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Identification and relative abundances of mRNA for a gene encoding the vWD domain and three Kazal-type domains in the ovary of giant freshwater prawns, *Macrobrachium rosenbergii*

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ABSTRACT

Understanding *Macrobrachium rosenbergii* ovarian maturation control at the genome level is an important aspect for increasing larvae production. In this study, an ovarian maturation related gene, *M. rosenbergii* vWD domain and three Kazal-type domains of a gene (*MrvWD-Kazal*) have been studied. The *MrvWD-Kazal* gene was isolated using a rapid amplification of cDNA end (RACE) method and the relative abundances of *MrvWD-Kazal* mRNA in the ovary, hepatopancreas, stomach, intestine and gill were determined by using the quantitative PCR technique. The *MrvWD-Kazal* gene is composed of 2194 bp with an open reading frame (ORF) of 1998 bp encoding 665 amino acids and has great similarity to the *M. nipponense* vWD-Kazal gene (91%). The qPCR analyses indicated the relative abundance of *MrvWD-Kazal* mRNA transcript varied among different stages of ovarian function ($P < 0.05$), but there were no differences abundance in hepatopancreas, stomach, intestine and gill ($P > 0.05$). In the ovary, relative abundance of *MrvWD-Kazal* mRNA transcript gradually increased with ovarian maturation from Stages 1 (Spent; 1.00-fold), to 2 (Proliferative; 3.47-fold) to 3 (Premature; 6.18-fold) and decreased at Stage 4 (Mature; 1.31-fold). Differential relative abundances of *MrvWD-Kazal* mRNA transcript in the ovary indicate the *MrvWD-Kazal* protein may have an important function in ovarian maturation of *M. rosenbergii*. The results of this study also indicate the *MrvWD-Kazal* is not involved in regulation of the reproductive related function of the hepatopancreas, digestive system (stomach and intestine) and respiratory system (gill).

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* is one of the most favourable *Macrobrachium* species for aquaculture enterprises (Nhan, 2009) due the high demand for the meat at markets, disease resistance, ease of culturing and opportunity to produce and develop these prawns in cost efficient way (FAO, 2002). In fact, *M. rosenbergii* can be easily breed in captivity, do not

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require a large volume of water in the production system and because its reproductive cycle can be managed for an extended period of time (Cavalli et al., 1999). As the demand for *M. rosenbergii* increases, it is usually harvested from its natural habitat and cultured in ponds or hatcheries to maintain the greatest inventory as a food source. In aquaculture systems, larva are reared until reaches the adult developmental stage before being commercialized. Larval stock production is dependent on mature female broodstock as these broodstocks are the only potential source for breeding. The eyestalk ablation technique has been used commercially to induce ovarian maturation of broodstock (Choy, 1987). The use of the eyestalk ablation technique has some undesireable outcomes such as losses to death as a consequence of using this technique and lesser than optimal seed ratios for the broodstock (Choy, 1987). Molecular approaches have been developed, therefore, for identifing ovarian maturation related genes which can be potentially used as molecular markers for inducing ovarian maturation in broodstock.

Ovarian maturation consists of activation of a complex series of cellular mechanisms that involve genes regulating the oocyte developmental stages (Qiu and Yamano, 2005). One of the genes that is involved in ovarian maturation is vWD-Kazal. The vWD-Kazal gene was first isolated in the ovary of *M. nipponense* and named *MnvWD-Kazal.* The variable expression of the *MnvWD-Kazal* gene in different ovarian maturation stages indicated that the *MnvWD-Kazal* protein may involved in ovarian maturation of *M. nipponense* (Zhang et al., 2011). The von Willebrand factor (vWF) is a multimetric plasma glycoprotein consisting of subunits jointed by disulphide bonds (Sadler et al., 1985). The vWF protein consists of four domains which are the von Willebrand factors A, B, C and D (Baker, 1988). The von Willebrand factor D (vWD) can be found in the vitellogenin (Vtg) of crustaceans, vertebrates and insects (Raviv et al., 2006; Finn, 2007; Cabrera et al., 2009; Kawakami et al., 2009; Shu et al., 2009; Tiu et al., 2009). The vWD protein functions through binding of the oocyte membrane receptor to Vtg which leads to its uptake in the oocyte (Baker, 1988). Baker (1988) also suggested Vtg provide a nutrient source for embryo development, whereas vWF is also involved in the blood clotting process by mediating platelet binding to vascular walls. The vWF protein functions in haemostasis by promoting the adherence of platelets at sites where the epithelium is injured. This binding site is localized to the D domain of vWD which is homologous to Vtg that is found in humans, rats, nematodes, chickens and frogs.

