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Hepatopancreas but not ovary is the site of vitellogenin synthesis in female fresh water crab, *Oziothelphusa senex senex*

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ABSTRACT

The objective of the present study was to explore the site of synthesis of vitellogenin (Vtg) in fresh water edible crab, *Oziothelphusa senex senex*. *Vtg* cDNA fragments were isolated from the hepatopancreas of female crabs using RT-PCR method, and the deduced amino acid sequence of *O. senex senex* showed more than 60% identity with other brachyuran Vtg sequences. RT-PCR analysis showed that *Vtg* mRNA can be detected only in hepatopancreas of female *Oziothelphusa* but not in other tissues including eyestalks, Y-organs, mandibular organs, thoracic ganglion, hypodermis and ovary. Antibodies were raised against vitellin purified from the ovary of *O. senex senex*. Immunoprecipitation analysis revealed the presence of Vtg in the hepatopancreas of vitellogenic stage I females and in the hemolymph, hepatopancreas and ovary extracts from vitellogenic stage II females but absent in hemolymph and hepatopancreas extract of males. These results suggest that Vtg is synthesized only in hepatopancreas but not in the ovaries of *O. senex senex*. In addition, Vtg synthesized in hepatopancreas is transported to ovary through hemolymph.

1. Introduction

Vitellogenesis is hormone regulated phase in female reproductive cycle of crustaceans, involving synthesis and accumulation of yolk proteins in the ovary. The major yolk protein, vitellin contains proteins, carbohydrates and phospholipids and serves as the nutritive material for embryogenesis and early larva [1–5]. In addition, carotenoid, thyroxine, retinol and riboflavin can be taken into oocytes during vitellogenesis by the carrier of vitellogenin (Vtg) [6–9]. The site of synthesis of Vtg (i.e. precursor of vitellin) is still controversial in crustaceans.

Vtg is synthesized in the liver of vertebrates [10], fat bodies of insects [11] and intestine of nematodes [12], and then transported to ovary through circulation. In crustaceans, Vtg is postulated to be synthesized by extra-ovarian tissues and then transported through the hemolymph to the developing oocytes [13–15]. Detection of Vtg in hemolymph supported the above hypothesis [16]. Vtg receptor (VtgR) is detected on the oocytes and it is proposed that Vtg internalization is receptor mediated endocytotic process [16]. Several studies have demonstrated that Vtg is synthesized exclusively in hepatopancreas of *Macrobrachium rosenbergii* [17], *Pandalus hypsinotus* [18] and *Charybdis feriatus* [19]. While other studies have shown that Vtg is synthesized in the ovaries of *Penaeus semisulcatus* [20] and *Callinectes sapdius* [21] respectively. Recently, few studies also confirmed both ovary and hepatopancreas were identified as sites of vitellogenesis but contribution of ovary is relatively less [22–25]. These variable results may reflect the existence of multiple sites of Vtg synthesis, species specificity, or may be a manifestation of differences in the methodologies adopted. Immunological detection, radiolabeling of proteins and expression of mRNA have been employed by several scientists to determine the site of vitellogenesis in crustaceans [16]. The present experiments were carried out to contribute to the discussion on this controversy using immunological and RT-PCR methodologies in fresh water edible crab, *O. senex senex* as model organism.

2. Materials and methods

2.1. Collection and maintenance of animals

Intact female crabs, *O. senex senex* (NCBI, Taxon ID: 324906) were collected from the rice fields and irrigation canals around Tirupati (13° 36′ N, 79° 21′ E), Andhra Pradesh, India. Crabs were housed 6–8 per glass aquaria (length:width:height = 60:30:30 cm) with sufficient ambient medium (salinity: 0.5 ppt) and water was

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replaced daily. They were maintained under controlled laboratory conditions (temperature 27 ± 1 °C and a light period of 12 h) for at least 1 week before being used for experimentation. During their sojourn, the crabs were fed with sheep meat daily *ad libitum*. Vitellogenic stage I and stage II female and adult male crabs were used in the present study.

