH activity.

4-7. AGH in other isopod crustacean species

Thereafter, we examined the distribution of this AGH molecule among phylogenetically close and remote species by using the anti-AGH antisera as described above. Immunohistochemical studies revealed that AGs of species belonging to Armadillidiidae, Porcellionidae and Scyphacidae families were immunostained using these antisera (Hasegawa *et al.* 2002). On the other hand, no positive signal was observed in AGs of species belonging to Tylidae and Ligiidae. These observations suggested that structural similarity of AGH might exist among some terrestrial isopods, although AGH seemed to harbor a higher degree of species specificity.

In order to clarify the molecular diversity of AGH in relation to the degree of immunostaining and hormonal activity, cDNAs encoding AGH precursors were cloned from two Porcellionidae species, *Porcellio scaber* and *Porcellio dilatatus*, by PCR techniques (Ohira *et al.* 2003b). The deduced amino acid sequences predicted from the nucleotide sequences of cDNA encoding *P. scaber* AGH consisted of a signal peptide (21 amino acid residues), a B chain (44 residues), an Arg–Arg dibasic site, a C peptide (45 residues), a Lys–Arg dibasic site, and an A chain (31 residues) (**Fig. 39**). The predicted structure of the AGH precursor protein of *P. dilatatus* was quite similar to that of *P. scaber*, except for the length of C peptide which consisted of 46 residues in *P. dilatatus*. The deduced amino acid sequences of AGH precursors of

A chain   B chain
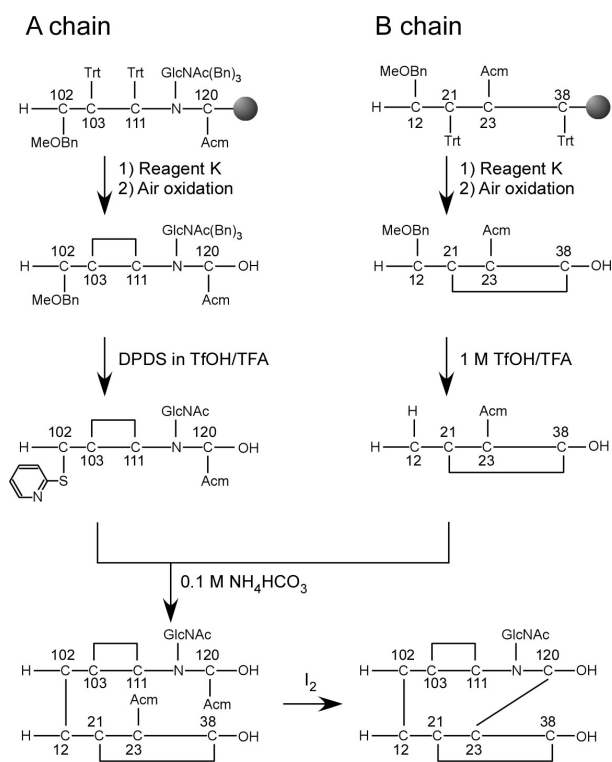
Fig. 38. Synthesis procedure for AGH by selective disulfide formation. Reprinted with permission from *Biochemistry*, **49**, Katayama *et al*., Correct disulfide pairing is required for the biological activity of crustacean androgenic gland hormone (AGH): Synthetic studies of AGH, 1798–1807, Scheme 3, © 2010, American Chemical Society.

these two species shared high sequence identity with that of *A. vulgare* AGH (65% and 63% identity with those of *P. scaber* and *P. dilatatus*, respectively). The *N*-glycosylation site at the A chain was also conserved among these species. Such structural similarity is highly consistent with the results obtained from immunohistochemical studies (Hasegawa *et al*. 2002). In order to better understand the structural and functional diversity of AGHs in other crustacean species, further experimentation is required.

4-8. AGH of decapod crustacean species

The role of AG has also been studied in decapod crustaceans. In the giant freshwater prawn *Macrobrachium rosenbergii*, the implantation of AGs into young female prawns caused these genetic females to become phenotypic reproductive males (Nagamine *et al*. 1980). In the red-claw crayfish *Cherax quadricarinatus*, the implantation of hypertrophied AGs obtained from bilaterally deeyestalked males into immature female animals induced male-specific morphologies and inhibited female secondary sex characteristics in recipient females (Khalaila *et al*. 2001). These results indicate that the AGs in decapod species also control sexual

differentiation. Recently, cDNA libraries were constructed by using suppression subtractive hybridization of the AGs of *C. quadricarinatus* and *M. rosenbergii* (Manor *et al*. 2007; Ventura *et al*. 2009). Expressed sequence tags from these libraries were sequenced and then one each AG-specific gene of *C. quadricarinatus* and *M. rosenbergii* was detected. The gene product of both animals consisted of a signal peptide, a B chain, a C peptide and an A chain. Since the organization of these gene products was the same as that of the vertebrate insulin precursor, they were named insulin-like AG factor (IAG). *C. quadricarinatus* IAG and *M. rosenbergii* IAG possessed six and eight cysteine residues, respectively, in their predicted mature peptides (A and B chains). The positions of the six cysteine residues of *C. quadricarinatus* IAG were the same as six of the eight cysteine residues in *M. rosenbergii* IAG (Manor *et al*. 2007; Ventura *et al*. 2009). Furthermore, the six cysteine residues were well-conserved within the insulin superfamily, including isopod AGHs. Therefore, the two IAG molecules have been thought to be putative AGHs in decapod species. However, *C. quadricarinatus* IAG and *M. rosenbergii* IAG showed low sequence similarities with the three isopod AGHs and shared only a little similarity with each other.

In order to obtain information of amino acid sequences of additional IAG molecules, we attempted to clone cDNAs encoding IAG precursors from three palaemonid species *Macrobrachium lar*, *Palaemon paucidens* and *Palaemon pacificus*, and a penaeid species *Marsupenaeus japonicus* (Banzai *et al*. 2011, 2012). The four IAG precursors consisted of a signal peptide, a B chain, a C peptide and an A chain (**Fig. 40**). This organization is in agreement with those of the known isopod AGH and decapod IAG precursors as mentioned above. *M. lar* IAG, *P. paucidens* IAG and *P. pacificus* IAG showed moderate sequence identities to *M. rosenbergii* IAG (72, 69 and 49%, respectively). *M. japonicus* IAG had considerable sequence similarity only to *P. monodon* IAG (68%), which was recently revealed by another research group and deposited in the GenBank database (accession No. GU208677). These results are consistent with the following phylogenetic relationship. *M. lar*, *P. paucidens*, *P. pacificus* and *M. rosenbergii* are freshwater shrimps of the family Palaemonidae, and *M. japonicus* and *P. monodon* are marine shrimps of the family Penaeidae. In isopods, however, sequence identities of AGH precursors between different families, the Armadilliidae and the Porcellionidae, are clearly higher (over 60% as described above) than those of IAG between different decapod families (around 30%). This may be due to the differences in the molecular evolutionary pathway between IAG and AGH. In order to clarify this point, further studies characterizing amino acid sequences of additional IAGs and AGHs are required.

