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Biochemical Analysis and Immunohistochemical Examination of a GnRH-like Immunoreactive Peptide in the Central Nervous System of a Decapod Crustacean, the Kuruma Prawn (Marsupenaeus japonicus)

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We examined whether a gonadotropin-releasing hormone (GnRH)-like peptide exists in the central nervous system (CNS) of the kuruma prawn, Marsupenaeus japonicus, by reverse-phase high performance liquid chromatography (rpHPLC) combined with time-resolved fluoroimmunoassay (TR-FIA) analysis and by immunohistochemistry. The displacement curve obtained for serially diluted extracts of the kuruma prawn brain paralleled the chicken GnRH-II (cGnRH-II) standard curve obtained by cGnRH-II TR-FIA using the anti-cGnRH-II antibody, which cross-reacts not only with cGnRH-II but also with lamprey GnRH-II (lGnRH-II) and octopus GnRH (octGnRH). Extracts of kuruma prawn brains and eyestalks showed a similar retention time to synthetic lGnRH-II and octGnRH in rpHPLC combined with TR-FIA analysis. Using this antibody, we detected GnRH-like-immunoreactive (ir) cell bodies in the anterior-most part of the supraesophageal ganglion (brain), the protocerebrum. Furthermore, GnRH-like-ir fibers were observed in the protocerebrum and deutocerebrum. In the eyestalk, GnRH-like-ir cell bodies were detected in the medulla interna, and GnRH-like-ir fibers were distributed in the medulla interna, medulla externa, and lamina ganglionalis. In the thoracic ganglion, GnRH-like-ir fibers, but not GnRH-like-ir cell bodies, were detected. No GnRH-like-ir cell bodies or fibers were detected in the abdominal ganglion or ovary. Thus, we have shown the existence and distribution of a GnRH-like peptide in the CNS of the kuruma prawn.

Key words: GnRH neuron, brain, eyestalk, immunohistochemistry, HPLC, invertebrate

INTRODUCTION

Gonadal maturation in vertebrates is primarily regulated by the brain-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of pituitary gonadotropin (GTH), which stimulates the secretion of steroid hormones from the gonads. Recent studies have shown that two or three molecular forms of GnRH exist even within the same species (Oka, 1997; Okuzawa and Kobayashi, 1999; Okubo and Nagahama, 2008).

In addition to the peptides present in vertebrate species, GnRH peptides have been isolated and their sequences determined in the protochordates Chelyosoma productum (Powell et al., 1996) and Ciona intestinalis (Adams et al., 2003), and the octopus Octopus vulgaris (Iwakoshi et al., 2002). Moreover, the full-length cDNA of a GnRH-like mole-

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A et al., 2008b). Interestingly, the GnRH forms present in the brains of these two crustaceans are different, and GnRH immunoreactivity was not detected in the eyestalk, where a number of neurohormones are synthesized.

In this study, we examined whether a GnRH-like peptide exists in the CNS of the decapod crustacean, the kuruma prawn (Marsupenaeus japonicas), which is the most commercially important species in shrimp aquaculture in Japan, by reverse-phase high performance liquid chromatography (rpHPLC) combined with time-resolved fluorimunnoassay (TR-FIA) analysis and by immunohistochemistry. A preliminary experiment showed that a rabbit polyclonal antibody (aCII6) raised against chicken GnRH-II (cGnRH-II) immunostained the kuruma prawn brain.

**MATERIALS AND METHODS**

**Kuruma prawns**

Subadult (immature prepuberal) female kuruma prawns were purchased from a local fish farm in Okinawa Prefecture, Japan. Their body weights ranged from approximately 20 to 60 g.

**TR-FIA**

First, we examined whether a GnRH-like peptide exists in the kuruma prawn brain by performing a TR-FIA for cGnRH-II using aCII6 (Amano et al., 2004). Senthilkumaran et al. (1999) previously reported the cross-reactivities of aCII6 against salmon GnRH (sGnRH), seabream GnRH, IgGnRH-I, mammalian GnRH (mGnRH), cGnRH-I, and catfish GnRH. In this study, we further examined the cross-reactivities of aCII6 against tunicate GnRH-I (tGnRH-I), tGnRH-II, IgGnRH-I, and IgGnRH-II, dogfish GnRH, herring GnRH, medaka GnRH (pejerrey GnRH), whitefish GnRH, frog GnRH, guinea pig GnRH, and octGnRH. The cross-reactivities of aCII6 against tunicate GnRH-II (tGnRH-II) were relatively high, as summarized in Table 1.