The Kazal-type protease inhibitor belongs to the family of serine protease inhibitors and it consists of several Kazal-type domains (Li et al., 2008). Male specific Kazal-type has been identified in mammals and invertebrates including *M. nipponense* and *M. rosenbergii* (Li et al., 2008). The Kazal-type protein was detected in the male but not the female reproductive system of *M. rosenbergii* and is localized in epithelial cells and is present during the male maturation process (Li et al., 2008). The Kazal-type protein in male reproduction suppresses sperm gelatinolytic activity and binding of compounds to the sperm base (Li et al., 2008). Zaneveld et al. (1973) proposed that an action of the Kazal-type protein is inhibition of fertilization by participating in enzyme activities of sperm. Because the Kazal-type protein is also expressed during male maturation, the Kazal-type protein is also involved in maintainence of the internal stucture of the male reproductive tract and is directly involved in the fertilization process (Cao et al., 2007; Li et al., 2008). Unfortunately, the functions of the Kazal-type protein in the regulation of ovarian maturation in female *M. rosenbergii* is still unclear. In this study, a gene encoding the vWD domain and three Kazal-type domains was cloned and the relative abundances of the mRNA for this gene was assessed in different ovarian maturation stages of *M. rosenbergii*. The relative abundances of the mRNA for the cloned gene in different stages of ovarian development and in various tissues was also evaluated. The present research on the structure and relative abundances of the *MrvWD-Kazal* mRNA transcript will provide fundamental knowledge about the *MrvWD-Kazal* complex in female *M. rosenbergii*.

2. Materials and methods

2.1. Tissue collection, RNA isolation and reverse transcription

The *M. rosenbergii* (n = 40) were obtained from fisherman at Sungai Manir, Kuala Terengganu, Malaysia (5°18′39.98″ N, 103°5′23.12″ E). Four ovarian maturation stages [Stages 1: Spent (n = 10), 2: Proliferative (n = 10), 3: Premature (n = 10) and 4: Mature (n = 10)] were determined based on descriptions from the previous study of Chengal et al. (2013). Five dissected tissues consisting of the ovary, hepatopancreas, stomach, intestine and gill were placed in a 1.5 mL centrifugation tube and stored in -80 °C. Total RNA was extracted and purified using the innuPREP Mini Kit following the manufacturer's protocol (Analytic Jena, Germany). The concentration and purity of total RNA were spectrophotometrically measured. Total RNA in a concentration of 100 ng/μL was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit by Applied Biosystems (USA) following the manufacturer's protocol.

2.2. RACE PCR and gene cloning

The RACE PCR and gene cloning were conducted using a SMARTer® RACE 5′/3′ Kit (Clontech Laboratories, USA) according to the instructions of manufacrurer's. The 5′end cDNA was identified using a gene specific primer (FS-5GSP2: 5′-TAGGCAGGCATCTGGC AATGTTAG-3′) using the procedures described by Zhang et al. (2011). The 3′end cDNA was identified using a designed gene specific primer (FZ-3GSP: 5′-GGCTTCATTCAAGTGTCTC-3′). The PCR reactions were conducted in conditions of 94 °C during the pre-denaturation period for 3 min, 95 °C for 30 s (30 cycles), 65 °C for 40 s, 72 °C for 1 min and 72 °C for 10 min. The PCR fragments were subjected to electrophoresis using 1.5% agarose gel and eluted from the gel. The amplified cDNA fragments were cloned into the pU19 vector (TaKaRa, Japan) using the manufacturer's protocol.

2.3. Nucleotide sequence analysis

Nucleotide sequences were assembled and searched against reference sequences in the GenBank using BLASTX and BLASTN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein prediction was performed using the ORF Finder Tool (https://www.ncbi.nlm.nih.gov/orffinder/). The p*I* value and molecular weight of the deduced *MrvWD-Kazal* protein were examined using ProtParam (http://www.expasy.org/tools/protparam.html). The protein and signal peptide in the deduced *MrvWD-Kazal* were predicted using SMART (http://smart.embl-heidelberg.de).

2.4. Quantitative PCR analysis

The quantitative PCR was used to determine relative abundance of the mRNA transcript of *M. rosenbergii* vWD-Kazal when there were different ovarian maturation stages in the ovary, hepatopancreas, stomach, intestine and gill. In the addition to the ovary, the hepatopancreas was studied as it is involved in metabolic processes in crustacean (Caceci et al., 1988; Bhavan and Geraldine, 2000). Bastos et al. (2009) reported that the hepatopancreas also has a significant functions in reproduction processes, nutrient absorbtion, energy storage, digestion and excretion. Meanwhile, the stomach and intestine were studied by Zhang et al. (2011) and there were indictions that vWD-Kazal may also have a function in the digestive system of *M. nipponense*. The relative abundance of the vWD-Kazal mRNA transcripts was also determined in the gill to confirm that there are no variation in vWD-Kazal gene expression because the gill participates in physiological processes including respiration of gas transportation and ionic osmotic regulation (Bhavan and Geraldine, 2000; Henry et al., 2012).