Fig. 39. Amino acid sequence alignment of *Porcellio scaber* AGH (Pos-AGH), *P. dilatatus* AGH (Pod-AGH) and *Armadillidium vulgare* AGH (Arv-AGH) precursors. Open boxes indicate the amino acids conserved among all precursors. Gray boxes indicate the positions of conserved Cys residues. The typical proteolytic cleavage motifs (R-X-K/R-R) and the putative *N*-linked glycosylation motif (N-X-S/T) are indicated by asterisks and symbols (+), respectively. Reprinted with permission from *Zool. Sci.*, **20**, Ohira *et al.*, Molecular cloning and expression analysis of cDNAs encoding androgenic gland hormone precursors from two Porcellionidae species, *Porcellio scaber* and *P. dilatatus*, 75–81, Fig. 3, © 2003, Zoological Society of Japan.

Fig. 40. Amino acid sequence alignment of *Macrobrachium lar* IAG (Malar), *Palaemon paucidens* IAG (Papau), *Palaemon pacificus* IAG (Papac), *Macrobrachium rosenbergii* IAG (Maros), *Cherax quadricarinatus* IAG (Chqua), *Penaeus monodon* IAG (Pemon), and *Marsupenaeus japonicus* IAG (Majap). Open and grey boxes indicate the positions of conserved Cys residues in all species and only in palaemonid species, respectively. The putative cleavage sites predicted by the ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/) are shown by arrows. The boundary between the signal peptide and the B chain is represented by a vertical dotted-line. Underlines indicate the putative *N*-linked glycosylation motifs (N-X-S/T). Reprinted with permission from *Jpn. Agric. Res. Q.*, **46**, Banzai *et al.*, Molecular cloning and expression analysis of cDNAs encoding insulin-like androgenic gland factor from three palaemonid species, *Macrobrachium lar*, *Palaemon paucidens* and *P. pacificus*, 105–114, Fig. 4, © 2012, Zoological Society of Japan.

In isopods, AGH has a single conserved *N*-glycosylation motif (N-X-S/T) in the A chain (Okuno *et al*. 1999; Ohira *et al*. 2003b). On the other hand, the positions and the number of *N*-glycosylation motifs are not conserved among decapod IAGs (**Fig. 40**). For instance, *M. lar* IAG has one *N*-glycosylation motif in the B chain, two in the C peptide, and one in the A chain. On the other hand, *P. pacificus* IAG has only one motif in the C peptide, which indicates a mature molecule comprising the B and A chains does not possess an *N*-linked glycan moiety. Although the *N*-linked glycan in the A chain of *A. vulgare* AGH is indispensable for biological activity (Okuno *et al*. 2002b), *N*-glycosylation of the A chain of decapod IAG is not likely to be essential for conferring function. Further experiments on the purification and the structural determination of native IAG are required to clarify this issue.

IAG has been thought of as a putative AGH in decapod species as described above. In *M. rosenbergii*, silencing of the IAG gene using RNA interference methods, temporally prevented the regeneration of male-specific sexual characteristics, and led to the arrest of spermatogenesis (Ventura *et al*. 2009). This correlates well with the above hypothesis; however, as of yet, there has been no direct evidence for IAG having AGH activity. However, we are now able to easily produce a heterodimeric mature *P. pacificus* IAG with selective disulfide bond formation in its synthetic A and B chains. Because *P. pacificus* IAG is the first example of a non-glycosylated IAG in its mature form, the synthetic *P. pacificus* IAG will be useful for *in vivo* and/or *in vitro* biological studies, which are expected to provide important information for understanding IAG function in decapod crustaceans.

5. Summary and Future Perspective

Shrimp aquaculture is a significant global industry worldwide. In order to achieve further development, more efficient seed production techniques are required. Therefore, it is desirable to gain a better understanding of shrimp reproduction. However, knowledge of vitellogenesis in shrimp is still limited, and it remains difficult to control reproduction under artificial conditions for a large number of commercially important species. Therefore, at present, it is common that fully mature shrimps for commercially important species are captured from the wild and are allowed to spawn in the hatchery. However, recently it is becoming increasingly difficult to obtain sufficient numbers of wild mature shrimps, because of overfishing and/or the presence of serious shrimp viral disease in many key areas. Indeed, the development of disease-resistant strains or strains harboring certain phenotypes is now necessary. For these reasons, we have been working

on the isolation of hormones regulating shrimp reproduction.

Eyestalk ablation is known to induce vitellogenesis, which permits us to presume that there exists a VIH in the eyestalks. VIHs were first isolated from the American lobster *H. americanus* (Soyez *et al*. 1991) and then from the terrestrial isopod *A. vulgare* (Gréve *et al*. 1999). Strangely enough, in the early 2,000's, VIH had not yet been isolated from shrimps. CHHs from *M. japonicus* were found to inhibit both transcription and translation in the developing ovary of another shrimp, *P. semisulcatus*, in a non-specific manner, while MIH from *M. japonicus* did not (Khayat *et al*. 1998). On the other hand, it can be presumed that the first function of VIH is vitellogenin (VG) synthesis. Recently, VG cDNAs have been cloned from several crustacean species (reviewed by Wilder *et al*., 2010). It might be possible to develop a VIH bioassay by looking at the reduction of VG mRNA expression levels in the producing organ, e.g., the ovary or hepatopancreas in response to various factors. Along these lines, we examined the *in vitro* VIH activity of *M. japonicus* CHH-family peptides based on quantitative PCR experiments on VG gene expression. As a result, we found that CHHs from *M. japonicus* suppressed VG gene expression, while *M. japonicus* MIH and MIH-B did not (Tsutsui *et al*. 2005a, 2013). Although we have thus far isolated major CHH-family peptides, we do not know whether VIH is one of the known CHH-family peptides, or an unknown peptide. Because, other minor CHH-family peptides, putative CHH and putative MIH-C, were found in *M. japonicus* by EST analysis (Yamano and Unuma 2006). In the future, we will again search for VIH in the sinus gland extracts of the kuruma prawn *M. japonicus*. Moreover, we will analyze hemolymph levels of these peptides during vitellogenesis to elucidate the real VIH molecules in *M. japonicus*.