The kuruma prawns were anesthetized in ice. Their brains were rapidly dissected, frozen in dry ice, and stored at –85°C until analysis. GnRH was extracted according to a previously reported method (Amano et al., 2004). Parallelism between the typical standard curve for cGnRH-II and the competition curves obtained for the kuruma prawn brain extracts was examined by using a 2-fold serially diluted standard and the brain extracts in an assay buffer (Amano et al., 2008).

**rpHPLC combined with TR-FIA**

For rpHPLC combined with TR-FIA analysis, 66 brains and 34 eyestalks (17 individuals) were extracted according to the method of Okuzawa et al. (1990), with slight modification. Frozen brain ganglion and eyestalk tissues were homogenized in 100:3 acetone : 1N HCl (v/v; 5 mL/g frozen tissue). The homogenate was stirred for 3 hr on dry ice and then centrifuged at 10,000×g for 30 min at 4°C. The precipitate was re-extracted in 80:20 acetone : 0.01 N HCl (v/v) in two-fifths the volume of the original extraction fluid, stirred for 5 min, and re-centrifuged. The combined supernatant was then extracted with petroleum ether to remove the acetone, lipids, and other hydrophobic substances. The ratio of filtrate (or aqueous phase) to petroleum ether was maintained at approximately 4:1 (v/v) for each of five successive extractions. The final aqueous phase was passed through a 0.22-μm filter (Millipore, Bedford, MA).

Since aCII6 also cross-reacts with IgGnRH-II and octGnRH (Table 1), the extracts of the brain and eyestalk were separated by rpHPLC under the same conditions used to separate cGnRH-II, IgGnRH-II, and octGnRH, as described below. The filtrate (800–900 μl) was injected through a 1-ml injection loop onto an Asahipak Gel ODP-50 column (0.46×25 cm; Asahi Chemical Industry, Kawasaki, Japan). Liquid chromatography was performed using a flow rate of 1 mL/minute and a high-pressure gradient system with two pumps (880-PU, JASCO, Tokyo, Japan) with 20 mM triethylamine acetate, pH 7.0 (TEAA) and acetonitrile containing 16 mM TEAA. The percentage of acetonitrile was increased linearly from 10% (0 min) to 50% (50 min). Fractions were collected every minute. The standard solution consisting of 1 μg of cGnRH-II, IgGnRH-II, and octGnRH in 200 μl of 0.1% trifluoroacetic acid was injected and fractionated as explained above. The fractions collected from the brain and eyestalk samples were freeze dried by using a vacuum centrifuge concentrator. The respective dried residues were re-dissolved in 200 μl of TR-FIA assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 μM diethylaminoethyl-N,N,N',N"-pentaacetic acid, 0.01% Tween-40, pH 7.2) and were assayed for cGnRH-II by TR-FIA (Amano et al., 2004).

**Immunohistochemistry**

For immunohistochemistry, the brain, eyestalk, thoracic ganglion, and abdominal ganglion were fixed in Bouin’s fluid for 24 hours at 4°C and subsequently rinsed in cold 70% ethanol, dehydrated through a graded series of ethanol concentrations, and embedded in Paraplast (Monoject, Sherwood Medical, St Louis, MO). Sagittal and frontal sections were cut at 8 μm and mounted on gelatinized slides. Immunohistochemistry tests were conducted according to the method of Amano et al. using aCII6 (Amano et al., 2008). A Histofine immunostaining kit (Nichirei, Tokyo, Japan) was used for the immunohistochemical reactions. The sections were counterstained with Mayer’s hematoxylin. To test the specificity of the immunoreactions, control sections were incubated with aCII6 that had been preabsorbed overnight at 4°C with an excess of cGnRH-II, IgGnRH-II or octGnRH (10 μg cGnRH-II, IgGnRH-II, or octGnRH in 1 mL antiserum). The subsequent procedure was identical to that used for the experimental sections. We followed the terminology for the neuronal cluster devised by Sandeman et al. (1992).

**RESULTS**

The displacement curve obtained for the serially diluted extracts of the kuruma prawn brain paralleled the cGnRH-II standard curve (Fig. 1).

