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Farnesoic acid and methyl farnesoate production during lobster reproduction: Possible functional correlation with retinoid X receptor expression

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ABSTRACT

Farnesoic acid (FA) and methyl farnesoate (MF) are juvenile hormone-related compounds secreted by the mandibular organ (MO) of crustaceans and play an important role in stimulation of ovarian maturation. To better understand how the MO activity influences female reproduction by secretion of FA and MF, the biosynthesis and release of these two compounds were measured *in vitro* by the incorporation of L-[^{3}H-methyl]methionine into MF and [2-^{14}C]acetate into FA by the MO of *Homarus americanus*. The production of FA is 7.5 times that of MF, and most FA and MF synthesized remained within the gland, and was not released into the surrounding medium. Most FA and MF were synthesized in the anterior fan-fold region of the MO. The rates of biosynthesis of FA and MF were stage-related, with maximal production occurring during secondary vitellogenesis (i.e. stages 4 and 5). A potential juvenoid receptor, retinoid X receptor (RXR), *HaRXR*, was characterized using PCR cloning techniques. *HaRXR* belongs to the nuclear hormone receptor superfamily and its deduced amino acid sequence shares a high homology to other RXRs of crustaceans, insects, and vertebrates. Transcripts of *HaRXR* can be detected in many tissues, and significant high expression level was detected in the MO, especially in the anterior fan-fold region. Expression of *HaRXR* was also related to reproductive stage, and maximal level of expression was observed at stage 4, in which secondary vitellogenesis is occurring. Changes in transcript level of *HaRXR* and the rates of FA/MF biosynthesis in the female reproductive cycle indicate that *HaRXR* and FA/MF may play important roles in crustacean reproduction.

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1. Introduction

Crustacean reproduction is under the endocrine control of juvenile hormone-related compounds secreted from the mandibular organ (MO), ecdysteroids (from the Y-organ), the family of CHH/MIH/GIH neuropeptides (from X-organ/sinus gland complex in eyestalk), and biogenic amines. Methyl farnesoate (MF), the immediate precursor of insect juvenile hormone III (JH III) [4,24,43] is synthesized and released from the MO in crustaceans. MF was initially isolated from the hemolymph of the crab *Libinia emarginata* [24] and was subsequently isolated from the MO or hemolymph of many other crustaceans, e.g. *L. emarginata*, *Homarus americanus*, *Cambarus bartonii*, *Cancer borealis*, and *Carcinus maenas* [4], *Scylla serrata* [43] and *Macrobrachium rosenbergii* [32]. MF was also demonstrated to have stimulatory effects on crustacean ovarian maturation, both *in vitro* [33] and *in vivo* [23].

Farnesoic acid (FA), the immediate precursor of MF [9,44], may also be involved in stimulating gonad maturation, either directly or indirectly. Recently, the stimulatory effect of FA on ovarian maturation has been demonstrated in *Charybdis feriatus* [27], *Metapenaeus ensis* [39], *Penaeus monodon* [40], and *H. americanus* [37,38], and this effect is more consistent and potent than that of MF. However, it is uncertain whether FA or MF is the major chemical mediator stimulating gonad maturation directly in crustaceans. It is also unclear how the MO regulates gonad maturation. To address these issues, MF/FA biosynthesis by the MO during the female reproductive cycle has been studied and is reported in this paper.

We have previously demonstrated synergism between FA and 20-hydroxyecdysone (20E) on vitellogenin (Vg) production by hepatopancreas of female lobster [37]. Such synergistic mechanisms may be explained by the model suggested in the mosquito *Aedes aegypti* [31]. In this species, there are several transcription factor binding sites located at the 5′ upstream promoter region of the Vg gene that regulate gene expression. These include binding sites for the ecdysone receptor (EcR) and ultraspiracle (USP), the insect homolog of the retinoid X receptor (RXR). Although EcR is known to bind ecdysteroids, there is no clear consensus regarding the identity of the USP ligand. However, USP has been suggested to be a juvenile hormone (JH) receptor in *Drosophila* and *Heliothis* [18]. Given that USP and EcR form a heterodimer which is involved in regulating Vg transcription in insects, the binding of juvenoids to lobster RXR could explain the synergism we observed previously between 20E and FA. At present, this hypothesis remains speculative. Therefore,

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to study the effectors activating Vg gene expression, and hence female ovarian maturation in crustaceans, our first step was to clone and characterize lobster USP/RXR, and to study its role in Vg production.