Eyestalk ablation also causes various physiological changes, such as decrease of carbohydrate levels in hemolymph, shortening the molt-interval, and promotion of the methyl farnesoate synthesis in mandibular organs. These phenomena are known to be associated with CHH-family peptides. In our experiments, although eight CHH-family peptides have been purified and characterized from *M. japonicus* eyestalks, we have demonstrated only three functions, CHH, MIH and VIH activities. On the other hand, mandibular organ-inhibiting hormone (MOIH) was shown to be a CHH-family peptide (Wainwright *et al*. 1996). In addition, it was recently revealed that CHH-family peptides are also involved in various processes such as ion transport to the gills and water uptake for the purpose of enlarging body size after molting (Chung *et al*. 1999; Spanings-Pierrot *et al*. 2000). In order to determine the intrinsic function(s) of each peptide, further studies might be required.

AGH is highly species-specific, and therefore we know the structures of only three terrestrial isopod species, which are phylogenetically very close to one another (Ohira *et al.* 2003b). As for the structure of AGH in decapod crustaceans, we know that similar to the case of isopod AGHs, IAG genes are expressed specifically in the AGs, and IAGs are presumed to belong to the insulin superfamily, strongly suggesting that IAG is an intrinsic decapod AGH. However, IAGs share little sequence similarity to isopod AGHs, and the *N*-glycosylation site which is significant for conferring AGH activity in *A. vulgare* is not conserved in decapod IAGs. In addition, direct evidence that IAG shows AGH activity has not yet been reported either *in vivo* or *in vitro*. In order to elucidate the biological activity of IAG, an assay system for AGH activity using decapod species is required.

Eyestalk hormones are involved in the control of various processes as mentioned above. Although eyestalk hormones have been studied extensively by many researchers, many problems still remain to be solved. One such unsolved problem is how AGs are regulated by the sinus glands. AG activity is thought to be under the control of a certain eyestalk hormone on the basis of finding that eyestalk ablation causes the enlargement of AG cells (Khalaila *et al.* 2002). Recently, it was reported that IAG gene expression in AG is enhanced by eyestalk ablation in *C. sapidus* (Chung *et al.* 2011). We need to further characterize sinus gland neuropeptides, both biologically and chemically, in order to clarify the endocrine mechanisms not only of sexual differentiation but also of growth and reproduction, the results of which we hope will lead to new techniques useful for shrimp aquaculture.

References

Abdu U, Davis C, Khalaila I, Sagi A. The vitellogenin cDNA of *Cherax quadricarinatus* encodes a lipoprotein with calcium binding ability, and its expression is induced following the removal of the androgenic gland in a sexually plastic system. *Gen. Comp. Endocrinol.* 2002; **127**: 263–272.

Abramowitz AA, Hisaw FL, Papandrea DN. The occurrence of a diabetogenic factor in the eyestalks of crustaceans. *Biol. Bull.* 1944; **86**: 1–5.

Aguilar MB, Soyez D, Falchetto R, Arnott D, Shabanowitz J, Hunt DF, Huberman A. Amino acid sequence of the minor isomorph of the crustacean hyperglycemic hormone (CHH-II) of the Mexican crayfish *Procambarus bouvieri* (Ortmann): presence of a ᴅ-amino acid. *Peptides* 1995; **16**: 1375–1383.

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

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

Asazuma H, Nagata S, Nagasawa H. Inhibitory effect of molt-inhibiting hormone on Phantom gene expression in the Y-organ of the kuruma prawn, *Marsupenaeus japonicus. Arch. Insect Biochem. Physiol.* 2009; **72**: 220–233.

Audsley N, McIntosh C, Phillips JE. Isolation of a neuropeptide from locust corpus cardiacum which influences ileal transport. *J. Exp. Biol.* 1992; **173**: 261–274.

Avarre JC, Michelis R, Tietz A, Lubzens E. Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complimentary DNAs. *Biol. Reprod.* 2003; **69**: 355–364.

Banzai K, Ishizaka N, Asahina K, Suitoh K, Izumi S, Ohira T. Molecular cloning of a cDNA encoding insulin-like androgenic gland factor from the kuruma prawn *Marsupenaeus japonicus* and analysis of its expression. *Fish. Sci.* 2011; **77**: 329–335.

Banzai K, Izumi S, Ohira T. Molecular cloning and expression analysis of cDNAs encoding insulin-like androgenic gland factor from three palaemonid species, *Macrobrachium lar*, *Palaemon paucidens* and *P. pacificus. Jpn. Agric. Res. Q.* 2012 (in press).

Bogerd J, Kooiman FP, Pijnenburg MA, Hekking LH, Oudejans RC, Van der Horst DJ. Molecular cloning of three distinct cDNAs, each encoding a different adipokinetic hormone precursor, of the migratory locust, *Locusta migratoria*. Differential expression of the distinct adipokinetic hormone precursor genes during flight activity. *J. Biol. Chem.* 1995; **270**: 23038–23043.

Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris. FEMS Microbiol.* 2000; **24**: 45–66.

Chang ES, Prestwich GD, Bruce MJ. Amino acid Sequence of a peptide with both molting-inhibiting and hyperglycemic activities in the lobster *Homarus*

americanus. Biochem. Biophys. Res. Commun. 1990; **171**: 818–826.

Charniaux-Cotton H. Decouverte chez un Crustace Amphipode (*Orchestia gammarella*) d'une glande endocrine responsable de la differenciation des caracteres sexuels primaires et secondaires male. *C.R. Acad. Sci. Paris* 1954; **239**: 780–782.

Charniaux-Cotton H. Endocrinology and genetics of sex differentiation in invertebrates. *C.R. Seances Soc. Biol. Fil.* 1967; **161**: 6–9.

