Immunohistochemical Identification of Hyperglycemic Hormone- and Molt-Inhibiting Hormone-Producing Cells in the Eyestalk of the Kuruma Prawn, Penaeus japonicus

Authors: Shih, Tung-Wei, Suzuki, Yuzuru, Nagasawa, Hiromichi, and Aida, Katsumi

Source: Zoological Science, 15(3) : 389-397

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.15.389

ZOOLOGICAL SCIENCE **15**: 389–397 (1998)

Immunohistochemical Identification of Hyperglycemic Hormone- and Molt-Inhibiting Hormone-Producing Cells in the Eyestalk of the Kuruma Prawn, *Penaeus japonicus*

Tung-Wei Shih[1], Yuzuru Suzuki[1], Hiromichi Nagasawa[2,†] and Katsumi Aida[1]*

[1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*
[2]*Ocean Research Institute, The University of Tokyo, Nakano-ku, Tokyo 164, Japan*

ABSTRACT—This study deals with the localization of crustacean hyperglycemic hormone (CHH, Pej-SGP-III) and molt-inhibiting hormone (MIH, Pej-SGP-IV) in the eyestalk of the kuruma prawn *Penaeus japonicus* using immunohistochemistry. High-titer and highly specific antisera were raised in rabbits against synthetic Pej-SGP-III C-terminal peptide (Glu-Glu-His-Met-Ala-Ala-Met-Gln-Thr-Val-NH_2) and Pej-SGP-IV C-terminal peptide (Val-Trp-Ile-Ser-Ile-Leu-Asn-Ala-Gly-Gln-OH), both of which were conjugated with bovine serum albumin by a cross linker. Eyestalks were removed from mature male prawns at the intermolt stage of the molting cycle and fixed in Bouin's solution. Serial sections stained immunohistochemically showed that neurosecretory cells of Pej-SGP-III and Pej-SGP-IV were located in the same cluster of the medulla terminalis ganglionic X-organ (MTGX), and that three kinds of neurosecretory cells, which were stained with anti-Pej-SGP-III antiserum and/or anti-Pej-SGP-IV antiserum were present. The number of neurosecretory cells which stained with both antisera was much fewer than that of neurosecretory cells which stained with one of the antisera only. The axon and axon terminals in the sinus gland were also stained and the staining density of the sinus gland was always deeper than that of the neurosecretory cells.

INTRODUCTION

The crustacean neurosecretory system, which is comprised principally of the X-organ/sinus gland complex, is located in the eyestalk of decapod crustaceans and produces a range of neuropeptides. These neuropeptides which are known to have important roles in physiological regulation are first synthesized in the X-organ and then transferred to the sinus gland, from where they are released into hemolymph (Keller, 1992).

Among these eyestalk peptides, CHH (crustacean hyperglycemic hormone) -family peptides have been most extensively studied. This peptide family consists of some structurally similar peptides with two common characteristics in that they contain 72-76 amino acid residues and have similar amino acid sequences with conserved six cysteine residues. These CHH-family peptides include CHH (Leuven *et al.*,1982; Tensen *et al.*, 1989; Yasuda *et al.*, 1994), MIH (molt-inhibiting hormone) (Chang *et al.*, 1990; Aguilar *et al.*, 1996; Terauchi *et al.*, 1996; Webster and Keller, 1986), VIH (vitellogenesis-inhibiting hormone) or GIH (gonad-inhibiting hormone) (Soyez *et al.*, 1991; Aguilar *et al.*, 1992) and MOIH (mandibular organ-inhibiting hormone) (Wainwright *et al.*, 1996). Possibly due to structural similarity between MIH and CHH, MIH of *Homarus americanus* also exhibits a low hyperglycemic activity (Chang *et al.*, 1990) and CHH of *Carcinus maenas* shows very weak MIH activity (Webster and Keller, 1986).

Immunohistochemical studies on CHH-, MIH- and GIH-producing cells have been carried out in several crustacean species. By using antisera against CHH and MIH of *C. maenas*, CHH- and MIH-producing cells were localized in the eyestalks of *C. maenas* (Jaros and Keller, 1979), *Liocarcinus puber*, *Cancer pagurus*, *Uca pugilator* and *Maja squinado* (Dircksen *et al.*, 1988). CHH-producing cells were found in the eyestalks of *H. gammarus*, *Orconectes limosus*, *Nephrops norvegicus*, *Palinurus vulgaris*, *Macropipus puber*, *Palaemon serratus* (Gorgels-Kallen *et al.*, 1982) and the larvae and postlarvae of *Astacus leptodactylus* (Gorgels-Kallen and Meij, 1985) by using antiserum against the CHH of *A. leptodactylus*. CHH-

* Corresponding author: Tel. +81-3-3812-2111 ext. 5287; FAX. +81-3-3812-0529.
† Present address: Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

and VIH-producing cells were observed in the eyestalks of *H. americanus* by using antiserum against the CHH of *A. leptodactylus* and antiserum against the VIH of *H. americanus* (Kallen and Meusy, 1989). CHH- and GIH-producing cells were located by using antisera raised against CHH or GIH of *H. americanus* (Rotllant *et al.*, 1993). These neurosecretory cells are located in the medulla terminalis ganglionic X-organ (MTGX) and all of the antisera used in these studies were raised against native peptides.

In the kuruma prawn, *Penaeus japonicus*, six molecular species of CHH (Pej-SGP-I, II, III, V, VI and VII) and a MIH (Pej-SGP-IV) have been found in the eyestalk (Yang, 1997; Yang *et al.*, 1997). The complete amino acid sequences of these peptides have been determined and their bioactivities were investigated by *in vivo* or *in vitro* assay. The homologies between any two of CHHs (Pej-SGP-I, II, III, V, VI, and VII) were about 65-99% and the homologies between MIH (Pej-SGP-IV) and CHHs were about 30%. As in the case of the CHHs of *C. maenas* and MIH of *H. americanus*, some of these peptides exhibited a certain degree of overlapping biological activity (Yang *et al.*, 1996, 1997). The similarities in sequence between CHHs and MIH were considerably low, which permitted us to classify these peptides into two types, Type I and II (Yang *et al.*, 1996). However, it is still unknown where these neuropeptides are synthesized.

