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Short communication

Primary structure of a novel gonadotropin-releasing hormone (GnRH) in the ovary of red swamp crayfish *Procambarus clarkii*

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is a neuropeptide known to regulate and maintain reproductive functions and conserved in both vertebrate and invertebrate species. In this study, a GnRH peptide was isolated from the ovaries of red swamp crayfish, *Procambarus clarkii*, by using chromatographic and immunological methods. The form was proven to be a novel form of GnRH by determining its primary structure by chemical sequencing and mass spectrometry, and was designated as *P. clarkii* GnRH (pcGnRH). The primary structure of pcGnRH is pQSYHFSLGWKP-NH_2, which is different from the known forms of the vertebrate and invertebrate GnRH family. Synthetic pcGnRH was biologically active, as it showed stimulative effects on ovarian maturation to crayfish. Our data supported the hypothesis that in crustacean there is GnRH synthesized locally in the ovary and playing an important role in the regulation of oocyte development. This is the first report on the isolation and characterization of GnRH-like peptide in crustacean.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a conserved neuropeptide known to regulate and maintain reproductive functions in both vertebrates and invertebrates. New peptide sequences identified in invertebrates from annelids to tunicates reveal GnRH-like peptides of 10–12 amino acids. Presently, GnRH is characterized into 30 isoforms: 15 isoforms in vertebrates and 15 isoforms in invertebrates (Roch et al., 2011; Sun et al., 2012). This peptide displays special modifications, including N-pyroglutamyl and C-amidated residues, for molecular stability and receptor recognition (Lindemans et al., 2011).

Extensive studies have demonstrated the presence and distribution of GnRH-like immunoreactivities in the central nervous system (CNS) or ovary of various decapod crustacean species, such as *Portunus pelagicus* (Saetan et al., 2013), *Litopenaeus vannamei* (Tinikul et al., 2011), *Marsupenaeus japonicas* (Amano et al., 2009), *Penaeus monodon* (Ngernsoungnern et al., 2008a), and *Macrobrachium rosenbergii* (Ngernsoungnern et al., 2008b). Moreover, the effects of GnRH isoforms on the reproduction to several decapod crustacean species have been demonstrated, e.g. Ngernsoungnern et al. (2008a, 2008b) have demonstrated that synthetic lamprey (l) GnRH-I, lGnRH-III and octopus (oct) GnRH could shorten the cycle of ovarian maturation and spawning of *P. monodon* and *M. rosenbergii*; and Saetan et al. (2013) have shown that synthetic lGnRH-III peptide could significantly induce ovarian maturation of blue crab, *P. pelagicus*. However, to date, no GnRH-like peptides have been biochemically isolated and sequenced from crustaceans (Saetan et al., 2013; Sun et al., 2012).

The red swamp crayfish *Procambarus clarkii* is an important economic species in China, and has been artificially propagated in recent years (Guan et al., 2013; Shui et al., 2012). In the present study, we determined the primary structure of a novel GnRH peptide in the ovary of this crayfish and assessed the biological activity of the novel GnRH on ovarian maturation. This is the first report on the isolation and characterization of GnRH-like peptide in crustaceans.

2. Materials and methods

2.1. Animals and ovarian peptide extraction

Domesticated female *P. clarkii* (30–45 g body weight [BW]) were collected from the Jiangsu Baolong Breed Aquatics Company in Dafeng city, Jiangsu province, China. Crayfishes were maintained in 40-liter aquaria in filtered aerated water at room temperature (25 °C). A commercially available diet for crayfish was fed to the animals twice daily.

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Since eyestalk ablation possibly increases the expression level of GnRH and activates the GnRH signaling pathway in the ovary of decapod crustaceans (Guan et al., 2013; Uawisetwathana et al., 2011), ovaries were carefully removed from crayfishes on 1 day after unilateral eyestalk ablation and subsequently stored at −70 °C. Crayfish ovaries were extracted based on the protocol described in the previous studies (Adams et al., 2002; Pati and Habibi, 1998). Briefly, frozen ovaries (~30 g) along with dry ice were powdered with a precooled pestle and mortar held on dry ice. The powdered ovary was added to acetone: 1 N HCl (100:3 vol:vol) in a ratio of 1 g frozen tissue: 5 ml extraction fluid. The extraction mixture was stirred on ice for 3 h. Every hour, the mixture was homogenized for 1 min with a polytron. The mixture was filtered through Whatman #1 filter paper after 3 h, and the residue was further homogenized and reextracted in acetone: 0.01 N HCl (4:1 vol:vol) for 5 min and then refiltered. The combined filtrates were refiltered and extracted with petroleum ether to remove the hydrophobic substances; the ratio of the filtrate (aqueous phase) to the petroleum ether was maintained at 4:1 (vol:vol) for each extraction. Extraction was repeated four to five times until the volume of the aqueous phase became constant. Following the last extraction, the separation funnel was placed on ice for 30 min to allow the pigments present in the ovary to form an interphase between the ether and aqueous phases. The final aqueous phase was separated and centrifuged for 20 min at 17,000 ×g (4 °C), and the clear supernatant was purified using Waters Sep-Pak C-18 columns (4 ml/column) (Waters Associates, USA). The Sep-Pak columns were washed with 0.1% trifluoroacetic acid (TFA, 20 ml/Sep-Pak), and the absorbed proteins/peptides eluted with acetonitrile: organopure water (3:2 vol:vol; 4 ml/Sep-Pak). The eluted material (ovarian extract) was aliquoted in 5–10 g frozen tissue equivalents per vial, lyophilized and stored at −20 °C until further characterization. Once the presence of GnRH-like substance in the ovarian extract was established (see below), a bulk extraction was carried out from 855.6 g of frozen pooled ovaries (obtained from approximately 1800 crayfishes) using identical protocols.