2.2. Hemolymph and tissue collection

Hemolymph was collected by withdrawing from the sinuses at the base of the third walking leg with a syringe and mixed (1:2) with anticoagulant (containing 1.4% Na_2HPO_4, 1.3% KH_2PO_4, 0.3% ethylene diamine tetra acetic acid (EDTA), 2% dextrose, and 0.25% sodium citrate). The hemolymph samples were stored at −40 °C, after centrifugation at 4000*g* for 10 min at 4 °C. The tissues (ovary and hepatopancreas) were isolated and homogenized in phosphate buffer saline (PBS) (10 mM sodium phosphate, pH 7.7, 0.01% EDTA, 0.1 M NaCl, 0.1 mM phenyl methyl sulfonyl fluoride).

2.3. Vtg isolation

Vtg was isolated from the ovaries of crab *O. senex senex* by the method described by Tsukimura et al. [26]. The quality of isolated ovarian Vtg was determined using discontinuous SDS–PAGE. The purified Vtg was stored at −20 °C until further use.

2.4. Ouchterlony double-immunodiffusion

Double immunodiffusion experiments were performed using 1% agarose gels [27] using the antibodies raised against Vtg. The preparation of *Oziothelphusa* Vtg antibodies was described elsewhere [28]. After loading the samples, the plates were placed in a moist chamber for 16 h and observed for precipitin bands.

2.5. Total RNA isolation and first-strand cDNA synthesis

Hepatopancreas and ovary of *O. senex senex* were isolated, washed in ice cold crustacean physiological saline and immediately processed for RNA isolation. Total RNA was extracted from tissues according to manufacturer's protocol using RNA isolation kit (Fermentas, Lithuania). The concentration of extracted RNA was measured and 1 μg of total RNA was used for cDNA synthesis using Revert aid cDNA synthesis kit (Fermentas, Lithuania). cDNAs were kept for PCR reaction using gene specific primers i.e. β-actin and *Vtg* for expression studies in different tissues.

2.6. Cloning and sequencing of cDNAs encoding Vtg

The *Vtg* primers were designed based on the highly conserved nucleotides of *Vtg* sequences of *Eriocheir sinensis* (accession number: KC699915.1) and *Scylla paramamosain* (accession number: FJ812090.1) in the GenBank database. Primers were synthesized by Eurofins Genomics Pvt. Ltd. India and are shown in Table 1. The primers (0.1 nM) were used in standard PCR reactions (25 μl total volume) with an initial denaturation (94 °C, 4 min), and 38 amplification cycles of denaturation (94 °C, 30 s), annealing (49.6 °C for Vtg, 54.4 °C for β-actin, 45 s), extension (72 °C, 120 s). The final extension (72 °C, 6 min), is followed by cooling at 4 °C. After PCR amplification, an aliquot of reactions were separated and visualized on 2% agarose gel with ethidium bromide. After purification (GenElute PCR DNA Purification Kit, Sigma), the PCR products were sequenced by Sanger's dideoxy sequencing method at Eurofins Genomics Pvt. Ltd. India. Analysis of nucleotide sequence was performed using BLAST *N* and BLAST *X* programs, NCBI and phylogenic analysis was performed on amino acid sequences (using translated nucleotide query) of known brachyuran Vtg molecules using CLUSTAL W.

3. Results

3.1. Determination of molecular masses of Vtg sub-units

The purified ovarian Vtg was subjected to SDS–PAGE to determine the molecular mass. The purified ovarian Vtg was separated into three polypeptides, having molecular masses of 73.3, 84.3 and 100.0 kDa (data not shown).

3.2. Immunoprecipitation

Immunoprecipitation analysis revealed the formation of precipitin with the extract of hepatopancreas isolated from vitellogenic stage I females but not with extracts of hemolymph and ovary, whereas, the precipitin was observed with the extracts of hemolymph, ovary and hepatopancreas isolated from females in vitellogenic stage II, but not with the extracts of hemolymph and hepatopancreas of males (Table 2 and Fig. S1).