In the present study, we examined the localization of CHH (Pej-SGP-III) and MIH (Pej-SGP-IV) neurosecretory cells immunohistochemically. We attempted to produce highly specific antisera by using synthetic partial peptides in order to localize the CHH (Pej-SGP-III) and MIH (Pej-SGP-IV) neurosecretory cells.

MATERIALS AND METHODS

Animals

Mature male kuruma prawns, *P. japonicus*, were obtained from the Momoshima Station of the Japan Sea-Farming Association in Hiroshima Prefecture, Japan. The average body weight of the prawns was about 35 g and the GSI (gonadosomatic index) was about 1. Prawns were sampled during the daytime. Molt stage was determined according to Hong (1977). Prawns at the intermolt stage C were chosen for this experiment.

Eyestalk preparation

Eyestalks were cut from live prawns and fixed for 72 hr in Bouin's solution. Before dehydration, the exoskeleton was removed from the eyestalk. Dehydration, clearing, and embedding in paraffin were carried out by conventional methods.

Extraction and purification of CHH (Pej-SGP-III) and MIH (Pej-SGP-IV)

The extraction and purification of CHH (Pej-SGP-III) and MIH (Pej-SGP-IV) were done according to the methods reported previously (Yang *et al.*, 1995).

Preparation and purification of complexes of Pej-SGP-III (63-72)-BSA and Pej-SGP-IV (68-77)-BSA

C-terminal amino acid sequences of Pej-SGP-III (Glu-Glu-His-Met-Ala-Ala-Met-Gln-Thr-Val-NH_2) and -IV (Val-Trp-Ile-Ser-Ile-Leu-Asn-Ala-Gly-Gln-OH) were chosen as antigens. A cysteine residue was added to the N-terminus of each synthetic peptide. The two peptides were synthesized on a peptide synthesizer (Applied Biosystems model 430A). Deprotection and cleavage from the resin were carried out according to the manufacture's instructions. The resulting peptides were purified by reversed-phase HPLC using a Senshu Pak ODS-H-4151 column (10 $\phi \times$ 150 mm, Senshu Kagaku, Tokyo). The purified peptides were conjugated with a carrier protein, bovine serum albumin (BSA), which had been coupled with N-succinimidyl 3-(2-pyridyldithio)propionate (Pierce). The reaction products were purified on a PD-10 column (Pharmacia Biotech).

Preparation of antisera

About 200 μg each of two synthetic peptide-BSA complexes were injected into two rabbits at every injection. For the first and second injections, the complexes were dissolved in 0.5 ml of phosphate buffer (10 mM, pH 7.2, 0.9% NaCl) and emulsified with 0.5 ml of complete Freund's adjuvant (Difco Laboratory, Michigan, USA). For subsequent injections, the complexes were prepared by the same methods described above except for emulsifying with incomplete Freund's adjuvant (Wako, Osaka). Injections were done once every 2 weeks for 6 months. All the rabbits were immunized by subcutaneous and intra-

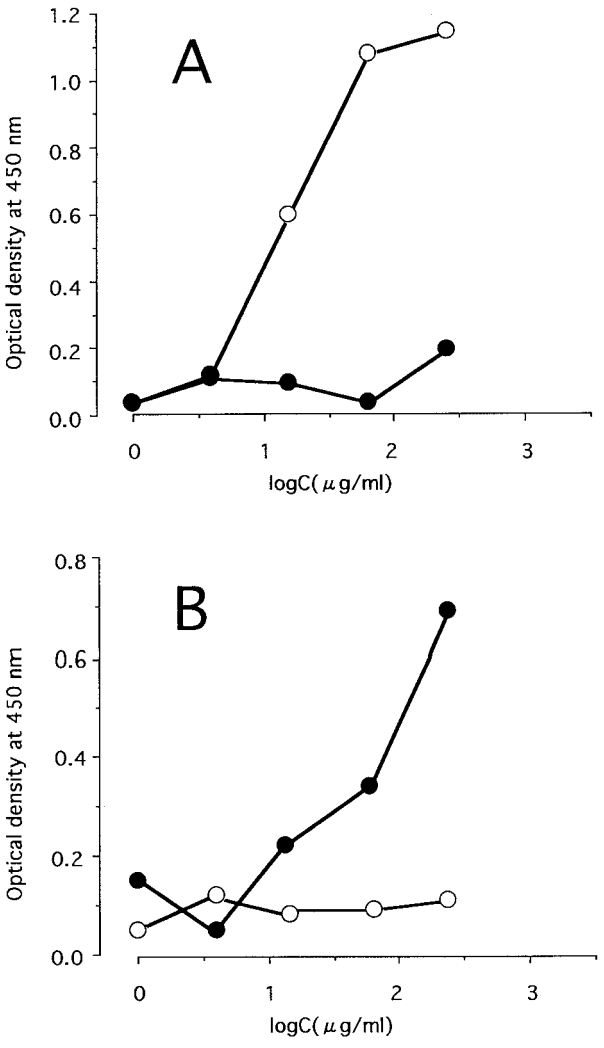

Fig. 1. Specificities of two antisera as determined by ELISA. (**A**) and (**B**) show the results using of anti-Pej-SGP-III and anti-Pej-SGP-IV antisera, respectively. Pej-SGP-III is indicated by open circles and Pej-SGP-IV by closed circles.