<table>
<thead>
<tr>
<th>GnRH forms</th>
<th>Cross-reactivity (%)</th>
<th>anti-cGnRH-II (aCII6)</th>
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<tr>
<td>Mammalian GnRH</td>
<td>&lt;0.01</td>
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<tr>
<td>Guinea pig GnRH</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Chicken GnRH-I</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>Chicken GnRH-II</td>
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<td></td>
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<tr>
<td>Frog GnRH</td>
<td>0.03</td>
<td></td>
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<tr>
<td>Salmon GnRH</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>Catfish GnRH</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Seabream GnRH</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Medaka GnRH</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Herring GnRH</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>Dogfish GnRH</td>
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<td></td>
</tr>
<tr>
<td>Whitefish GnRH</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Lamprey GnRH-I</td>
<td>&lt;0.01</td>
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<td>Lamprey GnRH-II</td>
<td>41.8</td>
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<td>Lamprey GnRH-III</td>
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<tr>
<td>Tunicate GnRH-I</td>
<td>0.23</td>
<td></td>
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<tr>
<td>Tunicate GnRH-II</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Octopus GnRH</td>
<td>47.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Cross-reactivities of aCII6 against various forms of GnRH. Cross-reactivity was measured at B/B0=50%. * According to Senthilkumaran et al. (1999).
rpHPLC combined with TR-FIA

The kuruma prawn brain and eyestalk extracts showed a similar retention time to synthetic cGnRH-II and octGnRH in rpHPLC combined with TR-FIA analysis, but did not show a similar retention time to cGnRH-II (Fig. 2).

Immunohistochemistry

The distribution of GnRH-like-ir cell bodies and fibers in the parasagittal and frontal sections of the brain is depicted in Fig. 3. The GnRH-like-ir cell bodies were detected in the anterior-most part of the brain, the protocerebrum. They were located in close proximity to but more caudal than the medium-sized neurons of neuronal cluster 6, which are located in the anterior-most part of the shrimp brain (Figs. 3A, B, 4A–G). The GnRH-like-ir cell bodies measured 11.2 ± 1.1 μm (n=10). GnRH-like-ir fibers were observed in the protocerebrum and deutocerebrum (Figs. 3A–D, 4A–G). No GnRH-like immunoreactivity was observed when the antiserum was preabsorbed overnight at 4°C with an excess of GnRH-like antigens.
of cGnRH-II (Fig. 3H), lGnRH-II, and octGnRH (data not shown).

In the eyestalk, GnRH-like-ir cell bodies were detected in the medulla interna (Fig. 5A, D–F). The GnRH-like-ir cell bodies measured 4.4±0.2 μm (n=10). A large number of GnRH-like-ir fibers were detected in the medulla interna (Fig. 5A, B, E) and lamina ganglonalis (Fig. 5C), and a few GnRH-like-ir fibers were observed in the medulla externa (Fig. 5D). No GnRH-like immunoreactivity was observed when the antiserum was preabsorbed overnight at 4°C with an excess amount of cGnRH-II (Fig. 5G), lGnRH-II, and octGnRH (data not shown).

In the thoracic ganglion, GnRH-like-ir fibers, but not GnRH-like-ir cell bodies, were detected in the neuropil (Fig. 6A–C). No GnRH-like immunoreactivity was observed when the antiserum was preabsorbed overnight at 4°C with an excess amount of cGnRH-II (Fig. 6G), lGnRH-II, and octGnRH.
excess amount of cGnRH-II (Fig. 6D). In the abdominal ganglion and the ovary, no GnRH-like-ir cell bodies or fibers were detected (data not shown).

**DISCUSSION**

The displacement curve obtained for the serially diluted extracts of the kuruma prawn brain paralleled the cGnRH-II standard curve. The kuruma prawn brain and eyestalk extracts showed a similar retention time to synthetic lGnRH-standard curve. The kuruma prawn brain and eyestalk extracts showed a similar retention time to synthetic lGnRH-standard curve. The kuruma prawn brain and eyestalk extracts paralleled the cGnRH-II (Ngernsoungnern A et al., 2008b), although the relationship among the peptides is also suggested in the giant freshwater prawn (Ngernsoungnern A et al., 2008a). However, it should be noted that no GnRH-I-like-ir cell bodies or fibers were detected in the CNS of the black tiger shrimp (Ngernsoungnern A et al., 2008a) or in that of the giant freshwater prawn (Ngernsoungnern P et al., 2008) or in that of the giant freshwater prawn (Ngernsoungnern A et al., 2008b). Thus, at present, the effect of endogenous GnRH in the CNS on ovarian maturation in crustaceans is unclear.

In summary, we detected and localized GnRH-like-ir peptide(s) in the CNS of the kuruma prawn by rpHPLC combined with TR-FIA analysis. GnRH-like peptides chromatographically and immunologically similar to Ngernsoungnern A et al., 2008b) or giant freshwater prawn (Ngernsoungnern A et al., 2008b). Thus, at present, the effect of endogenous GnRH in the CNS on ovarian maturation in crustaceans is unclear.

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