3.3. Characterization of cDNA encoding Vtg

The nucleotide sequence of the PCR product was determined and the amplicon size obtained was 700 base pairs (b.p.) for *Vtg*. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. Cloned *Vtg* sequence was submitted to GenBank, NCBI (accession number: KF563903.1). Analysis with CLUSTAL W for brachyuran Vtg sequences was given in Fig. 2. When the deduced amino acids was aligned with other known brachyuran Vtg amino sequences, the *O. senex senex* Vtg exhibited highest identity with *Callinectes sapidus* Vtg and *Charybdis feriata* Vtg (70% identity) followed by *E. sinensis*, *S. paramamosain* and *Protunus trituberculatus* Vtg (69% identity).

The phylogenetic tree based on the Vtg amino acid sequences of decapods was shown in Fig. 3. The results indicate clear speciation among prawns and shrimps, crayfishes and lobsters, marine crabs and freshwater crabs.

3.4. Expression of Vtg mRNA in different tissues of Oziothelphusa

Expression of the *Vtg* mRNA in different tissues was analyzed by semi quantitative RT-PCR. The *Vtg* cDNA fragment of the expected size 700 b.p. was detected only in the hepatopancreas of female, whereas it was not detected in ovary, eyestalks, Y organ, mandibular organ, thoracic ganglion, and hypodermis (Fig. 4). The β-actin was used as an internal control for each sample to indicate that same amount of total RNA was used.

Table 1
Primer sequences used for amplification of mRNA (*Vtg* and β-actin) using semiquantative RT-PCR, their respective GeneBank accession numbers and their amplicon size.

mRNA	Amplicon size in base pairs	GenBank accession numbers	Primer sequences
Vtg	700	KF563903	FP:CAGATATGTCCGCAACACA RP:GGATAAACCCACGAACCT
β-Actin	509	KF319125	FP:CAAGCGAGGTATCCTGACTCT RP:CCACGTTCATCTCACTCTCG

Table 2
Immunological reactivity of different tissue extracts with antisera raised against *Oziothelphusa* Vtg (also see Fig. S1).

Sample	Immuno reactivity		
	Vitellogenic stage I	Vitellogenic stage II	Male
Hepatopancreas extract	+	+	–
Hemolymph	–	+	–
Ovarian extract	–	+	