Chen YN, Tseng DY, Ho PY, Kuo CM. Site of vitellogenin synthesis determined from a cDNA encoding a vitellogenin fragment in the freshwater giant prawn, *Macrobrachium rosenbergii. Mol. Reprod. Dev.* 1999; **54**: 215–222.

Chung JS, Webster SG. Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH) in the crab, *Carcinus maenas. Eur. J. Biochem.* 2003; **270**: 3280–3288.

Chung JS, Webster SG. Dynamics of *in vivo* release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas. Endocrinology* 2005; **146**: 5545–5551.

Chung JS, Wilkinson MC, Webster SG. Determination of the amino acid sequence of the moult-inhibiting hormone from the edible crab, *Cancer pagurus. Neuropeptides* 1996; **30**: 95–101.

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

Chung JS, Manor R, Sagi A. Cloning of an insulin-like androgenic gland factor (*IAG*) from the blue crab, *Callinectes sapidus*: Implications for eyestalk regulation of *IAG* expression. *Gen. Comp. Endocrinol.* 2011; **173**: 4–10.

Dawson PE, Muir PW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. *Science* 1994; **266**: 776–779.

De Kleijn DP, Van Herp F. Molecular biology of neurohormone precursors in the eyestalk of Crustacea. *Comp. Biochem. Physiol. B* 1995; **112**: 573–579.

De Kleijn DP, Coenen T, Laverdure AM, Tensen CP, Van Herp F. Localization of messenger RNAs encoding crustacean hyperglycemic hormone and gonad inhibiting hormone in the X-organ sinus gland complex of the lobster *Homarus americanus. Neuroscience* 1992; **51**: 121–128.

De Kleijn DP, Linck B, Klein JM, Weidemann WM, Keller R, Van Herp F. Structure and localization of mRNA encoding a pigment dispersing hormone (PDH) in the eyestalk of the crayfish *Orconectes limosus. FEBS Lett.* 1993; **321**: 251–255.

De Kleijn DP, Janssen KP, Martens GJ, Van Herp F. Cloning and expression of two crustacean hyperglycemic-hormone mRNAs in the eyestalk of the crayfish *Orconectes limosus. Eur. J. Biochem.* 1994a; **224**: 623–629.

De Kleijn DP, Sleutels FJ, Martens GJ, Van Herp F. Cloning and expression of mRNA encoding prepro-gonad-inhibiting hormone (GIH) in the lobster *Homarus americanus. FEBS Lett.* 1994b; **353**: 255–258.

De Kleijn DP, De Leeuw EP, Van Den Berg MC, Martens GJ, Van Herp F. Cloning and expression of two mRNAs encoding structurally different crustacean hyperglycemic

hormone precursors in the lobster *Homarus americanus. Biochim. Biophys. Acta* 1995; **1260**: 62–66.

Desmoucelles-Carette C, Sellos D, Van Wormhoudt A. Molecular cloning of the precursors of pigment dispersing hormone in crustaceans. *Biochem. Biophys. Res. Commun.* 1996; **221**: 739–743.

Dircksen H, Böcking D, Heyn U, Mandel C, Chung JS, Baggerman G, Verhaert P, Daufeldt S, Plösch T, Jaros PP, Waelkens E, Keller R, Webster SG. 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.* 2001; **356**: 159–170.

Endo H, Nagasawa H, Watanabe T. Isolation of a cDNA encoding a CHH-family peptide from the silkworm *Bombyx mori. Insect Biochem. Mol. Biol.* 2000; **30**: 355–361.

Fernlund P. Structure of a light-adapting hormone from the shrimp, *Pandalus borealis. Biochim. Biophys. Acta* 1976; **439**: 17–25.

Fernlund P, Josefsson L. Crustacean color-change hormone: amino acid sequence and chemical synthesis. *Science* 1972; **177**: 173–175.

Fingerman M, Rao KR, Bartell CK. A proposed uniform method of reporting response values for crustacean chromatophorotropins: the standard integrated response. *Experientia* 1967; **23**: 962.

Gäde G. The adipokinetic neuropeptide of Mantodea. Sequence elucidation and evolutionary relationships. *Biol. Chem. Hoppe-Seyler* 1991; **372**: 193–201.

Gasparini S, Kiyatkin N, Drevet P, Boulain JC, Tacnet F, Ripoche P, Forest E, Grishin E, Ménez A. The low molecular weight protein which co-purifies with α-latrotoxin is structurally related to crustacean hyperglycemic hormones. *J. Biol. Chem.* 1994; **269**: 19803–19809.

Gaus G, Kleinholz LH, Kegel G, Keller R. Isolation and characterization of red-pigment-concentrating hormone (RPCH) from six crustacean species. *J. Comp. Physiol. B* 1990; **160**: 373–379.

Gilbert LI. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster. Mol. Cell. Endocrinol.* 2004; **215**: 1–10.

Gréve P, Sorokine O, Berges T, Lacombe C, Van Dorsselaer A, Martin G. Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). *Gen. Comp. Endocrinol.* 1999; **115**: 406–414.

Gu PL, Yu KL, Chan SM. Molecular characterization of an additional shrimp hyperglycemic hormone: cDNA cloning, gene organization, expression and biological assay of recombinant proteins. *FEBS Lett.* 2000; **472**: 122–128.

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

Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; **18**: 2714–2723.

Hampshire F, Horn DHS. Structure of crustecdysone a crustacean moulting hormone. *Chem. Commun.* 1966; **1966**: 37–38.

Hasegawa Y, Okuno A, Nagasawa H. Immunohistochemical

study of androgenic gland hormone: Localization in the male reproductive system and species specificity in the terrestrial isopods. *Gen. Comp. Endocrinol.* 2002; **125**: 218–225.

Hogben L, Slome D. The pigmentary effector system. VI. The dual character of endocrine co-ordination in amphibian colour change. *Proc. R. Soc. Lond. B* 1931; **108**: 10–53.

Holm M, Sander C. Protein structure comparison by alignment of distance matrices. *J. Mol. Biol.* 1993; **233**: 123–138.

Hoppe W, Huber R. Bestimmung des Sterin-Skeletts und seiner Orientierung mit diffuser Rontgenstreuung in Kristallen von Ecdyson. *Chem. Ber.* 1965; **98**: 2353–2360.

Huberman A, Hernandez-Arana A, Aguilar M, Rojo-Dominguez A. Secondary structure of a crustacean neuropeptide hormone family by means of CD. *Peptides* 1989; **10**: 1113–1115.