Ten samples from each different ovaries in the different maturation stages were used and qPCR was conducted using technical triplicates of each sample. The SYBR Green I qPCR assay was conducted in a 96-CFX (Bio-Rad, USA). The amplifications were performed in an eight-strip 0.2 µL tube in a 25 µL reaction volume containing 12.5 µL of 2X SYBR Green Master Mix (Bioline, UK), 1 µL of each FZ-RTF (5′-ATGGAAGAGCATCTTGTCTGAG-3′) and FZ-RTR primers (5′-TCCATTCACTGTATAACTGGAAGTC-3′) (8 µM), 1 µL of cDNA template and 9.5 µL of nuclease free water. The β-actin (Forward: 5′-CGTGACATCAAGGAGAAGCTGTG-3′; Reverse: 5′-TGACCGTCGGGGAAGCTC-3′) was amplified as a housekeeping gene using the procedures described by Mohamad et al. (2017) (β-actin stability were provided in Data in Brief). The thermal profile for SYBR Green RT-QPCR was 95 °C for 2 min followed by 40 cycles at 95 °C for 5 s and 62 °C for 20 s. The melting curve of vWD-Kazal and β-actin were constructed at the end of qPCR (melting curve results were provided in Data in Brief). The target gene transcript (vWD-Kazal) was normalized with a housekeeping gene transcript (β-actin) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For all tissues, Stage 1 was used to normalize the abundance of mRNA for the target gene.

2.5. Statistical analysis

Statistical analyses were conducted using the Statistical Package for Social Science (SPSS Version 15). The differences of *MrvWD-Kazal* relative abundances of mRNA transcript in the ovary, hepatopancreas, stomach, intestine and gill at different ovarian maturation stages were assessed using a one-way analysis of variance (ANOVA) followed by use of the Duncan's multiple range test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. MrvWD-Kazal gene sequence and structure of deduced protein analysis

The cDNA sequence of *MrvWD-Kazal* was 2194 bp in length containing an ORF of 1998 bp corresponding to a polynucleotide of 665 amino acids (Fig. 1). The initiation codon, ATG is at position 195 of the 5′untranslated region of the *MrvWD-Kazal* sequence. The 5′ UTRs of *MrvWD-Kazal* was 194 bp. The calculated p*I* and molecular weight of the deduced *MrvWD-Kazal* protein were 5.30 and 72.5 kDA, respectively. The *MrvWD-Kazal* structure was similar to vWD-Kazal reported in *M. nipponense* with the results from BLAST analyses indicating the structure of *MrvWD-Kazal* was highly similar to *M. nipponense* vWD-Kazal mRNA (HM132051.1) with 91% identity and an e-value = 1e-160. The nucleotide and deduced amino acid sequences were deposited in the NCBI database with an accession number of MF773969.1 and AXH21510.1, respectively.

3.2. MrvWD-Kazal mRNA transcript abundance in the ovary of M. rosenbergii

The relative abundances of *MrvWD-Kazal* mRNA transcript in the ovary at different ovarian maturation stages are depicted in Fig. 2. The relative abundances of *MrvWD-Kazal* mRNA transcript increased at Stage 2 of development by 3.47-fold ($P < 0.05$) compared to Stage 1 (1.00-fold). The relative abundance of *MrvWD-Kazal* mRNA was the greatest at Stage 3 of development being 6.18-fold greater than at Stage 2 ($P < 0.05$). The relative abundance of *MrvWD-Kazal* mRNA transcript, however, was less at Stage 4 than at Stage 3 of development (1.31-fold; $P < 0.05$).

3.3. MrvWD-Kazal mRNA transcript relative abundance in various tissues of M. rosenbergii

In Fig. 3 a–d, the relative abundance patterns of *MrvWD-Kazal* mRNA transcripts are depicted in the hepatopancreas, stomach,

(a)