+, immuno-cross reactivity.

```
cagatatgtccgcaacacaacaaactcgagcgcctttatttcgacgcgatagccttcgtc
 Q  I  C  P  Q  H  N  K  L  E  R  L  Y  F  D  A  I  A  F  V
cacgaaccagagtctgtcgagttgatggtcaaggaattgttggaaaaacgcgctaccggt
 H  E  P  E  S  V  E  L  M  V  K  E  L  L  E  K  R  A  T  G
actcgggctgccctctattctgccgcattcagcttcgtgtcccgccccaacatgaaagcc
 T  R  A  A  L  Y  S  A  A  F  S  F  V  S  R  P  N  M  K  A
attcaggcattagagcctcttttcagggccagcgaagcacacatgtcttcggcaaagctt
 I  Q  A  L  E  P  L  F  R  A  S  E  A  H  M  S  S  A  K  L
tccgcggcctccatggtcaacaagtactgtcgccaaaacccacactgttacgatgaagct
 S  A  A  S  M  V  N  K  Y  C  R  Q  N  P  H  C  Y  D  E  A
ccggtgaggaatttggcccaggctctcaagcacgacgttgaggaagatttctcgcccaac
 P  V  R  N  L  A  Q  A  L  K  H  D  V  E  E  D  F  S  P  N
agtaatgaggaatctcaggaaaaggctttgtcagccttcaagagcctgggcaacatgggc
 S  N  E  E  S  Q  E  K  A  L  S  A  F  K  S  L  G  N  M  G
gtcacgacccccgaagtgtccgaggcggtgatgcgctacgtgagaaaggagaacaagaaa
 V  T  T  P  E  V  S  E  A  V  M  R  Y  V  R  K  E  N  K  K
gtgaacattcgcgtggcagccgcacagtccttcaggctggcaaggtgtgaaagttcggtg
 V  N  I  R  V  A  A  A  Q  S  F  R  L  A  R  C  E  S  S  V
acgcagcaacttgttgactttgccctccggcccggaaagaacacggaggttcgcatcgcc
 T  Q  Q  L  V  D  F  A  L  R  P  G  K  N  T  E  V  R  I  A
tgctatttggcggctgttcgctgcgccaacttcgagcacctgcaggagattgtggccaac
 C  Y  L  A  A  V  R  C  A  N  F  E  H  L  Q  E  I  V  A  N
atctcctccgaggagaacaccc aggttcgtgggtttatcc
 I  S  S  E  E  N  T  Q  V  R  G  F  I
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Fig. 1. The nucleotide and deduced amino acid sequences of cDNA encoding *Vtg* fragment of *O. senex senex*. Locations of the primers were denoted by underlining with solid line.

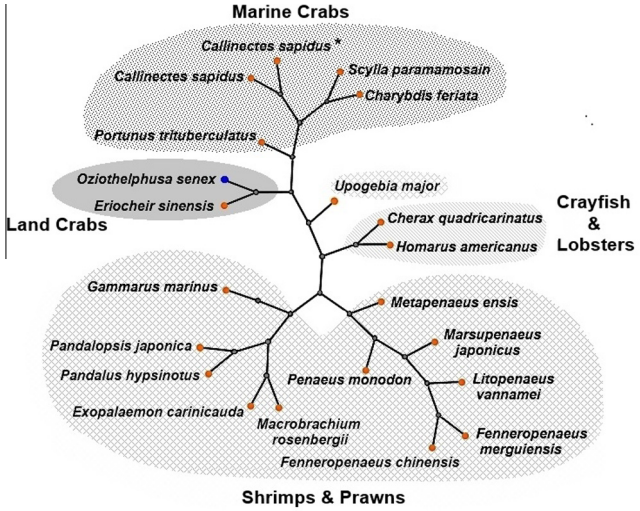

Fig. 3. Radiational tree of the Vtg family sequences built by the neighbor-joining method based on the CLUSTAL W. The sub groupings of Vtg family members among decapoda are shaded. Taxonomic name of the decapods, Vtg amino acid sequence used for phylogenetic analysis, with GenBank accession numbers are as follows: *Eriocheir sinensis:* AGM75775.1; *Scylla paramamosain*: AGN96208.1; *Charybdis feriatus*: AAU93694.1; *Callinectes sapidus:*∗ AEI59132.1; *Callinectes sapidus*: ABC41925.1; *Portunus trituberculatus*: AAX94762.1; *Marsupenaeus japonicus:* BAB01568.1; *Pandalopsis japonica*: ACU51164.1; *Homarus americanus*: ABO09863.1; *Pandalus hypsinotus*: BAD11098.1; *Fenneropenaeus chinensis*: ABC86571.1; *Fenneropenaeus merguiensis*: ACV32381.1; *Penaeus monodon*: ABB89953.1; *Upogebia major*: BAF91417.1; *Palaemon carinicauda*: AFM82474.1; *Cherax quadricarinatus*: AAG17936.1; *Metapenaeus ensis*: AAM48287.1; *Macrobrachium rosenbergii*: BAB69831.1; *Litopenaeus vannamei*: AAP37026.1; *Gammarus marinus*: AHK05985.1. ∗ represents Vtg precursor sequence.

4. Discussion

Purification, detection, cloning and expression of Vtg using immunological and molecular methodologies would permit characterization, and increase our understanding of the site of synthesis. To date, only few *Vtg* sequences have been cloned in brachyurans. Vtg is generally synthesized in extra ovarian sites like liver in vertebrates [10], fat bodies in insects [11] and intestine in

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Charybdis       QICPQHSRLESMYKDAIAFVHEPESVPVMVKELVEGRATGTLAALYSTGFYLVPRPDVKA