Huberman A, Aguilar MB, Brew K, Shabanowitz J, Hunt DF. 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* 1993; **14**: 7–16.

Jaros PP, Keller R. Immunocytochemical identification of hyperglycemic hormone-producing cells in the eyestalk of *Carcinus maenas. Cell Tissue Res.* 1979; **204**: 379–385.

Jasmani S, Kawazoe I, Shih TW, Suzuki Y, Aida K. Hemolymph vitellogenin levels during ovarian development in the kuruma prawn *Penaeus japonicus. Fish. Sci.* 2000; **66**: 535–539.

Katakura Y, Fujimaki Y, Unno K. Partial purification and characterization of androgenic gland hormone from the isopod crustacean, *Armadillidium vulgare. Annot. Zool. Japon.* 1975; **48**: 203–209.

Katayama H, Nagasawa H. Effect of a glycine residue insertion into crustacean hyperglycemic hormone on hormonal activity. *Zool. Sci.* 2004; **21**: 1121–1124.

Katayama H, Ohira T, Nagata K, Nagasawa H. 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.* 2001; **65**: 1832–1839.

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

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

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

Katayama H, Hojo H, Ohira T, Ishii A, Nozaki T, Goto K, Nakahara Y, Takahashi T, Hasegawa Y, Nagasawa H, Nakahara Y. Correct disulfide pairing is required for the biological activity of crustacean androgenic gland hormone (AGH): Synthetic studies of AGH. *Biochemistry* 2010; **49**: 1798–1807.

Kawakami A, Kataoka H, Oka T, Mizoguchi A, Kimura-Kawakami M, Adachi T, Iwami M, Nagasawa H, Suzuki A, Ishizaki H. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science* 1990; **247**: 1333–1335.

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

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

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

Khalaila I, Katz T, Abdu U, Yehezkel G, Sagi A. Effects of implantation of hypertrophied androgenic glands on sexual characters and physiology of the reproductive system in the female red claw crayfish, *Cherax quadricarinatus. Gen. Comp. Endocrinol.* 2001; **121**: 242–249.

Khalaila I, Manor R, Weil S, Granot Y, Keller R, Sagi A. The eyestalk-androgenic gland-testis endocrine axis in the crayfish, *Cherax quadricarinatus. Gen. Comp. Endocrinol.* 2002; **127**: 147–156.

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

Klein JM, De Kleijn DPV, Keller R, Weidemann WM. Molecular cloning of crustacean pigment dispersing hormone precursor. *Biochem. Biophys. Res. Commun.* 1992; **189**: 1509–1514.

Klein JM, De Kleijn DPV, Hünemeyer G, Keller R, Weidemann WM. Demonstration of the cellular expression of genes encoding molt-inhibiting hormone and crustacean hyperglycemic hormone in the eyestalk of the shore crab *Carcinus maenas. Cell Tissue Res.* 1993; **274**: 515–519.

Klein JM, Mohrherr CJ, Sleutels F, Riehm JP, Rao KR. Molecular cloning of two pigment-dispersing hormone (PDH) precursors in the blue crab *Callinectes sapidus* reveals a novel member of the PDH neuropeptide family. *Biochem. Biophys. Res. Commun.* 1994; **205**: 410–416.

Kromer-Metzger E, Lagueux M. Expression of the gene encoding an insulin-related peptide in *Locusta* (*Insecta, Orthoptera*). *Eur. J. Biochem.* 1994; **221**: 427–434.

Krungkasem C, Ohira T, Yang WJ, Abdullah R, Nagasawa H, Aida K. Identification of two distinct molt-inhibiting hormone-related peptides from the giant tiger prawn *Penaeus monodon. Mar. Biotechnol.* 2002; **4**: 132–140.

Lacombe C, Gréve P, Martin G. Overview on the subgrouping of the crustacean hyperglycemic hormone family. *Neuropeptides* 1999; **33**: 71–80.

Leuven RS, Jaros PP, Van Herp F, Keller R. Species or group specificity in biological and immunological studies of crustacean hyperglycemic hormone. *Gen. Comp. Endocrinol.* 1982; **46**: 288–296.

Mak ASC, Choi CL, Tiu SHK, Hui JHL, He JG, Tobe SS, Chan SM. Vitellogenesis in the red crab *Charybdis*

feriatus: Hepatopancreas-specific expression and farnesoic acid stimulation of vitellogenin gene expression. *Mol. Reprod. Dev.* 2005; **70**: 288–300.

Manor R, Weil S, Oren S, Glazer L, Aflalo ED, Ventura T, Chalifa-Caspi V, Lapidot M, Sagi A. Insulin and gender: an insulin-like gene expressed exclusively in the androgenic gland of the male crayfish. *Gen. Comp. Endocrinol.* 2007; **150**: 326–336.

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

Marco HG, Avarre JC, Lubzens E, Gäde G. In search of a vitellogenesis-inhibiting hormone from the eyestalks of the South African spiny lobster, *Jasus lalandii*. *Invert. Reprod. Dev.* 2002; **41**: 143–150.

Martin G, Juchault P, Sorokine O, van Dorsselaer A. Purification and characterization of androgenic hormone from the terrestrial isopod *Armadillidium vulgare* Latr. (Crustacea, Oniscidea). *Gen. Comp. Endocrinol.* 1990; **80**: 349–354.

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

Martin G, Sorokine O, Moniatte M, Van Dorsselaer A. The androgenic gland hormone of the crustacean isopod *Armadillidium vulgare*. *Ann. N.Y. Acad. Sci.* 1998; **839**: 111–117.

Martin G, Sorokine O, Moniatte M, Bulet P, Hetru C, Van Dorsselaer A. The structure of a glycosylated protein hormone responsible for sex determination in the isopod, *Armadillidium vulgare*. *Eur. J. Biochem.* 1999; **262**: 727–736.

Mettulio R, Giulianini PG, Ferrero EA, Lorenzon S, Edomi P. Functional analysis of crustacean hyperglycemic hormone by *in vivo* assay with wild-type and mutant recombinant proteins. *Regul. Pept.* 2004; **119**: 189–197.

Mordue W, Stone JV. Comparison of the biological activities of an insect and a crustacean neurohormone that are structurally similar. *Nature* 1976; **264**: 287–289.