2. Materials and methods

2.1. Animals

Homarus americanus were purchased from a local supermarket throughout the year. They were acclimated for 1 week at 15 °C, in circulating sea water with salinity at 32–35 ppm (made using Instant Ocean), in a 12:12 light:dark photoperiod. The reproductive stage was assessed on the basis of color and size of ovaries, and oocyte diameter [1,38]. Dissection of animals was performed in ice-cold lobster saline (460 mM NaCl, 13 mM KCl, 13 mM $CaCl_2$, 10 mM Hepes, 1.7 mM D-glucose, pH 7.4) in a sterile environment.

2.2. Radiochemical assay

The radiochemical assay (RCA), as described previously [9,22] was used to measure the rate FA and MF biosynthesis by the MO. [2-^{14}C]Acetate (1.6 mCi mmol^{-1}, American Radiolabeled Chemicals Inc., USA) and L-[methyl-^{3}H]-methionine (84 Ci mmol^{-1}; Amersham Pharmacia Biotech Inc., USA) were used to determine the biosynthesis of FA and MF, respectively. The medium was diluted with unlabeled methionine to a final concentration of 50 mM final specific radioactivity of methionine was ~1400 μCi/μmol and that of acetate was ~1.5 μCi/μmol in the medium. The MOs were cut transversely into four sections (I–IV) of approximately equal width (Fig. 1). Each section was further divided into eight equal parts by cutting perpendicularly to the first cut, and one part from each section was used for the assay. Each fragment was rinsed and incubated in a glass tubes of 50 μL Medium 199 (in 1× lobster saline, lacking methionine and supplemented with 20 mg/mL Ficoll, (to maintain osmotic balance). The incubation was carried out at 28 °C with gentle shaking, for 3 h. After incubation, the MO fragments and the media were extracted separately, to determine the amount of FA or MF synthesized within the fragment (content) and released into the medium (release). The extracts were dried under a gentle stream of N_2 and analyzed by thin layer chromatography as described previously [9,41]. The radioactivity was measured by liquid scintillation spectrometry.

2.3. ELISA

The Vg content in hemolymph was measured by direct enzyme-linked immunosorbent assay (*ELISA*). Hemolymph was withdrawn and diluted in 100 mM carbonate buffer (pH 9.6) containing 1× protease inhibitor cocktail (Roche, Germany). Samples were frozen at −70 °C until used.

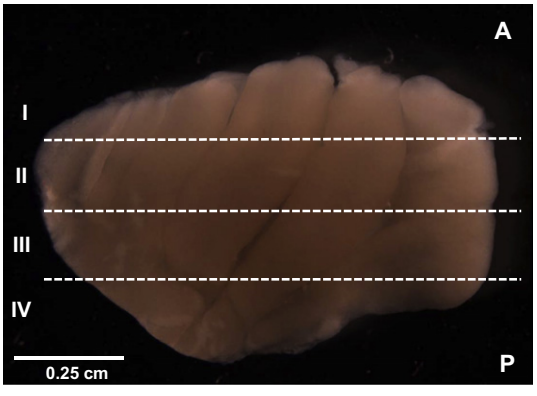

Fig. 1. The mandibular organ is divided into four regions (I– IV) as indicated. Region I is the anterior part (A), which is the fan-folded edge of the gland, whereas region IV is the posterior part (P).