Scylla          QICPQHSRLESLYMDAIAFVHEPESVPVMVKELVEGRATGTLAALYSTAFYLVPRPDVTA
Callinectes     QICPQHSRLESMFMDAIAFVHEPESVPVMVKELVEGRATGTLAALYSAAFYLVPRPDVTA
Protunus        QICPQHTKLESLFMDAIAFVHEPESVPVMVKELVEGRTTGTLAALYSTAFYLVPRPDVKA
Oziothelphusa   QICPQHNKLERLYFDAIAFVHEPESVELMVKELLEKRATGTRAALYSAAFSFVSRPNMKA
Eriocheir       QICPQHNKLKSLYFDAIAFVHEPESVIIMAKELAEGRATGARAAIYSAAFYLVPRPNMEA
                ******.:*: :: ************ :*.*** * *:**: **:**:.* :*.**:: *

Charybdis       IRALEPLFKS-NADLSSAKLAAASMVNTYCRHKPHCYEESPVRNLAQALKQKIEEDLSSS
Scylla          IRALEPLFKS-NADLSSAKLAAASMVNTYCRHKPHCHEESHVRNLVQALKQKIEEDLASS
Callinectes     IRALEPLFKS-SAKLSSAKLAAASMVNTYCRHKPHCYDESPVRNLALALKQKIEEDLSSS
Protunus        IQALEPLFKS-SADLSSAKLAAASMVNTYCRHKPHCYNETPVRNLAQALKQKIEEDLSSS
Oziothelphusa   IQALEPLFRASEAHMSSAKLSAASMVNKYCRQNPHCYDEAPVRNLAQALKHDVEEDFSPN
Eriocheir       VQALEPLFRADEPYLSSAKLAAASMVNRYCRHNPQCYNEATVKSLPQAMKQMIQNDLSAS
                ::******:: .. :*****:****** ***::*:*::*: *:.*  *:*: :::*::..

Charybdis       SREETQKQALSALKSLGNMGVMTPETADKVILYMENENKKVSTRVAAAQAFRLTKCQRLV
Scylla          SSEETQRQTLSAFKSLGNMGVMTPEAADKVILYMEKENKKVSNRVAAAQAFRLTKCQRPV
Callinectes     SSEDTQEQALSALKSLGNMGVMTPEVVDKVILYMENENKKVSIRVAAAQAFRLTKCQRQV
Protunus        S-EDTQKQALSALKSLGNMGVMTPEVAEKVVLYMENENKKVSARSR-CTSLQADRVSRPV
Oziothelphusa   SNEESQEKALSAFKSLGNMGVTTPEVSEAVMRYVRKENKKVNIRVAAAQSFRLARCESSV
Eriocheir       NNEESEKKALAAFKSLGNMGVMTHDVAEVLIRYMHSEQKKVNNRVAAVQAFRLSKCNREV
                . *:::.::*:*:******** * :. : :: *:..*:***. *     :::  : .  *

Charybdis       TQKLVQYALRPGQNTEVRIAAYLAAVRCANYEDLQHIVTKISYEENTQVRGFI
Scylla          TQKLVQYALRPEQHTEVRIAAYLAAVRCANYEDLQNIVTKISYEENTQVRGFI
Callinectes     TQKLVQYALRPEENTEVRIAAYLAAVRCAKYEDLEEIVTKISYEENTQVRGFI
Protunus        TQKLVHYALRPEENTEVRIAAYLAAVRCANYEDLQEIVTKISYEENTQVRGFI
Oziothelphusa   TQQLVDFALRPGKNTEVRIACYLAAVRCANFEHLQEIVANISSEENTQVRGFI
Eriocheir       TQKLVDFAVRPGKNTEVRIAAYLTALRCVDYEDLQTIVTKISVEENTQVRGFI
                **:**.:*:** ::******.**:*:**..:*.*: **::** **********
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Fig. 2. Multiple sequence alignment of Vtg amino acid sequences of brachyurans by CLUSTAL W. Taxonomic name of crabs, Vtg amino acid sequence used for phylogenetic analysis, with GenBank accession numbers are as follows: *Eriocheir sinensis:* AGM75775.1; *Scylla paramamosain*: AGN96208.1; *Charybdis feriatus*: AAU93694.1; *Callinectes sapidus*: ABC41925.1; *Portunus trituberculatus*: AAX94762.1. *Oziothelphusa senex senex*: KF563903.1. The symbol at the bottom represents the highly conserved position in the alignment table. An asterisk (∗) indicates that all residues in a column are identical. A colon (:) indicates that very similar residues are found at this position. The full stop (.) mark indicates position where more or less similar residues are found in a column.

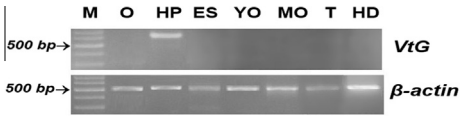

Fig. 4. Expression studies of *Vtg* in different tissues of *O. senex senex*. M: Marker; O: Ovary; HP: Hepatopancreas; ES: Eyestalk; YO: Y Organ; MO: Mandibular Organ; T: Thoracic ganglion; HD: Hypodermis.