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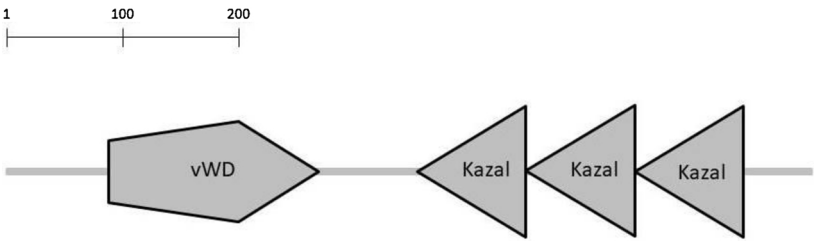
                                                                  A A A C A A A G A T T T A T   14
TTGAAGTCTC CATAAGGCGA ATGGGGAGAT GCACGTTCAT GACACCCGGG TTGTGAGTGC   74
CTGTCGCCGA TCTACGGCAG TCCTCACACG ATGTGACGTC ATTGCTTAAA GTATAGCAAA  134
ACTTACATCG GAATCCTTGG TGTGGAAATG CTCAGAGCAA CTTAATCAGT GCTGTAGTGT  194
ATGTTTTTGG TGTTTGTCTG TCTTCTGGTT TTTAACGTTG GAAATATGGA TAAAGGTTTG  254
M F L V F V C L L V F N V G N M D K G L   20
ACGTTCATTC  TCCTGACAC TGATTACCG TGAATGTTGC CGAATCTAGA GTGATACCAAGT  314
T F I L L T L I T V N V A E S R V I P S   40
GTTTCTGATC TAAATATCTT GCCAGTCCAT AGAGGATGCT GTTTTGGTTA CCAAAACTAT  374
V S D L N I L P V H R G C C F G Y Q N Y   60
GATCACCTGG ATGTAGTACT AACATTGCCA GATGCCTGCC TAAGACTCAT TTGTAACAAT  434
D H L D V V L T L P D A C L R L I C N N   80
GGGGAGATTG AAGCACAATA TGTTGGAAAG CCAGGGCAGA GGGACTGTTG TGAATTCAAT  494
G E I E A Q Y V G K P G Q R D C C E F N  100
GGCCTGCTTT ATGCTGATAA GTCGGAACTA CCTCTCCAGT GCTTCACTTT TGAGTGTAGG  554
G L L Y A D K S E L P L Q C F T F E C R  120
AAAGGTCATT GGTACACTAA TGGCACAATT CATGACTGCT GCAAGCACTG TGCTCTGTAC  614
K G H W Y T N G T I H D C C K H C A L Y  140
AACGATCCCC ACATAAAAAC TTTTGATGGG CACAGCTATG ATTGGCATGG CACTTGTAAT  674
N P D H I K T F D G H S Y D W H G T C N  160
TATTCTGTAG CGCAGCCATC CTTCACCCAT TCCCCAGAAT TTGGAATATT TAGTGACTTT  734
Y S V A Q P S F T H S P E F G I F S D F  180
TCAGCATGTA ATGGAAGAGC ATCTTGTCTG AGCAGGACTA CTTTTAAGGA TAACTCCAAT  794
S A C N G R A S C L S R T T F K D N S N  200
ACTGTGATTG TTTTTGACCA TGGCATGACT TCCAGTTATA CAGTGAATGG AGAGCTAAAG  854
T V I V F D H G M T S S Y T V N G E L K  220
ACAGTGACTT CATTAGGTGT GAGCCAAGTA ACCAGTTCAG TAGGGATACA CCCAGTGCTG  914
T V T S L G V S Q V T S S V G I H P V L  240
GTGTGGAAAT GCAATCAGTG TCTGGCTATG GCTGGTTCAT CACAGTTAAC AATTTTAGTA  974
V W K C N Q C L A M A G S S Q L T I L V  260
TGTCCAAACA GAATGGATGT CTGGGCTCAC CCAAGTCACA CTGGAAACTT GAATGGACTC 1034
C P N R M D V W A H P S H T G N L N G L  280
TGCGGCCATT TCAGCTCTTA CAAGGATGAT GATTTTACCA CCAGGAATAA TATTGTTGAG 1094
C G H F S S Y K D D D F T T R N N I V E  300
CCTCTGACCA AGTTCCCTAT GGCGTTCCCT TCCTCATGGA TGACTAGTGA CCAAGGCAAT 1154
P L T K F P M A F P S S W M T S D Q G N  320
CCAGCTTGCA GTGATCCATG CCCAGAGTGT TTCAAAAGTG AAACGGAGGA TCCGTGTAAA 1214
P A C S D P C P E C F K S E T E D P C K  340
GCAGGTGAGA AGGCAAAGAA AATATATCGA GAAAGATGCA GGAAATCTTT GCGAGATGTG 1274
A G E K A K K I Y R E R C R K S L R D V  360
ATTCAAGACA ATGATAACCT GTCTCAGGTG CATCTTGATG CTTGTGCATT TGATGTTTGT 1334
I Q D N D N L S Q V H L D A C A F D V C  380
GTCATGATCC AAAATGGTGC AGGTGATAAG GATGTGGAAG AATGGCTTAG ACAGCTTTTG 1394
V M I Q N G A G D K D V E E W L R Q L L  400
GAGGTTGTCA GAATGACAAG ATTTGTTGGT GGTGGTAGTA TCTGTGATCC TTGTTCGAAA 1454
E V V R M T R F V G G G S I C D P C S K  420
GGCTGCAATA AACTTTACGA TCCTGTCTGT GGCACAGACG GTAAAACTTA TGGTAATCCA 1514
G C N K L Y D P V C G T D G K T Y G N P  440
TGTATTCTAG AAGTAGCCAT TTGCAATGAC AAGACCCTTG GCTTGAAGCA TAAAGGCGAA 1574
C I L E V A I C N D K T L G L K H K G E  460
TGTGGAGATA CATGCGTACA GGCTTGTCCT GAAAATTATG ATCCAGTTTG TGGGACTGAT 1634
C G D T C V Q A C P E N Y D P V C G T D  480
GGAAGAACTT ACGGTAACAA CTGTGCTCTA GGTGTAGCCT CTTGTATGAA CAAGTGCATT 1694
G R T Y G N N C A L G V A S C M N K C I  500
GGCTTCAAAC ATGTTGGAGA ATGTAGTCCT GGGTGTCCCA TTGAGATATG TACCCTAGAA 1754
G F K H V G E C S P G C P I E I C T L E  520
TATAGACCAA TTTGTGGTTC CAATGGTGTG ACTTACGGAA ACGAATGTAG CTTCAATTCT 1814
Y R P I C G S N G V T Y G N E C S F N S  540
GCTAAGTGTG ACAACCCTTG TTTGGAGAAG AGACATGATG GGGAGTGTTT GAGCCAGATT 1874