2.2. Reverse-phase HPLC purification of GnRH

Fifty-five fractions of 1 ml were collected from the Sep-Pak C-18 column extraction and an aliquot of 10 μl taken for immuno-dot blotting (IDB) assay. Immunoreactive GnRH (ir-GnRH) fractions were pooled, concentrated under vacuum and injected onto an ODS-C18 Supelcosil column (Supelco; Sigma, USA) connected to a Shimadzu Model SCL-10A HPLC apparatus with a SPD-10AV UV detector (Shimadzu, Japan). Four successive reverse-phase HPLC (RP-HPLC) steps were performed. Column types, solvents and conditions were summarized (Table 1). The elution profile was monitored continuously at 280 nm, except in step 4, which was monitored at 210 nm. The flow rate was kept at 1 ml/min. Aliquots of 10 μl from each fraction were used for IDB to determine ir-GnRH. Each injection of the tissue extract was preceded by a blank run, in which the mobile phase was injected. The blank fractions were assayed by IDB under the same conditions as the samples to ensure that the column was not contaminated.

2.3. Immuno-dot blotting assay

Aliquots of 10 μl from fractions collected at each successive step in the RP-HPLC purification were assayed for ir-GnRH by IDB method described previously with minor modifications (Tinikul et al., 2011). The rabbit's polyclonal antibody against lGnRH-I (1:1000 final dilution) prepared in our laboratory previously was used in this assay, and all procedures were approved by the Jiangnan University Animal Ethics Committee. Briefly, the samples were spotted onto nitrocellulose membranes and dried at room temperature for 45 min. Non-specific bindings were blocked by the blocking solution containing 4% skimmed milk in TPBS (0.1% Tween-20 in 0.1 M phosphate-buffered saline) at room temperature for 2 h. After being washed, the membranes were incubated in the primary antibody (anti-lGnRH-I), which had been pre-absorbed with BSA at a concentration of 150 mg/ml, and then incubated overnight at 4 °C. Subsequently, the membranes were washed three times with TPBS at room temperature for 10 min each. Then, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, USA), diluted at 1:4000 in blocking solution, was added to the membranes, which were further incubated at room temperature for 2 h. The membranes were gently washed three times in TPBS. Signals were then developed by using an enhanced chemiluminescence kit (Amersham Biosciences, USA), and the membranes were exposed to film, before being developed. Controls were performed by substituting primary antibodies with pre-immune rabbit sera.

2.4. Primary structure characterization and pcGnRH peptide synthesis

An aliquot of the peptide purified by RP-HPLC on a diphenyl column was injected into a microbore C-18 column using 0.05% TFA and acetonitrile for elution. Sequencing was initially attempted on 10% of the sample. Failure of this sequencing indicated a blocked N-terminus. Subsequent sequencing was carried out on the remaining material after treatment with pyroglutamyl aminopeptidase (Sigma, USA) and microbore HPLC purification (Adams et al., 2002). This was followed by separation and sequence analysis by automated Edman degradation on a PEABI Procise 494 protein sequencer (Applied Biosystems, USA). The molecular weight of the pcGnRH peptide was determined by a nanoflow electrospray ionization time-of-flight mass spectrometry (nano-ESI-TOF-MS) (Micromass UK, UK). pcGnRH and octGnRH peptides were synthesized with a solid-phase peptide synthesizer using GenScript's FlexPeptide™ technology (GenScript China, China).

Table 1
RP-HPLC steps of GnRH purification from the ovarian extract of *P. clarkii*.

Step	Column type	Mobile phase		Sequential elution conditions			Fractions pooled for next step
		A	B	Initial % B	Final % B	Time (min) in gradient	
1	ODS-C18 Supelcosil	0.25 M TEAF (pH 6.5)	100% ACN	17	17	10	54–55
				17	25	15	
				25	45	35	
2	ODS-C18 Supelcosil	0.15 M TEAP (pH 2.5)	100% ACN	17	17	10	33–35
				17	25	15	
				25	45	35	
3	ODS-C18 Supelcosil	0.05% TFA (pH 2.0)	0.05% TFA/80% ACN	5	5	10	33–36
				5	50	30	
				50	100	10	
				100	100	10	
4	Vydac Diphenyl	0.05% TFA (pH 2.0)	0.05% TFA/80% ACN	5	5	10	29–31
				5	20	10	
				20	80	40	