Muir TW, Sondhi D, Cole PA. Expressed protein ligation: A general method for protein engineering. *Proc. Natl. Acad. Sci. USA* 1998; **95**: 6705–6710.

Nagai C, Asazuma H, Nagata S, Ohira T, Nagasawa H. A convenient method for preparation of biologically active recombinant CHH of the kuruma prawn, *Marsupenaeus japonicus*, using the bacterial expression system. *Peptides* 2009; **30**: 507–517.

Nagamine C, Knight AW, Maggenti A, Paxman G. Masculinization of female *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) by androgenic gland implantation. *Gen. Comp. Endcrinol.* 1980; **41**: 442–457.

Nagasawa H, Hasegawa Y, Haino-Fukushima K, Hatayama H, Yanagisawa T, Katakura Y. Isolation and structural determination of seminal vesicle-specific peptides of the terrestrial isopod, *Armadillidium vulgare*. *Biosci. Biotechnol. Biochem.* 1995; **59**: 1246–1250.

Nagasawa H, Yang WJ, Shimizu H, Aida K, Tsutsumi H, Terauchi A, Sonobe H. Isolation and amino acid sequence of a molt-inhibiting hormone from the American crayfish, *Procambarus clarkii*. *Biosci. Biotechnol. Biochem.* 1996;

60: 554–556.

Nagasawa H, Yang WJ, Aida K, Sonobe H. Chemical and biological characterization of neuropeptides in the sinus glands of the kuruma prawn, *Penaeus japonicus*. *In*: Shimonishi Y (ed). *Peptide Science—Present and Future*. Kluwer Academic Publishers, Great Britain. 1999; 453–454.

Nakatsuji T, Sonobe H. Measurement of molt-inhibiting hormone titer in hemolymph of the American crayfish, *Procambarus clarkii*. *Zool. Sci.* 2003; **20**: 999–1001.

Nakatsuji T, Sonobe H. Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish, *Procambarus clarkii*. *Gen. Comp. Endocrinol.* 2004; **135**: 358–364.

Nakatsuji T, Sonobe H, Watson RD. Molt-inhibiting hormone-mediated regulation of ecdysteroid synthesis in Y-organ of the crayfish (*Procambarus clarkii*): involvement of cyclic GMP and cyclic nucleotide phosphodiesterase. *Mol. Cell. Endocrinol.* 2006; **253**: 76–82.

Nakatsuji T, Lee CY, Watson RD. Crustacean molt-inhibiting hormone: Structure, function, and cellular mode of action. *Comp. Biochem. Physiol. A* 2009; **152**: 139–148.

Ohira T. Molecular biological studies on crustacean hyperglycemic hormone-family peptides of the kuruma prawn *Penaeus japonicus*. PhD Thesis, The University of Tokyo, Tokyo, 2000.

Ohira T, Watanabe T, Nagasawa H, Aida K. Cloning and sequence analysis of a cDNA encoding a crustacean hyperglycemic hormone from the Kuruma prawn *Penaeus japonicus*. *Mol. Mar. Biol. Biotechnol.* 1997a; **6**: 59–63.

Ohira T, Watanabe T, Nagasawa H, Aida K. Molecular cloning of cDNAs encoding four crustacean hyperglycemic hormones and a molt-inhibiting hormone from the kuruma prawn *Penaeus japonicus*. *In*: Kawashima S, Kikuyama S (eds). *Proceedings of the XIII International Congress of Comparative Endocrinology*. Monduzzi Editore, Bologna. 1997b; 83–86.

Ohira T, Watanabe T, Nagasawa H, Aida K. Molecular cloning of a molt-inhibiting hormone cDNA from the kuruma prawn *Penaeus japonicus*. *Zool. Sci.* 1997c; **14**: 785–789.

Ohira T, Nishimura T, Sonobe H, Okuno A, Watanabe T, Nagasawa H, Kawazoe I, Aida K. Expression of a recombinant molt-inhibiting hormone of the kuruma prawn *Penaeus japonicus* in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* 1999; **63**: 1576–1581.

Ohira T, Nagasawa H, Aida K. Molecular cloning of cDNAs encoding two pigment-dispersing hormones and two corresponding genes from the kuruma prawn (*Penaeus japonicus*). *Mar. Biotechnol.* 2002; **4**: 463–470.

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

Ohira T, Hasegawa Y, Tominaga S, Okuno A, Nagasawa H. Molecular cloning and expression analysis of cDNAs encoding androgenic gland hormone precursors from two Porcellionidae species, *Porcellio scaber* and *P. dilatatus*. *Zool. Sci.* 2003b; **20**: 75–81.

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

259–268.

Ohira T, Okumura T, Suzuki M, Yajima Y, Tsutsui N, Wilder MN, Nagasawa H. Production and characterization of recombinant vitellogenesis-inhibiting hormone from the American lobster *Homarus americanus*. *Peptides* 2006; **27**: 1251–1258.

Okumura T, Ohira T, Katayama H, Nagasawa H. *In vivo* effects of a recombinant molt-inhibiting hormone on molt interval and hemolymph ecdysteroid level in the kuruma prawn, *Marsupenaeus japonicus*. *Zool. Sci.* 2005; **22**: 317–320.

Okuno A, Hasegawa Y, Nagasawa H. Purification and properties of androgenic gland hormone from the terrestrial isopod *Armadillidium vungare*. *Zool. Sci.* 1997; **14**: 837–842.

Okuno A, Hasegawa Y, Ohira T, Katakura Y, Nagasawa H. Characterization and cDNA cloning of androgenic gland hormone of the terrestrial isopod *Armadillidium vulgare*. *Biochem. Biophys. Res. Commun.* 1999; **264**: 419–423.

Okuno A, Hasegawa Y, Ohira T, Nagasawa H. Immunological identification of crustacean androgenic gland hormone, a glycopeptide. *Peptides* 2001; **22**: 175–181.

Okuno A, Yang WJ, Jayasankar V, Saido-Sakanaka H, Huong DTT, Jasmani S, Atmomarsono M, Subramoniam T, Tsutsui N, Ohira T, Kawazoe I, Aida K, Wilder MN. Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. *J. Exp. Zool.* 2002a; **292**: 417–429.

Okuno A, Hasegawa Y, Nishiyama M, Ohira T, Kurihara M, Mastumoto S, Nagasawa H. Preparation of an active recombinant peptide of crustacean androgenic gland hormone. *Peptides* 2002b; **23**: 567–572.