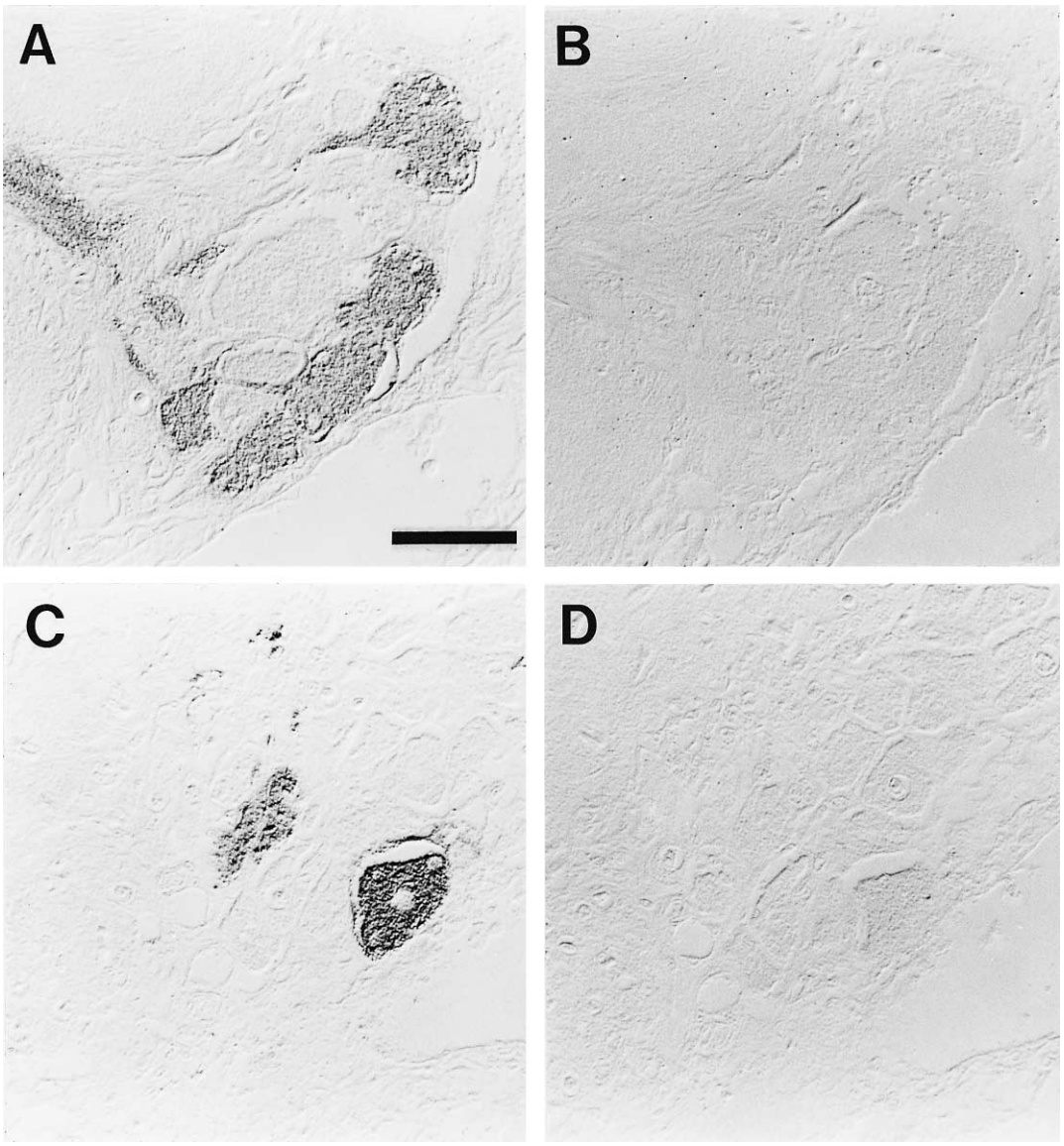

Fig. 2. Pre-absorption test of Pej-SGP-III and -IV antisera by using homologous peptides. (**A**) Pej-SGP-III-immunopositive neurosecretory cells in the MTGX. (**B**) control to **A**, an adjacent section stained by antiserum pre-absorbed with Pej-SGP-III. (**C**) Pej-SGP-IV-immunopositive neurosecretory cells in the MTGX. (**D**) control to **C**, an adjacent section stained by antiserum pre-absorbed with Pej-SGP-IV. Scale bar represents 50 μm.

dermal routes except for the first injection when the samples were injected into the inguinal lymph nodes of the rabbits. Three days after the last injection, the rabbits were exsanguinated under anaesthesia by injecting pentobarbital sodium (Diamabot LTD, USA). The blood was kept at 4°C over night and centrifuged. The antisera were stored at -80°C.

Enzyme-linked immunosorbent assay (ELISA)

The purified Pej-SGP-III (0.98 - 250 ng/ml) and -IV (0.98 - 250 ng/ml) were serially diluted with coating buffer consisting of 0.015 M Na_2CO_3, 0.35 M $NaHCO_3$ and 0.05% NaN_3, and were coated onto 96-well microplates (MaxiSorp™, InterMed Nunc) at 4°C overnight. The plates were washed four times with washing buffer (0.07 M Na_2HPO_4, 0.04 M KH_2PO_4, 0.04 M NaCl and 0.05% Tween-20), and blocked with blocking buffer (10 mM Na_2HPO_4, 0.15 M NaCl and 0.5% gelatin) for 2 hr at room temperature. The plates were then washed again with washing buffer. Anti-Pej-SGP-III (1:100,000 dilution) and anti-Pej-SGP-IV (1:100,000 dilution) antisera were added to the wells and incubated for 2 hr and then the plates were washed as mentioned above. Biotinylated anti-rabbit IgG(H+L) goat IgG (1:1000 dilution, Vector) was applied into wells, which were incubated for 2 hr. The plates were then washed. A macromolecular complex of avidin and biotinylated horseradish peroxidase (ABC Kit, Vector) was added to each well, which was incubated for 2 hr and then washed. Thereafter, 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB, 0.42 M) dissolved in 0.1 M acetate buffer containing 1.3 mM H_2O_2 (pH 5.5) was added to each well and the plates were incubated for 10-15 min. The reaction was stopped by the addition of 50 μl of 2 M sulfuric acid. The optical density of each well was measured at 450 nm on a Microplate Reader (Tosoh).