A K C D N P C L E K R H D G E C L S Q I  560
GGAAGGTCAA GCTCCCAAGA AAGTCCTGAT CTTGATGATA ACTTGGATCT TGGAAAAATC 1934
G R S S S Q E S P D L D D N L D L G K I  580
TTGAATGCAG AAAATGCTGT TAATGCTGAG AATGCTGTTA ATGCAGAGAA TGCCATCCAT 1994
L N A E N A V N A E N A V N A E N A I H  600
GCCAAAAATG CCGTGAGTGC AGAGAACGCC GACAATGCAG TACATGCTAA AAATGCTGGG 2054
A K N A V S A E N A D N A V H A K N A G  620
AATGCTGAAA ATGCTGTCAA TGCAGAAAAT GCAGTAAATG CTATCAATGC AGTGTTTGCC 2114
N A E N A V N A E N A V N A I N A V F A  640
CTAAACTTAG TTAAATCAGA CACTGCTGAA AAGCATATAG TTCCCCCGTT GAGATTAGCA 2174
L N L V K S D T A E K H I V P P L R L A  660
CCCACAGGGT CTGAAGGGGA                                             2194
P T G S E G G                                                     667
```

(b)



(caption on next page)

Fig. 1. cDNA sequence and the 2D deduced amino acid structure of the *MrvWD-Kazal* gene domain; (a) cDNA and deduced amino acid sequence of *MrvWD-Kazal*; Start (ATG) is illustrated in boldface; Nucleotide sequence is displayed in the 5′-3′ directions and numbered to the right; Deduced amino acid sequence is shown in the single letter amino acid code; vWD domain is shadowed and three Kazal-type domains are underlined; (b) 2D deduced amino acid structure of *MrvWD-Kazal* domain.

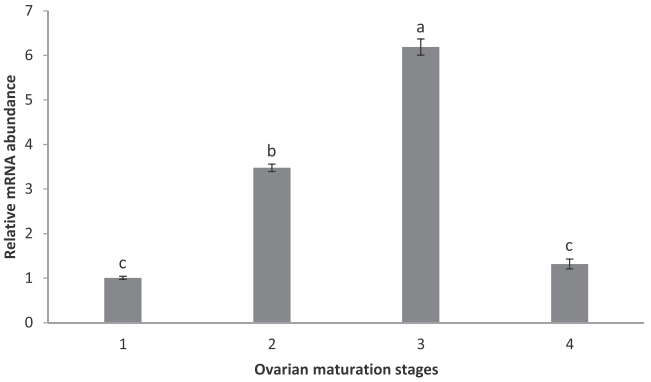


Fig. 2. Relative abundance of *MrvWD-Kazal* mRNA transcript in the ovary at different ovarian maturation stages; Stage 1-Spent, Stage 2-Proliferative, Stage 3-Premature, Stage 4-Mature; Y-axis represents the relative mRNA transcript abundance of *MrvWD-Kazal*/ β-actin; Data shown as means ± SE (standard error) for triplicates of ten different individuals; Different lowercase letters (a, b and c) indicated difference; same lowercase letters indicate no significant difference between different developmental stages.

intestine and gill at different ovarian maturation stages, respectively. There are no significant differences in *MrvWD-Kazal* mRNA relative abundances in the hepatopancreas (Stage 1: 1.00-fold, Stage 2: 1.24-fold, Stage 3: 1.20-fold, Stage 4: 1.25-fold), stomach (Stage 1: 1.00-fold, Stage 2: 1.02-fold, Stage 3: 1.04-fold, Stage 4: 1.06-fold), intestine (Stage 1: 1.00-fold, Stage 2: 1.26-fold, Stage 3: 1.10-fold, Stage 4: 1.08-fold) and gill (Stage 1: 1.00-fold, Stage 2: 1.06-fold, Stage 3: 0.97-fold, Stage 4: 1.09-fold) during different ovarian maturational stages ($P > 0.05$).

4. Discussion

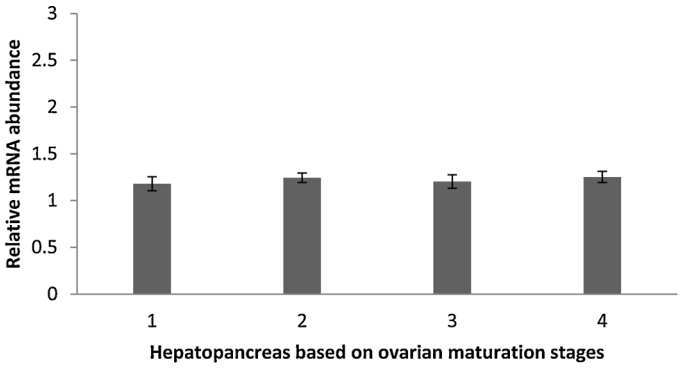
The *MrvWD-Kazal* mRNA sequence consisted of 2194 bp with an ORF of 1998 bp corresponding to 665 amino acids and the value of p*I* and molecular weight of deduced protein sequence was 5.30 and 72.5 kDA compared to the vWD-Kazal that was isolated from the *M. nipponense* ovary which is 2713 bp containing 2574 bp in ORF (857 amino acids; p*I* = 4.89; 92.7 kDA). the BLAST results indicated that the 2194 bp sequence was similar to vWD-Kazal of *M. nipponense* (HM132051.1) with an e-value = 1e-160 and 91% identity and the name provided was *MrvWD-Kazal*. The *MrvWD-Kazal* protein is comprised of four conserved regions which are the vWD domain and three Kazal-type domains.

The qPCR results indicated that the relative abundance of *MrvWD-Kazal* mRNA transcripts changed markedly at different stages of ovarian development (Fig. 2). There was differential relative abundance of *MrvWD-Kazal* mRNA transcript in a sequential pattern in the ovarian tissues of *M. rosenbergii* at ovarian maturational stages with the relative abundances increasing from Stage 1 to 3 and markedly decreasing at Stage 4. The relative abundance of *MrvWD-Kazal* mRNA transcript started to increase at Stage 2 as the vWD binding to the oocyte membrane receptor to Vtg increased which resulted in an uptake of Vtg into the oocyte resulting in enhanced oocyte development before ovarian maturation was complete (Baker, 1988).