Papadopoulos NM, Hess WC. Determination of neuraminic (sialic) acid, glucose and fructose in spinal fluid. *Arch. Biochem. Biophys.* 1960; **88**: 167–171.

Park JH, Hall JC. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J. Biol. Rhythms*. 1998; **13**: 219–228.

Park JH, Helfrich-Forster C, Lee G, Liu L, Rosbash M, Hall JC. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 2000; **97**: 3608–3613.

Phillips JM, Rao KR, Riehm JP, Morgan WT. Isolation and characterization of a pigment-dispersing hormone from the shrimp, *Penaeus aztecus*. *Soc. Neurosci. Abstr.* 1988; **14**: 534.

Rao KR, Riehm JP. The pigment-dispersing hormone family: Chemistry structure-activity relations, and distribution. *Biol. Bull.* 1989; **177**: 225–229.

Rao KR, Riehm JP. Pigment-dispersing hormones. *Ann. N.Y. Acad. Sci.* 1993; **680**: 78–88.

Rao KR, Kleinhlz LH, Riehm JP. Characterization of three forms of pigment-dispersing hormone from the shrimp, *Pandalus jordani*. *Soc. Neurosci. Abstr.* 1989; **15**: 367.

Raviv S, Parnes S, Segall C, Davis C, Sagi A. Complete sequence of *Litopenaeus vannamei* (Crustacea: Decapoda) vitellogenin cDNA and its expression in endocrinologically induced sub-adult females. *Gen. Comp. Endocrinol.* 2006; **145**: 39–50.

Rewitz KF, Gilbert LI. Daphnia Halloween genes encode cytochrome P450s mediating the synthesis of the arthropod molting hormones: evolutionary implications. *BMC Evol. Biol.* 2008; **36**: 188–199.

Rotllant G, De Kleijn D, Charmantier-Daures M, Charmantier G, Van Herp F. Localization of crustacean hyperglycemic hormone (CHH) and gonad-inhibiting hormone (GIH) in the eyestalk of *Homarus gammarus* larvae by immunocytochemistry and *in situ* hybridization. *Cell Tissue Res.* 1993; **271**: 507–512.

Saidi BN, Besse SD, Webster SG, Sedlmeier D, Lachaise F. Involvement of cAMP and cGMP in the mode of action of molt-inhibiting hormone (MIH) a neuropeptide which inhibits steroidogenesis in a crab. *Mol. Cell. Endocrinol.* 1994; **102**: 53–61.

Santos EA, Keller R. Crustacean hyperglycemic hormone (CHH) and the regulation of carbohydrate metabolism: current perspectives. *Comp. Biochem. Physiol. A* 1993; **106**: 405–411.

Schütt C, Nöthiger R. Structure, function and evolution of sex-determining systems in Dipteran insects. *Development* 2000; **127**: 667–677.

Shih TW, Suzuki Y, Nagasawa H, Aida K. Immunohistochemical localization of crustacean hyperglycemic hormones (CHHs) and a molt-inhibiting hormone (MIH) in the eyestalk of *Penaeus japonicus*. *In*: Kawashima S, Kikuyama S (eds). *Proceedings of the XIII International Congress of Comparative Endocrinology*. Monduzzi Editore, Bologna. 1997; 87–91.

Shih TW, Suzuki Y, Nagasawa H, Aida K. Immunohistochemical identification of hyperglycemic hormone- and molt-inhibiting hormone-producing cells in the eyestalk of the kuruma prawn, *Penaeus japonicus*. *Zool. Sci.* 1998; **15**: 389–397.

Sonobe H, Kamba M, Ohta K, Ikeda M, Naya Y. *In vitro* secretion of ecdysteroids by Y-organ of the crayfish, *Procambarus clarkii*. *Experientia* 1991; **47**: 949–952.

Soyez D. Occurrence and diversity of neuropeptides from the crustacean hyperglycemic hormone family in arthropods. *Ann. N.Y. Acad. Sci.* 1997; **814**: 319–323.

Soyez D, Van Deijnen JE, Martin M. Isolation and characterization of a vitellogenesis-inhibiting factor from sinus glands of the lobster, *Homarus americanus*. *J. Exp. Zool.* 1987; **244**: 479–484.

Soyez D, Le Caer JP, Noel PY, Rossier J. Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster *Homarus americanus*. *Neuropeptides* 1991; **20**: 25–32.

Soyez D, Van Herp F, Rossier J, Le Caer JP, Tensen CP, Lafont R. Evidence for a conformational polymorphism of invertebrate neurohormones. ᴅ-Amino acid residue in crustacean hyperglycemic peptides. *J. Biol. Chem.* 1994; **269**: 18295–18298.

Spanings-Pierrot C, Soyez D, Van Herp F, Gompel M, Skaret G, Grousset E, Charmantier G. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol.* 2000; **119**: 340–350.

Suzuki S, Yamasaki K. Sex reversal by implantations of ethanol-treated androgenic glands of female isopods, *Armadillidium vulgare* (Malacostraca, Crustacea). *Gen. Comp. Endocrinol.* 1998; **111**: 367–375.

Suzuki S, Yamasaki K, Katakura Y. Vitellogenin synthesis

in andrectomized male of the terrestrial isopoda, *Armadillidium vulgare* (Malacostracan crustacea). *Gen. Comp. Endocrinol.* 1990; **77**: 283–291.

Tensen CP, Verhoeven AH, Gaus G, Janssen KP, Keller R, Van Herp F. Isolation and amino acid sequence of crustacean hyperglycemic hormone precursor-related peptides. *Peptides* 1991; **12**: 673–681.

Treerattrakool S, Udomkit A, Eurwilaichitr L, Sonthayanon B, Panyim S. Expression of biologically active crustacean hyperglycemic hormone (CHH) of *Penaeus monodon* in *Pichia pastoris. Mar. Biotechnol.* 2003; **5**: 373–379.

Truman JW. Hormonal control of insect ecdysis: endocrine cascades for coordinating behavior with physiology. *Vitam. Horm.* 2005; **73**: 1–30.

Tsang WS, Quackenbush LS, Chow BKC, Tiu SHK, He JG, Chan SM. Organization of the shrimp vitellogenin gene: evidence of multiple genes and tissue specific expression by the ovary and hepatopancreas. *Gene* 2003; **303**: 99–109.