Immunohistochemistry

Serial paraffin sections (10 μm) were deparaffinized and washed with distilled water by conventional procedures. In order to eliminate

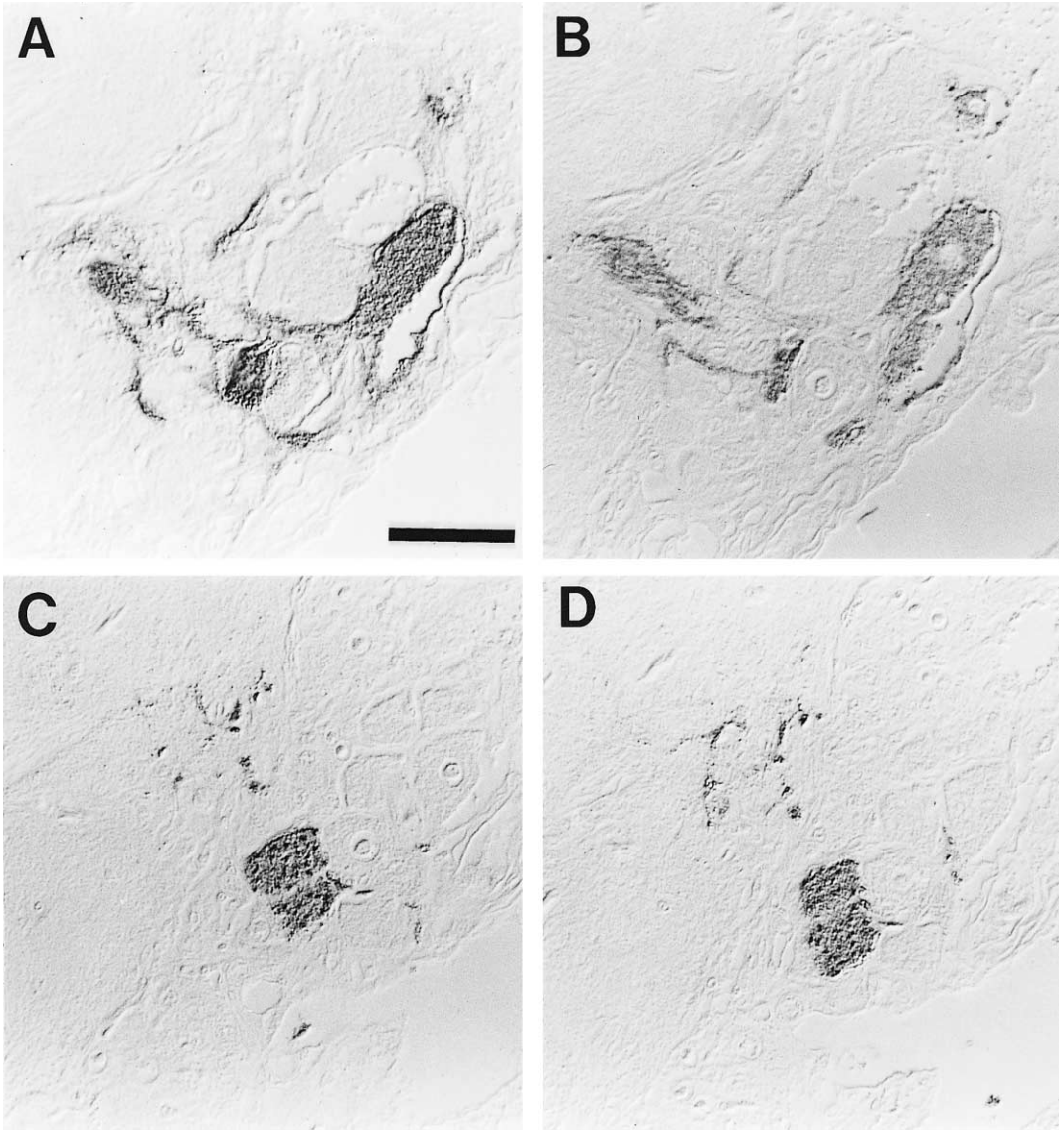

Fig. 3. Pre-absorption test of Pej-SGP-III and -IV antisera by using heterologous peptides. (**A**) Pej-SGP-III-immunopositive neurosecretory cells in the MTGX. (**B**) control to **A**, an adjacent section stained by antiserum pre-absorbed with Pej-SGP-IV. (**C**) Pej-SGP-IV-immunopositive neurosecretory cells in the MTGX. (**D**) control to **C**, an adjacent section stained by antiserum pre-absorbed with Pej-SGP-III. Scale bar represents 50 μm.

the effects of endogenous peroxidase, sections were treated with 0.3% H_2O_2 dissolved in 10 mM phosphate buffer containing 0.9% NaCl (pH 7.2) for 30 min at room temperature. This phosphate buffer was also used for the dilution of antisera and reagents described below. All procedures were done at room temperature except for incubation with the first antisera. After blocking with 10% normal goat serum (NGS) for 30 min, sections were incubated separately with the anti-Pej-SGP-III or -IV antisera (1:100,000 dilution) overnight at 4°C. Biotinylated goat anti-rabbit IgG (H+L) (1:1000 dilution, Vector) was applied to the slides for 1 hr. Subsequently, avidin and biotinylated horseradish peroxidase (ABC Kit, Vector) was added onto the sections and the slides were incubated for 30 min. Finally, 0.025% of 3,3'-diaminobenzidine (DAB, Sigma) containing 0.006% H_2O_2 was added as a substrate for color development. Excess substrate was removed by washing with distilled water before dehydrating and mounting slides. The staining reaction was stopped at 5 min for anti-Pej-SGP-III antiserum and at 10 min for anti-Pej-SGP-IV antiserum. Between each step, slides were washed twice for 5 min with phosphate buffer.

Examination of specificity of antisera

In order to investigate the specificity of the antisera, anti-Pej-SGP-III antiserum (1:100,000 dilution) was pre-incubated separately with 1 μg/ml of Pej-SGP-I, II, III, IV, V, VI, or VII at room temperature for 2 hr. Similarly, anti-Pej-SGP-IV antiserum (1:20,000 dilution) was pre-incubated separately with 1 μg/ml each peptide. Subsequently, these antisera were applied to consecutive eyestalk sections for immunohistochemistry.