nematodes [12]. Recent studies on crustacean vitellogenesis showed that Vtg is synthesized in both hepatopancreas and ovary of *C. sapidus* [24], *S. paramamosain* [25], *P. trituberculatus* [22], *E. sinensis* [23], *Carcinus maenas* [29], *Penaeus monodon* [30,31], *Metapenaeus ensis* [32], and *Marsupenaeus japonicus* [33] but studies of Mak et al. [19] demonstrated that Vtg is exclusively synthesized in hepatopancreas of *C. feriatus*. Zmora et al. [24] and Jia et al. [25] showed that relative contribution of ovary in vitellogenesis is very less when compared to hepatopancreas.

In the present study, deduced vitellogenin amino acid sequence of *Oziothelphusa* is having more than 50% identity with other decapod Vtg sequences. RT-PCR analysis revealed that *Vtg* is expressed in hepatopancreas but expression is completely absent in other tissues including ovaries of *Oziothelphusa*. These results are in conformity with the results of Mak et al. [19] in *C. feriatus* and Raviv et al. [34] in *Litopenaeus vannaemei*. *Vtg* expression is completely absent in different stages of ovaries in *Oziothelphusa* (data not shown). Further, our unpublished data on vitellogenesis of *Scylla serrata* also showed that Vtg synthesized only in hepatopancreas. Though RT-PCR analysis revealed the presence of *Vtg* mRNA in the vitellogenic ovary of *Scylla*, sequencing data indicated the presence of intron with a stop codon in the middle of the cloned mRNA. Piecing these evidences together, it can be concluded that vitellogenin is synthesized exclusively in the hepatopancreas of the crabs, *O. senex senex* and *Scylla serrata*.

Immunodiffusion studies of the present study showed that immunoreactive peptides for Vtg is present only in hepatopancreas extract of vitellogenic stage I females but not in the hemolymph and ovarian extract. Whereas, similar studies using hemolymph, hepatopancreas and ovarian extract of vitellogenic stage II female revealed the presence of Vtg in all the tissues but absent in hepatopancreas extract and hemolymph of male crab. These results infer that Vtg is female specific protein synthesized only in the hepatopancreas transported through hemolymph and finally accumulates in ovary. Our RT-PCR analysis also supports the above hypothesis. It is already established that Vtg synthesized in hepatopancreas is cleaved at a consensus motif (RXXR) by subtilisin-like protein [16] and then these two Vtg subunits were released into hemolymph. Secondary cleavage of Vtg takes place in hemolymph and then these subunits were translocated into ovary through VtgR. In *Oziothelphusa* isolation of Vtg from vitellogenic ovaries also revealed the presence of three protein bands with molecular weight i.e. 73.3, 84.3 and 100.0 kDa [28]. From the above results it can be concluded that Vtg is exclusively synthesized in the hepatopancreas of *Oziothelphusa* and transported to ovary through hemolymph.

Further, phylogenetic analysis indicated that the Vtg of decapods shows a clear speciation event during course of evolution among prawns and shrimps, crayfishes and lobsters, marine crabs and fresh water crabs. It seems apparent that Vtg domain might be used to know the ancestry between decapod crustaceans. Present study is the first report on cloning of *Vtg* in the fresh water crabs to the best of our knowledge.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.148.

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