Tseng DY, Chen YN, Kou GH, Lo CF, Kuo CM. Hepatopancreas is the extraovarian site of vitellogenin synthesis in black tiger shrimp, *Penaeus monodon. Comp. Biochem. Physiol. A* 2001; **129**: 909–917.

Tsutsui N, Kawazoe I, Ohira T, Jasmani S, Yang WJ, Wilder MN, Aida K. Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus. Zool. Sci.* 2000; **17**: 651–660.

Tsutsui N, Saido-Sakanaka H, Yang WJ, Jayasankar V, Jasmani S, Okuno A, Ohira T, Okumura T, Aida K, Wilder MN. Molecular characterization of a cDNA encoding vitellogenin in the coonstriped shrimp, *Pandalus hypsinotus* and site of vitellogenin mRNA expression. *J. Exp. Zool.* 2004; **301A**: 802–814.

Tsutsui N, Katayama H, Ohira T, Nagasawa H, Wilder MN, Aida K. The effects of crustacean hyperglycemic hormone-family peptides on vitellogenin gene expression in the kuruma prawn, *Marsupenaeus japonicus. Gen. Comp. Endocrinol.* 2005a; **144**: 232–239.

Tsutsui N, Kim YK, Jasmani S, Ohira T, Wilder MN, Aida K. The dynamics of vitellogenin gene expression differs between intact and eyestalk ablated kuruma prawn, *Penaeus* (*Marsupenaeus*) *japonicus. Fish. Sci.* 2005b; **71**: 249–256.

Tsutsui N, Ohira T, Kawazoe I, Takahashi A, Wilder MN. Purification of sinus gland peptides having vitellogenesis-inhibiting activity from the whiteleg shrimp *Litopenaeus vannamei. Mar. Biotechnol.* 2007; **9**: 360–369.

Tsutsui N, Nagakura-Nakamura A, Nagai C, Ohira T, Wilder MN, Nagasawa H. The *ex vivo* effects of eyestalk peptides on ovarian vitellogenin gene expression in the kuruma prawn *Marsupenaeus japonicus. Fish. Sci.* 2013; **79**: 33–38.

Udomkit A, Chooluck S, Sonthayanon B, Panyim S. Molecular cloning of a cDNA encoding a member of CHH/MIH/GIH family from *Penaeus monodon* and analysis of its gene structure. *J. Exp. Mar. Biol. Ecol.* 2000; **244**: 145–156.

Ventura T, Manor R, Aflalo ED, Weil S, Raviv S, Glazer L, Sagi A. Temporal silencing of an androgenic gland-specific insulin-like gene affecting phenotypical gender differences and spermatogenesis. *Endocrinology* 2009; **150**: 1278–1286.

Von Heijne G. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 1986; **14**: 4683–4690.

Wainwright G, Webster SG, Wilkinson MC, Chung JS, Rees HH. Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus. J. Biol. Chem.* 1996; **271**: 12749–12754.

Warrier S, Subramoniam T. Receptor mediated yolk protein uptake in the crab *Scylla serrata*: crustacean vitellogenin receptor recognizes related mammalian serum lipoproteins. *Mol. Reprod. Dev.* 2002; **61**: 536–548.

Webster SG. Amino acid sequence of putative moult-inhibiting hormone from the crab *Carcinus maenas. Proc. R. Soc. Lond. B* 1991; **244**: 247–252.

Webster SG, Keller R. Purification, characterization and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). *J. Comp. Physiol. B* 1986; **156**: 617–624.

Weidemann W, Gromoll J, Keller R. Cloning and sequence analysis of cDNA for precursor of a crustacean hyperglycemic hormone. *FEBS Lett.* 1989; **257**: 31–34.

Wilder MN, Okumura T, Tsutsui N. Reproductive mechanisms in Crustacea focusing on selected prawn species: vitellogenin structure, processing and synthetic control. *Aqua-BioScience Monographs* 2010; **3**: 73–110.

Yamano K, Unuma T. Expressed sequence tags from eyestalk of kuruma prawn, *Marsupenaeus japonicus. Comp. Biochem. Physiol. A* 2006; **143**: 155–161.

Yang F, Xu HT, Dai ZM, Yang WJ. Molecular characterization and expression analysis of vitellogenin in the marine crab *Portunus trituberculatus. Comp. Biochem. Physiol. B* 2005; **142**: 456–464.

Yang WJ. Structures and functions of peptide hormones in the sinus gland of the kuruma prawn *Penaeus japonicus*. PhD Thesis, The University of Tokyo, Tokyo, 1997.

Yang WJ, Aida K, Nagasawa H. Amino acid sequences of a hyperglycaemic hormone and its related peptides from the Kuruma prawn, *Penaeus japonicus. Aquaculture* 1995; **135**: 205–212.

Yang WJ, Aida K, Terauchi A, Sonobe H, Nagasawa H. Amino acid sequence of a peptide with molt-inhibiting activity from the kuruma prawn *Penaeus japonicus. Peptides* 1996; **17**: 197–202.

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

Yang WJ, Aida K, Nagasawa H. Characterization of chromatophorotropic neuropeptides from the kuruma prawn *Penaeus japonicus. Gen. Comp. Endocrinol.* 1999; **114**: 415–424.

Yang WJ, Ohira T, Tsutsui N, Subramoniam T, Huong DTT, Aida K, Wilder MN. Determination of amino acid sequence and site of mRNA expression of four vitellins in the giant freshwater prawn, *Macrobrachium rosenbergii. J. Exp. Zool.* 2000; **287**: 413–422.

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

Zeleny C. Compensatory regulation. *J. Exp. Zool.* 1905; **2**: 1–102.

Zheng J, Lee CY, Watson RD. Molecular cloning of a putative receptor quanylyl cyclase from Y-organs of the blue crab, *Callinectes sapidus*. *Gen. Comp. Endocrinol.* 2006; **146**: 329–336.

Zheng J, Nakatsuji T, Roer RD, Watson RD. Studies of a receptor guanylyl cyclase cloned from Yorgans of the blue crab (*Callinectes sapidus*), and its possible functional link to ecdysteroidogenesis. *Gen. Comp. Endocrinol.* 2008; **155**: 780–788.

Zmora N, Trant JM, Zohar JS, Chung JS. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 2: An ovarian stage dependent involvement. *Saline System* 2009; **5**: 6.