To check the non-specific binding of the antisera, the primary antiserum was replaced by each of the pre-immuned rabbit sera, anti-BSA rabbit antiserum, and biotinylated anti-rabbit IgG(H+L) goat IgG for immunohistochemistry as described above.

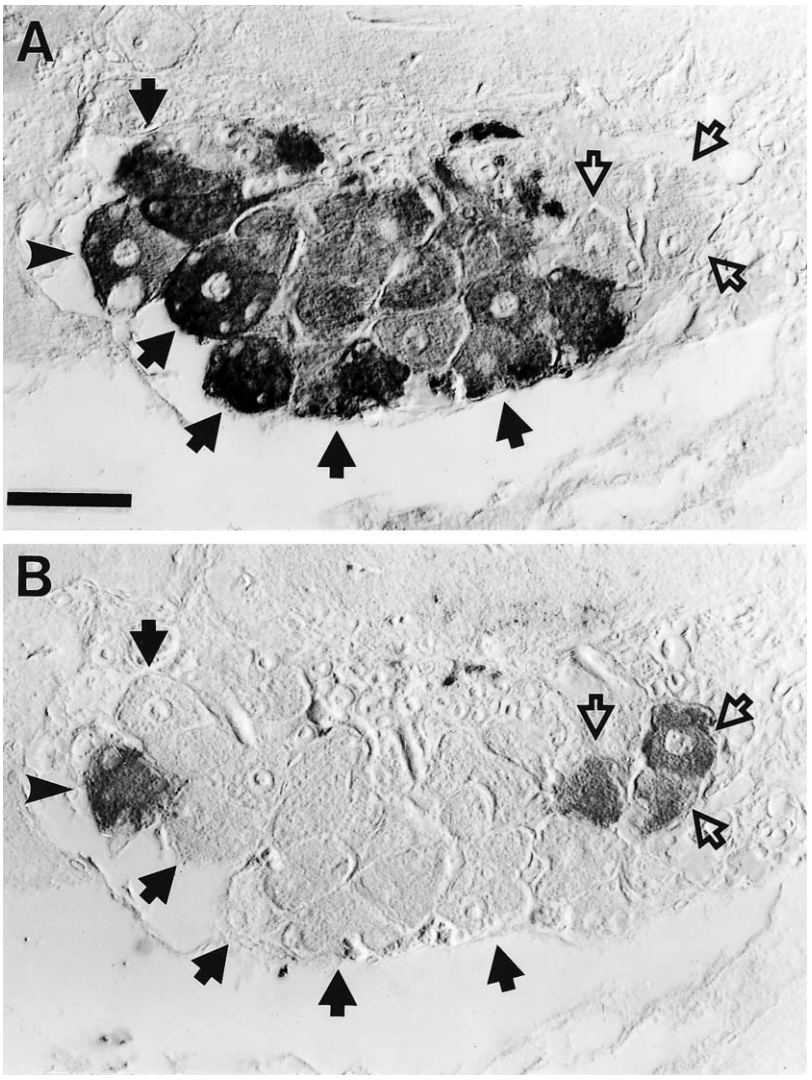

Fig. 4. Pej-SGP-III- and Pej-SGP-IV-immunopositive neurosecretory cells. (**A**) Pej-SGP-III-immunopositive neurosecretory cells in the MTGX. (**B**) Pej-SGP-IV-immunopositive neurosecretory cells in the MTGX. Arrows indicate Pej-SGP-III (➧), Pej-SGP-IV (⇨) and Pej-SGP-III and -IV (►) neurosecretory cells. Scale bar represents 50 μm.

RESULTS

Specificity of antisera

The specificity of antisera raised against Pej-SGP-III and -IV was examined by ELISA. The optical density of the solution after enzymatic reaction containing anti-Pej-SGP-III antiserum increased in a dose-dependent manner when the concentration of purified Pej-SGP-III increased from 0.98 ng/ml to 250 ng/ml. However, the similar response was not obtained even when the concentration of Pej-SGP-IV was increased to 250 ng/ml (Fig. 1A). Similarly, a dose-dependent response to the anti-Pej-SGP-IV antiserum was obtained only with purified Pej-SGP-IV, but not with purified Pej-SGP-III (Fig. 1B).

Anti-Pej-SGP-III and anti-Pej-SGP-IV antisera could be pre-absorbed completely by their respective peptides (Fig. 2B and D) but not by the heterologous peptide (Fig. 3B and D, for Pej-SGP-III and IV, respectively. Data not shown for others). Non-specific binding was not observed when the primary antibody was substituted by pre-immuned rabbit sera, anti-BSA rabbit antiserum or biotinylated anti-rabbit IgG(H+L) goat IgG (data not shown).

Localization of Pej-SGP-III and -IV neurosecretory cells and the sinus gland

The immunoreactive neurosecretory cells of Pej-SGP-III and -IV formed a cluster, which was situated at the side of the medulla terminalis ganglion (MT) near to the rostrum and attached onto the surface of the MT. There were three kinds of immunopositive neurosecretory cells in this cluster that produced Pej-SGP-III, -IV or both (Fig. 4A and B). Most of the Pej-SGP-IV neurosecretory cells existed at the periphery of a cluster of Pej-SGP-III neurosecretory cells and were more adjacent to the MT. By examining the immunopositive neurosecretory cells on every consecutive section, it was revealed that the number of Pej-SGP-III cells was most abundant (40-60) among the three kinds of immunoreactive neurosecretory