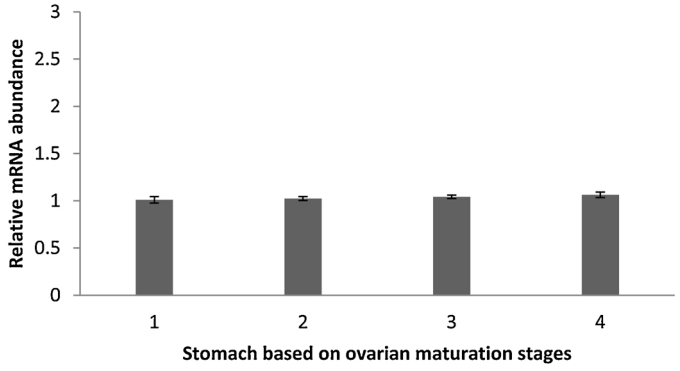
The relatively greater abundance of *MrvWD-Kazal* mRNA transcript at Stage 3 was due to the proteolysis-protective properties of vWD as this is an important process for the protection of Vtg during differential proteolysis of the egg yolk protein in the final stage of oocyte maturation (Finn, 2007). The relative abundance of *MrvWD-Kazal* mRNA was markedly less at Stage 4 than 3 as the meiosis of oocytes ceased and ovarian maturation was completed (Gao et al., 2006; Meeratana and Sobhon, 2007; Zhang et al., 2010). The trend of change in relative abundances of *MrvWD-Kazal* mRNA transcript at different ovarian maturation stages in *M. rosenbergii* was concomitant with the changes in relative abundances of *MnvWD-Kazal* in *M. nipponense* where the trend change for *MnvWD-Kazal* mRNA transcript at each ovarian maturation stage indicated *MnvWD-Kazal* was associated with oogenesis in *M. nipponense* (Zhang et al., 2011).

The trend changes in relative abundance of *MrvWD-Kazal* was also similar to the Ubc9 mRNA transcript in the ovary of *M. nipponense* where the relative abundance of Ubc9 mRNA transcript increased from Stage 1 and was greatest at Stage 3 of development before decreasing at Stage 4 (Zhang et al., 2010). The Ubc9 protein is involved in the regulation of meiosis and mitosis processes in the ovary of *M. nipponense* (Watanabe et al., 1996; Sakaguchi et al., 2007). The lesser relative abundances of Ubc9 mRNA transcript at Stage 1 coincided with the mitosis stage in the oogania (Zhang et al., 2010). The meiosis of oocytes is initiated at Stage 2 of ovarian development and continues until Stage 4 (Gao et al., 2006; Meeratana and Sobhon, 2007). The concomitant change in the relative abundance of Ubc9 mRNA transcript with the meiosis process is indicative that the Ubc9 protein is involved in ovarian maturations of *M. nipponense* (Zhang et al., 2010).

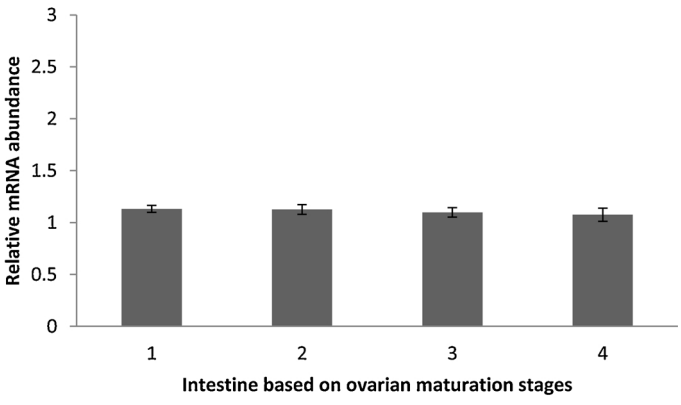
(a)



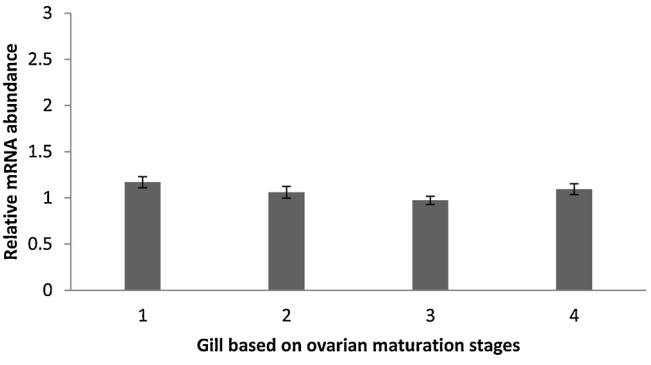
(b)



(c)



(d)



(caption on next page)

Fig. 3. Relative abundance of *MrvWD-Kazal* mRNA transcript in various tissues at different ovarian maturation stages; Stage 1-Spent, Stage 2-Proliferative, Stage 3-Premature, Stage 4-Mature; Y-axis represents the mRNA abundance ratios relative to the abundance of *MrvWD-Kazal*/ β-actin mRNA while x-axis represents the ovarian maturation stage; Data shown are means ± SE (standard error) of triplicates of ten different individuals.

In addition, the relative abundance of *MrvWD-Kazal* mRNA is similar trend with the relative abundances of mRNAs that encode proteins that are involved in adipose differentation such as adipose differentiation-related protein (ADRP) in the ovary of the black tiger prawn, *Penaeus monodon*. Sittikankaew et al. (2010) reported that, the relative abundance of ADRP in *P. monodon* was increased in Stages 1 and 2 of ovarian development before reaching the maximum at Stage 3. The relative abundance of ADRP mRNA transcript markedly decreased at Stage 4. The ADRP protein is involved in regulation of ovarian maturation processes where the abundances of ADRP increased when the accumulation capacity of oocytes to uptake long chain fatty acids is increase (Sittikankaew et al., 2010). Hence, the trends of changes in the relative abundances of *MrvWD-Kazal* mRNA transcript is similar to the changes in the relative abundances of Ubc9 and ADRP mRNA transcripts indicating the probable importance of the vWD-Kazal protein in ovarian maturation of *M. rosenbergii*.