The polyclonal antibody anti-HaVn was generated as described previously [38] and was further purified by passing through a Hi-Trap Protein G HP affinity column (GE Healthcare, USA) according to the manufacturer's instructions. Concentration of IgG of purified anti-HaVn was measured by the Bradford assay. IgG was stored at −70 °C until use.

The wells of a 96-well plate were coated with 15 μg of hemolymph protein in 50 μL of 100 mM bicarbonate/carbonate buffer (pH 9.6) at room temperature for 2 h. Vitellin purified from *H. americanus* ovary (0.16 μg to 5 μg/mL) was used as the standard. After coating, the wells were rinsed three times in PBS-T (1× PBS with 0.1% (v/v) Tween 20 added) and incubated with 100 μL blocking solution (3% BSA in 1× PBS) at 4 °C overnight. The wells were rinsed again (three times in PBST), and 100 μL of 0.3 ng/mL purified anti-HaVn IgG in 1% BSA was added and incubated at room temperature. The primary antibody was washed off (five times in PBST) after 2 h incubation; 100 μL of goat-anti-rabbit-HRP conjugate (Bio-Rad, USA ; 1:25,000 diluted in 1% BSA) were added and incubated for 1 h at room temperature. After rinsing (five times in PBST), 100 μL of tetramethylbenzidine solution (TMB, Invitrogen, USA) was added to each well, and the color was developed at room temperature. The color development was stopped by adding 100 μL of 1 N HCl after 10 min. The intensity of color of each well was measured using a SpectraMax Plus 384 microplate reader at OD_{450nm}.

2.4. cDNA cloning of HaRXR

A pair of MOs was homogenized in 2 mL TRIreagent (Sigma–Aldrich, USA) according to the manufacturer's instruction. Two micrograms of total RNA was reverse-transcribed in 1× first strand buffer, 0.5 mM dNTP mix, 1 μg oligo dT_{12}, 10 U RNase inhibitor (Invitrogen, USA), and 100 U MMLV-RT (Sigma, USA), at 37 °C for 2 h. Degenerate primers (DegF1; DegF2 and DegR1, Table 1) were designed based on the highly-conserved DNA binding domain of USP/RXR from other crustaceans and insects. Semi-nested PCR amplification was performed with denaturation at 95 °C for 1 min, followed by 34 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. At the end of the PCR, the reaction was further incubated at 72 °C for 10 min. The same thermal cycles were used in both rounds of reaction. The amplified PCR product after the second round PCR was subcloned into the pJET1.2 vector (Fermentas, USA), and sequencing reactions were performed at the DNA sequencing facility at the Analytical Genetics Technology Centre of Princess Margaret Hospital, Toronto, ON.

To obtain the full-length cDNA sequence of *HaRXR*, 5′ and 3′RACE (Rapid Amplification of cDNA Ends; Roche, Germany) were performed according to the manufacturer's instruction. Primers

Table 1
Primers used in the PCR cloning of HaRXR.

Primer name	Sequence (5′ → 3′)
HaRXR-DegF1	CGI GCI AGT GGN AAR CAY TA
HaRXR-DegF2	ATC CIC CTA AYC ATC CRC TSA
HaRXR-DegR1	CKC TTG TCI ATG ATR CAR TT
HaRXR-5′RACE-R1	CAG AAC TGG CAA CGA T
HaRXR-5′RACE-R2	GTC TCT TGT CAA TAG TGC AGG ATC T
HaRXR-5′RACE-R3	ACT GTT CGT TTG AAG AAT CCC TTA C
HaRXR-3′RACE-F1	GTC TAC AGC TGT GAG GGT TGT AAG G
HaRXR-3′RACE-F2	GTA AGG ATC TCA CTT ACG CCT GTC
HaRXR-3′RACE-F3	AGT GGG CTA AAC AGA TCC CAC ACT
HaRXR-3′RACE-F4	AGC TAC TGG ACT TGT GGT GCA CAG
HaRXR-ORF-F	GTC GGC ATG TCA GGG TCA CTG GAT
HaRXR-ORF-R	ATC ACT TCT TAA CTT GAT GGG G

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