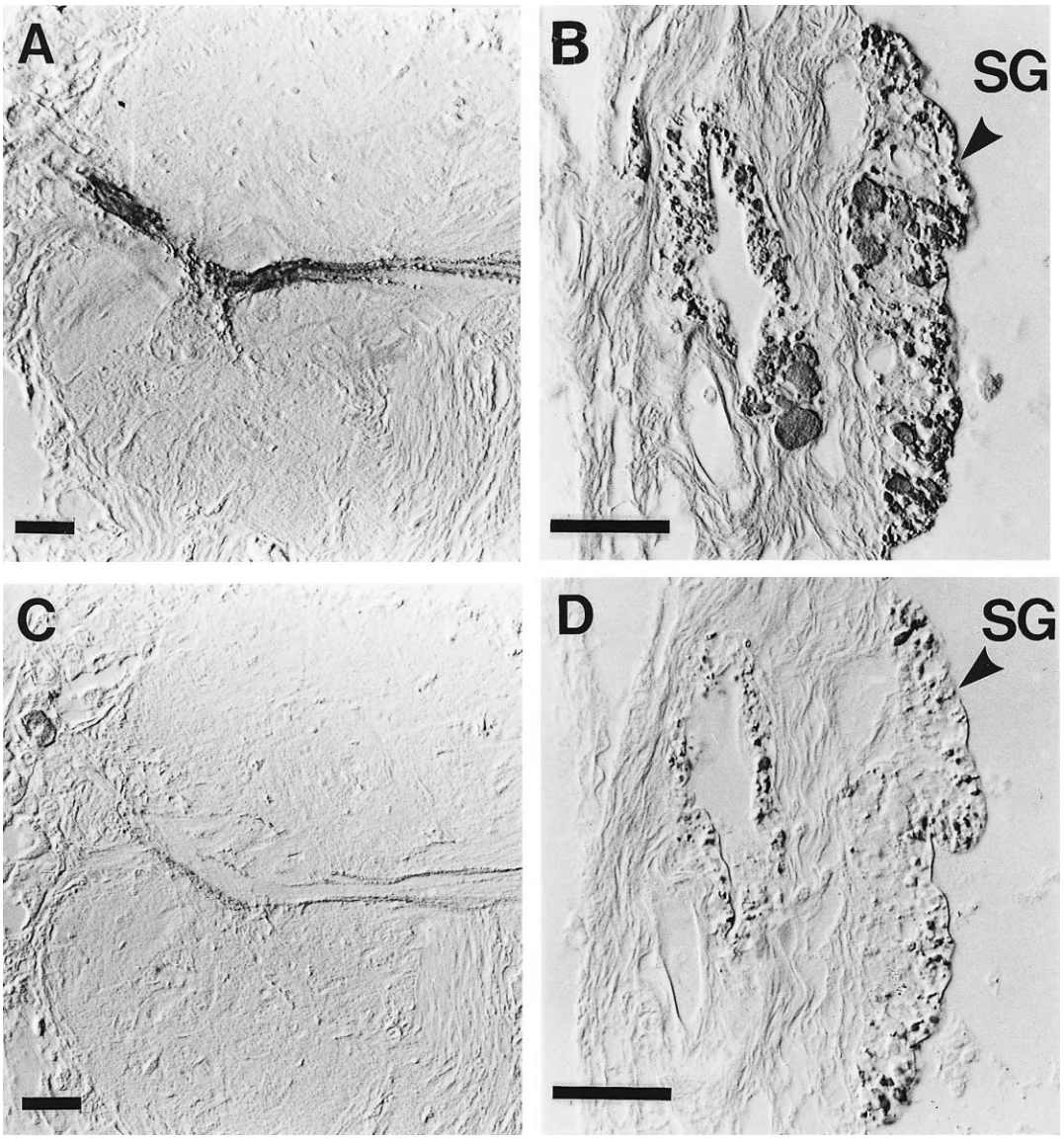

Fig. 5. Axon profiles and terminals of Pej-SGP-III and Pej-SGP-IV neurosecretory cells. (**A**) Pej-SGP-III axon profile. (**B**) Pej-SGP-IV axon profile. (**C**) Pej-SGP-III axon terminals (the sinus gland). (**D**) Pej-SGP-IV axon terminals (the sinus gland). SG, sinus gland. Scale bar represents 50 μm.

cells. Pej-SGP-IV cells were less abundant (10-20) than Pej-SGP-III cells. The number of neurosecretory cells immunoreactive to both antisera was much fewer (1-5) than those immunoreactive to one or the other. The diameter of these immunoreactive neurosecretory cells and nuclei were estimated to be about 30-50 μm and 10-11 μm.

The sinus gland was located between the MT and the medulla internalis ganglion (MI). Its position was opposite to the immunoreactive neurosecretory cells across the MT and it did not attach to the MT. Axons originating from the neurosecretory cells of Pej-SGP-III and -IV were observed to use the same track, penetrating the MT and extending to the sinus gland (Fig. 5A and B). Axon profiles and terminals of Pej-SGP-III neurosecretory cells were larger and much more abundant than those of Pej-SGP-IV neurosecretory cells (Fig. 5A-D). The staining density of the sinus gland was always deeper than that of the neurosecretory cells (Fig. 6A-B).

A composite diagram of the immunopositive neurosecretory cells in the eyestalk is schematically illustrated in Fig. 7.

DISCUSSION

In this experiment, we undertook a strategy to use C-terminal synthetic peptides rather than intact peptides as antigens for the following two reasons. Firstly, the sequence homology among seven peptides, Pej-SGP-I~VII, ranged from 30% to 99%. Except for the highest homology of 99% between Pej-SGP-V and -VI, the homologies between any two of these peptides were 30-90%. High similarities were found in the center of these molecules, which harbored six cysteine