The vWD protein has an important function in cellular recognition such as in mucin and zonadhesin actions where the vWD domain provides a site for anchor that facilitates the binding of Vtg to its Vtg receptor (Finn et al., 2007). The globular structure of vWF also indicates that this protein has the lubricant characteristics in mucins and adhesive function of other proteins including the zonadhesins (Desseyn et al., 1997; Gipson et al., 1997; Toribara et al., 1997; Sadler, 1998). The vWD protein has adhesive properties and functions at the oocyte membrane receptor by facilitating the binding of Vtg which leads to uptake of Vtg into the oocyte (Opresko and Wiley, 1987).

In male *M. nipponense*, the Kazal-type protein suppresses sperm maturation and is involved in the fertilization process by inhibiting actions of sperm protease (Jalkanen et al., 2006; Li et al., 2008, 2009). Serine protease inhibitor in *Xenopus laevis*, EP45, is also involved in meiotic division, embryonic development and interference in development and oocytes quality (Haspel et al., 1993; Martei et al., 2009). According to Zhang et al. (2011), there is no report on the association of Kazal protease inhibitor in the oocyte maturation process, however the changes in *MnvWD-Kazal* mRNA relative abundance with the oocyte maturation process implies a relationship between these two processes in ovarian maturation of *M. nipponense*.

In the hepatopancreas, reproductive hormones such as the gonadotropin hormones (GtH) induce the secretion of 17β-estradiol which result in stimulation of hepatic cells to synthesize the egg yolk protein precursor of Vtg (Matsubara et al., 1994). The Vtg will be transported to the ovaries and there is uptake by the developing oocytes. The Vtg is the precursor egg yolk that is present in female fish, amphibians, reptiles, birds and invertebrates (Sumpter and Jobling, 1995) that is normally present in females undergoing oogenesis. The Vtg is important in reproduction processes because Vtg is the main source of nutrients for the developing embryos (Mommensen and Walsh, 1998).

The Vtg is produced in the hepatopancreas as a precursor of yolk protein, which is then processed into smaller molecules and accumulates in oocytes as vitellin (Om, 2014). The Vtg gene was expressed in the hepatopancreas of pleocyemata species (Abdu et al., 2002; Okuno et al., 2002). Meanwhile, in dendrobranchiata species, the Vtg gene was expressed in the hepatopancreas and ovary (Tseng et al., 2001; Tsutsui et al., 2000; Avarre et al., 2003; Tsang et al., 2003). The sub-epidermal adipose tissue is known as a site of Vtg synthesis in decapods (Tom et al., 1987; Han et al., 1994; Rani and Subramoniam, 1997). Briefly, the hepatopancreas functions are associated with ovarian maturation of crustaceans including the *Macrobrachium* species. In *M. rosenbergii*, Vtg was detected in the hepatopancreas at all ovarian maturation stages and ovarian maturation is facilitated as a result of functions of the hepatopancreas (Ara and Damrongphol, 2014). The relative abundance of Vtg mRNA increased from Stage 1 to 4 due to the increased in yolk protein accumulation with the developing ovarian maturation stage (Chengal et al., 2013). In the present study, there were marked changes in the relative abundance of *MrvWD-Kazal* mRNA transcript in the *M. rosenbergii* ovary from Stage 1 to 4 [Fig. 3 (a)] of ovarian development, however, there were no changes in relative abundance of *MrvWD-Kazal* mRNA transcript in the in hepatopancreas indicating the *MrvWD-Kazal* protein is not involved in metabolism during the period of ovarian maturation in *M. rosenbergii*.