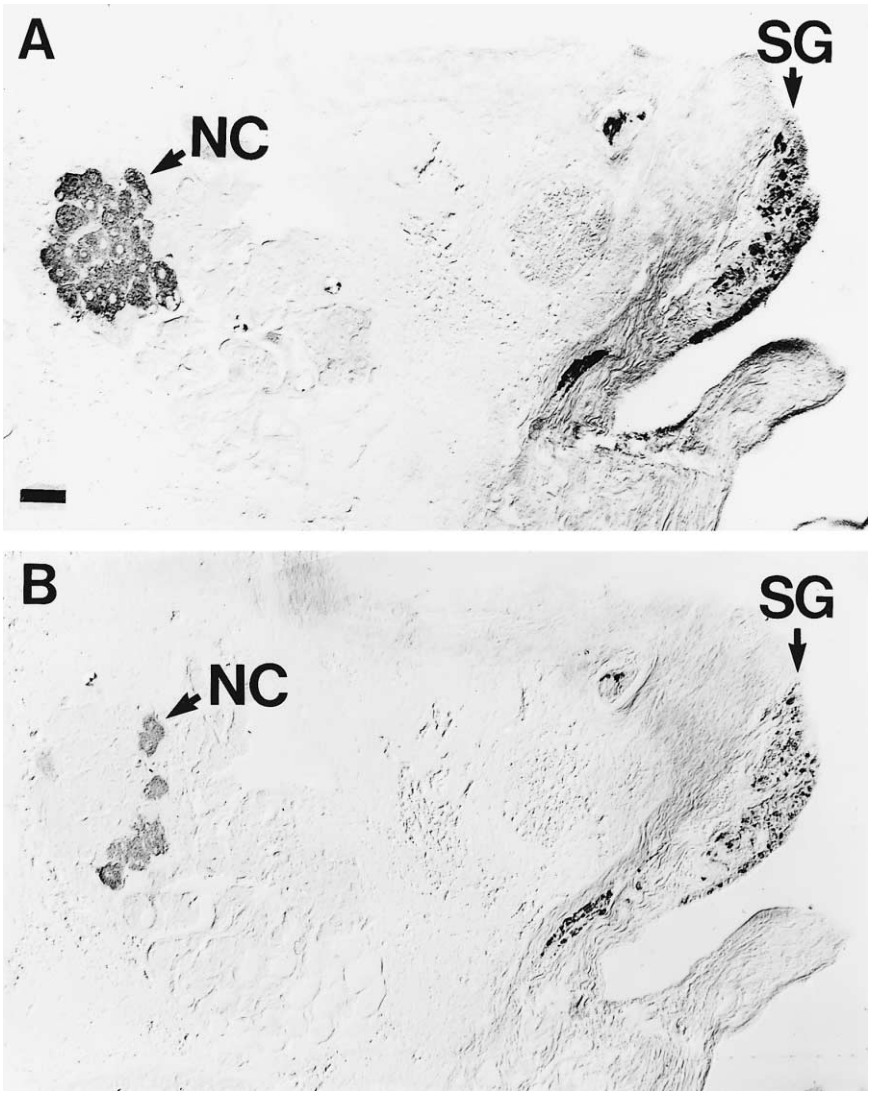

Fig. 6. The staining densities of neurosecretory cells and the sinus gland. (**A**) Pej-SGP-III neurosecretory cells and the sinus gland. (**B**) Pej-SGP-IV neurosecretory cells and the sinus gland. NC, neurosecretory cells; SG, sinus gland. Scale bar represents 50 μm.

residues, while relatively low similarities were found in the C-terminal part. Thus, the selection of the C-terminus as an antigen was expected to produce highly specific antibodies. Secondly, we considered the putative three-dimensional structures of these peptides. There are six cysteine residues in each peptide, which were found to form three intra-molecular disulfide bonds between Cys^{7} and Cys^{43}, Cys^{23} and Cys^{39}, and Cys^{26} and Cys^{52} in Pej-SGP-III (Yang *et al*., 1995). For Pej-SGP-IV, they were presumed to form disulfide bridges between Cys^{7} and Cys^{44}, Cys^{24} and Cys^{40}, and Cys^{27} and Cys^{53}, though there was no experimental evidence. Thus, these peptides would be expected to form a rather constrained three-dimensional structure from Cys^{7} to Cys^{52} (Cys^{53} in Pej-SGP-IV) resulting from the formation of the three disulfide bonds. By contrast, the other parts outside of Cys^{7} and Cys^{52} seemed to be rather flexible. The C-terminal part was thought to be more flexible than the the N-terminal part due to the longer peptide chain and, therefore, more suitable for usage as an antigen. To these authors' knowledge, this was the first application of synthetic peptides as antigens to produce antisera in order to study CHH-family peptides.

Immunostaining by anti-Pej-SGP-III antiserum could be abolished completely by pre-incubation of the antiserum with Pej-SGP-III but not with heterologous peptides, Pej-SGP-I, II, IV, V, VI and VII. Similarly, the immunostaining of anti-Pej-SGP-IV antiserum was inhibited by pre-incubation with Pej-SGP-IV but not with other heterologous peptides, Pej-SGP-I, II, III, V, VI and VII. The specificity of the two antisera was satisfactory.

Pej-SGP-III and -IV neurosecretory cells were found in the same cluster and exclusively in the MTGX. The results were similar to those from other crustacean species such as *C. maenas*, *L. puber*, *C. pagurus*, *U. pugilator* and *M. squinadao* (Dircksen *et al*., 1988). All these data showed that neurosecretory cells producing CHH-family peptides were localized in the MTGX. In this experiment, we found many cell

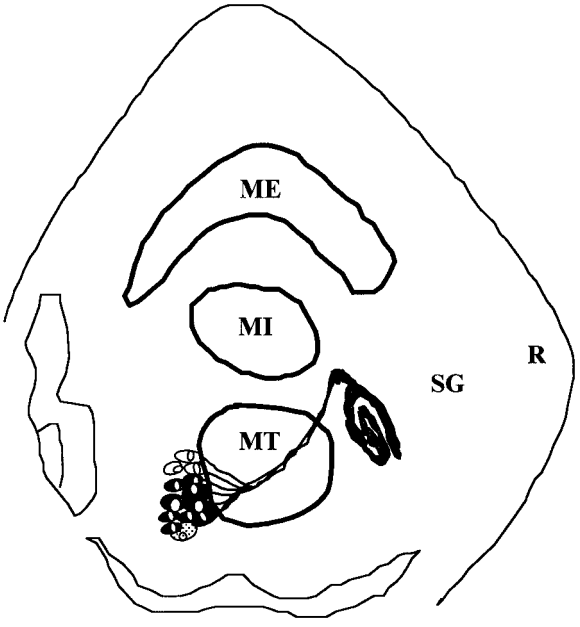

Fig. 7. Schematic representation of Pej-SGP-III and -IV neurosecretory cells in the right eyestalk. ME, medulla externa; MI, medulla interna; MT, medulla terminalis; SG, sinus gland; R, Retina.

clusters centered around the medulla externalis ganglion (ME), MI and MT, and not all of the neurosecretory cells in the MTGX could be stained by the antisera. It could not be ruled out that neurosecretory cells producing the other five peptides (Pej-SGP-I, -II, -V, -VI and VII) may exist in other clusters.

Three kinds of immuno-positive neurosecretory cells, which were stained by either anti-Pej-SGP-III, anti-Pej-SGP-IV antisera or by both, could be observed in the same cluster. Co-localization of two neuropeptides has been reported in previous the studies of CHH- and GIH- producing cells in *H. gammarus* (Rotllant *et al*., 1993) and *H. americanus* (De Kleijn *et al*., 1992) by immunohistochemistry and *in situ* hybridization. In contrast, no co-localization of MIH and CHH was observed at either mRNA or peptide levels in *C. maenas* (Klein *et al*., 1993). In *P. japonicus*, there still remains two possibilities: that either the two peptides co-exist in some cells, or alternatively, that these antisera also recognized other peptides which we have not yet been characterized. More detailed examination at the mRNA level will be required in order to obtain definite conclusions.

The diameters of Pej-SGP-III and -IV neurosecretory cells ranged from 30 to 50 μm and those of the nuclei were 10-11 μm. Their sizes were smaller than the CHH neurosecretory cells of the adult lobster *H. gammarus* (Max: 78 μm; Min: 55 μm) (Gorgels-Kallen *et al*., 1982) but close to those of *C. maenas* (cells: 30 by 40 μm; nuclei: 10 μm) (Jaros and Keller, 1979). The number of the Pej-SGP-III neurosecretory cells was more than that of Pej-SGP-IV neurosecretory cells from examination of consecutive sections. It seemed reasonable as we purified the extract of the sinus gland, that Pej-SGP-III was always more abundant than -IV.

The staining density of the sinus gland was deeper than that of the neurosecretory cell bodies, suggesting that the peptide concentration in axon terminals was higher than that in the cell bodies. Moreover, not all of neurosecretory cells had the same staining density, which suggested differing concentrations of peptides in each neurosecretory cell. Various staining densities was probably reflective of the differing synthetic activity of an individual cell. The same phenomena has also been found in other crustacean species (De Kleijn *et al*., 1992; Dircksen *et al*., 1988; Gorgels-Kallen *et al*., 1982; Kallen and Meusy, 1989).

ACKNOWLEDGMENTS

We are grateful to Mr. J. Matsumoto, the Momoshima station, Japan Sea-Farming Association for supplying us with prawns. We also thank Dr. M. N. Wilder of Japan International Research Center for Agricultural Sciences for critical reading of this manuscript. This work was supported in part by Grants-in-Aid for Scientific Research (No. 07456054 and No. 07406009) from The Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Aguilar MB, Quackenbush LS, Hunt DT, Shabanowitz J, Huberman A (1992) Identification, purification and initial characterization of the vitellogenesis-inhibiting hormone from the Mexican crayfish, *Procambarus bouvieri* (Ortmann). Comp Biochem Physiol 102B: 491–498

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

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

De Kleijn DPV, Coenen T, Laverdure AM, Tensen CP, Van Herp F (1992) 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 51: 121–128

Dircksen H, Webster SG, Keller R (1988) Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting hormone and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. Cell Tissue Res 251: 3–12

Gorgels-Kallen JL, Van Herp F, Leuven RSEW (1982) A comparative immunocytochemical investigation of the crustacean hyperglycemic hormone (CHH) in the eyestalks of some decapod crustacea. J Morphol 174: 161–168

Gorgels-Kallen JL, Meij JTA (1985) Immunocytochemical study of the hyperglycemic hormone (CHH)-producing system in the eyestalk of the crayfish *Astacus leptodactylus* during larval and postlarval development. J Morphol 183: 155–163

Hong DI (1977) Studies on sexual maturation and spawning in prawn, *Penaeus japonicus* BATE. Thesis The University of Tokyo

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

Kallen J, Meusy J-J (1989) Do the neurohormones VIH (vitellogenesis inhibiting hormone) and CHH (crustacean hyperglycemic

hormone) of crustaceans have a common precursor? Immunolocalization of VIH and CHH in the X-organ sinus gland complex of the lobster, *Homarus americanus*. Invert Reprod Develop 16: 43–52

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

Klein JM, De Kleijn DPV, Huenemeyer G, Keller R, Weidemann W (1993) 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 274: 515–519

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

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

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

Tensen CP, Janssen KPC, Van Herp F (1989) Isolation, characterization and physiological specificity of the crustacean hyperglycemic factors from the sinus gland of the lobster, *Homarus americanus* (Milne-Edwards). Invert Reprod Develop 16: 155–164

Terauchi A, Tsutsumi H, Yang W-J, Aida K, Nagasawa H, Sonobe H (1996) A novel neuropeptide with molt-inhibiting activity from the sinus gland of the crayfish, *Procambarus clarkii*. Zool Sci 13: 295–298

Wainwright G, Webster SG, Wilkinson MC, Chung JS, Rees HH (1996) Struture and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*: Involvement in multihormonal regulation of growth and reproduction. J Biol Chem 271: 12749–12754

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

Yang W-J, Aida K, Nagasawa H (1995) Amino acid sequences of a hyperglycemic hormone and its related peptides from the Kuruma prawn, *Penaeus japonicus*. Aquaculture 135: 205–212

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

Yang W-J (1997) Structure and functions of peptide hormone in the sinus glands of the kuruma prawn *Penaeus japonicus*. Thesis The University of Tokyo

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

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

(Received December 17, 1997 / Accepted February 19, 1998)