Results of studies by Laskowski and Kato (1980) indicated that Kazal-type protein also participates in serine protease activities in the digestive system and inhibits the early maturation of insulin activating enzyme. In *M. nipponenese*, the relative abundance of vWD-Kazal was the greatest in the intestine which suggested vWD-Kazal may also have functions in the digestive system of *Macrobrachium* species (Zhang et al., 2011). In the present study, however, the relative abundances of *MrvWD-Kazal* mRNA transcripts were consistantly unchanged in the stomach and intestine throughout ovarian maturation stages [Fig. 3 (b and c)] as compared with the relative abundance of *MrvWD-Kazal* mRNA in ovary (Fig. 2). Based on this finding, it appears as though the *MrvWd-Kazal* protein has no functions in regulation of biological processes of the digestive tract (stomach and intestine) in *M. rosenbergii*.

The gill has important functions in physiological processes such as in respiratory gas transportation and ionic osmotic regulation (Bhavan and Geraldine, 2000; Henry et al., 2012). The results of the present study indicate there are basal relative abundances of *MrvWD-Kazal* mRNA transcript in the gill [Fig. 3 (d)], indicating that the *MrvWD-Kazal* protein in gill is not related to ovarian maturation. Results from studies of Ara and Damrongphol (2014) indicate there is Vtg protein expression in the hepatopancreas and ovary but not in the gill, which might be due to the significant function of the Vtg protein in the hepatopancreas is correlated with ovarian maturation of *M. rosenbergii*. The apparant lack of expression of the *MrvWd-Kazal* gene in the gill in the present study inconsistent with the findings indicating that this gene has significant functions in the *M. rosenbergii* gill such as in calreticulin actions that are involved in several important biological process such as growth, molting and stress response to temperature (Hirak et al., 2012).

5. Conclusion

In the present study, there was successful cloning of the cDNA of *MrvWD-Kazal* which was 2194 bp in length and contained an ORF of 1998 bp and is a polynucleotide of 665 amino acids. The relative abundances of *MrvWD-Kazal* mRNA transcript during ovarian maturation stages indicated there are actions of the *MrvWD-Kazal* protein in ovarian maturation processes of *M. rosenbergii*. The *MrvWD-Kazal* protein apparently functions in ovarian maturation and allows subsequent normal ovarian maturation processes to occur. Differential relative abundances of *MrvWD-Kazal* mRNA occur in the ovary but not in the other tissues evaluated in the present study. Because there is no significant difference in relative abundances of *MrvWD-Kazal* mRNA transcript in the hepatopancreas, stomach, intestine and gill during ovarian maturation stages, it is suggested that the *MrvWD-Kazal* gene is not involved in the regulation of reproduction-related changes in the hepatopancreas, digestive system (stomach and intestine) and respiratory system (gill).

Declaration of Competing Interest

None.

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