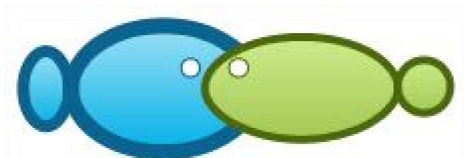

RNA expression of farnesoic acid O-methyl transferase in mandibular organ of intermolt and premolt mud crabs *Scylla olivacea*

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Abstract. Farnesoic acid O-methyl transferase (FAmeT) is an important enzyme for converting farnesoic acid into methyl farnesoate. Methyl farnesoate has a significant role in metamorphosis and reproduction of crustacean. The aims of this research were to determine the expression level of FAmeT RNA in intermolt and premolt stages and to characterize the sequence of FAmeT RNA in *Scylla olivacea.* The gene expression analysis was carried out through RNA extraction of mandibular organ then followed by RT-PCR, and for sequence analysis, purification of PCR product and sequencing was conducted. The thickness of bands of RNA FAmeT level in premolt was higher than intermolt. The thickness of bands in intermolt stage was 457.6±183.4 ng/μL, and in premolt stage was 502.8±155.8 ng/μL. FAmeT RNA sequence was identical with *S. serrata* and *S. paramamosain.*
Key Words: crustacean, seafood, enzyme, FAmeT, S-adenosyl-methionine.

Introduction. Farnesoic acid O-methil transferase (FAmeT) also known as S-adenosyl-methionine, which play a role in converting farnesoic acid (FA) into methyl farnosoate (MF) in the final step biosynthetic pathway (Borst et al 2001; Liu et al 2010). FAmeT was used to detect the presence of MF in several crustaceans e.g. *Metapenaeus ensis* (Gunawardene 2002) and American lobster *Homarus americanus* (Holford et al 2004).

FAmeT has been identified in some insects and crustaceans e.g. *Nilaparvata lugens* (Liu et al 2010), *Cancer pagurus* (Ruddell et al 2003), *Metapenaeus ensis* (Gunawardene 2002), *Portunus pelagicus, Scylla serrata* (Kuballa et al 2007), and *H. americanus* (Holford et al 2004). Estimated molecular weight of FAmeT in *H. americanus* is 38 KDa (Holford et al 2004).

Kuballa et al (2007) found that the cDNA sequence of FAmeT in swimming crab and mud crab *S. serrata* have three forms, i.e. long, medium and short sequences. In shrimps *Penaeus monodon, Fenneropenaeus merguiensis, Thenus orientalis* and *Cherax quadricarinatus* have 2 forms sequences, i.e. long and short. These finding indicated that FAmeT RNA in some crustaceans were multigenes group.

Methyl farnesoic (MF) is synthesized and secreted by mandibular organ (MO) in several crustaceans (Laufer et al 2002). MF is sesquiterpenoid compound (Borst et al 2001; Li et al 2010) and a member of juvenile hormone (JH) family in insects (Borst et al 2001; Holford et al 2004). The chemical structure of MF is nearly identical with juvenil hormone III (JH III) in insects. This similarity suggests that MF might have a similar role with JH III (Borst et al 2001). Several studies have demonstrated the MF role in crustaceans, e.g. MF stimulation of ecdisteroid secretion by Y-organs of the crab *in vitro* (Tamone & Chang 1993). The concentration of MF in MO has positive correlation with molting stages (Laufer et al 2002).

Mud crabs are well known in Indonesia as commercial fishery commodity, but studies are lacking. Whereas, the study of this species is very important for aquaculture

and development. In the present study we report the gene expression of FAmeT in intermolt and premolt stages and characterize the partial FAmeT RNA of mud crabs *Scylla olivacea.*

Material and Method

Animals. Adult male mud crabs *Scylla olivacea* intermolt and premolt stages were obtained from educational farms of Hasanuddin University, Bojo Village, Barru Regency, South Sulawesi Province, Indonesia. Experimental animals were immersed in cold water (10°C) and MO was removed from anesthetised crabs, and kept in freezer (-80°C) until RNA extraction.

RNA extraction. The FAmeT RNA was extracted using RNeasy Mini Kit (Qiagen), and amplificated by SuperScript III OneStep RT-PCR with Platinum Taq Polimerase (Invitrogen) according to manufacturer instruction. The concentration of RNA expression was measured using spectrophotometer in 260 and 280 nm. β-actin was used for qualitative test of extracted RNA.

Amplification of β-actin. Amplification was conducted by using SuperScript III OneStep RT-PCR with Platinum Taq Polimerase (Invitrogen) according to manufacturer instruction. β-actin gene was amplified by using primers β-actinF 5′-GAGCGAGAAATCGTTCGTGAC-3′ and β-actinR 5′-GGAAGGAAGGCTGGAAGAGAG-3′ (Zeng et al 2013). The PCR was performed in a 25 μL reaction volume and the PCR cycles were synthesis cDNA 45°C 30 minutes, pre-denaturation 95°C 30 sec, 50× (95°C during 10 sec, 60°C during 30 sec, 72°C during 20 sec), and 72°C during 10 minutes. The results of RT-PCR were visualized in 2% agarose gel in TAE buffer 1×.

Amplification and quantification of RNA. Amplification of FAmeT RNA used SuperScript III OneStep RT-PCR with Platinum Taq Polimerase (Invitrogen) according to manufacturer instruction. RT-PCR of FAmeT was carried out by using Primers FAmeTQ1 5′-GGCACGGACGAGAACAA-3′ and FAmeTQ2 5′-GCGACGCTGAAGGAGAT-3′ (Yang et al 2012). PCR cycles were (modification of Yang et al 2012) i.e. synthesis of cDNA 45°C during 30 minutes, pre-denaturation 94°C during 2 minutes, 35× (94°C 30 sec, 50°C during 1 minutes, 72°C during 30 minutes), and 72°C during 10 minutes. Then the results of RT-PCR were visualized in 1.5% agarose gel TAE buffer 1×. The concentration of RNA expression was measured by bands thickness in gel by software of UN-SCAN IT.

Gel purification. Purification was conducted to obtain the pure RNA as well as objective. NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) followed company instruction was used for this purification. The results of gel elution were confirmed by PCR.

Sequencing of RNA. Sequencing FAmeT RNA by PT. Genetika Science Jakarta and 1st Base Malaysia. The results of sequencing was compared with other crabs species. Similarity RNA of *S. olivacea* and the other species analysed by BLAST GenBank NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). While the difference of sequence position was performed by alignment used Bioedit software.

Results and Discussion

Expression of RNA. β-actin gene is constitutively express in cells, and called as house keeping gene. Amplification of β-actin primer resulted amplicon 202 bp, clearly showed in all sample of both stages (Figure 1A). The results of RT-PCR product of FAmeT RNA in intermolt and premolt phase show amplicon 450 bp (Figure 1B and 1C).

Measurement of concentrations showed the PCR product concentration difference between intermolt and premolt. The thickness of bands in intermolt stage was 457.6±183.4 ng/μL, and in premolt stage 502.8±155.8 ng/μL. The concentration changes of RNA expression increased during intermolt lead to premolt.

Increasing concentrations of PCR product of RNA FAmeT during intermolt towards premolt indicate RNA expression in *S. olivacea* increased just before molting although the increase is not significance.

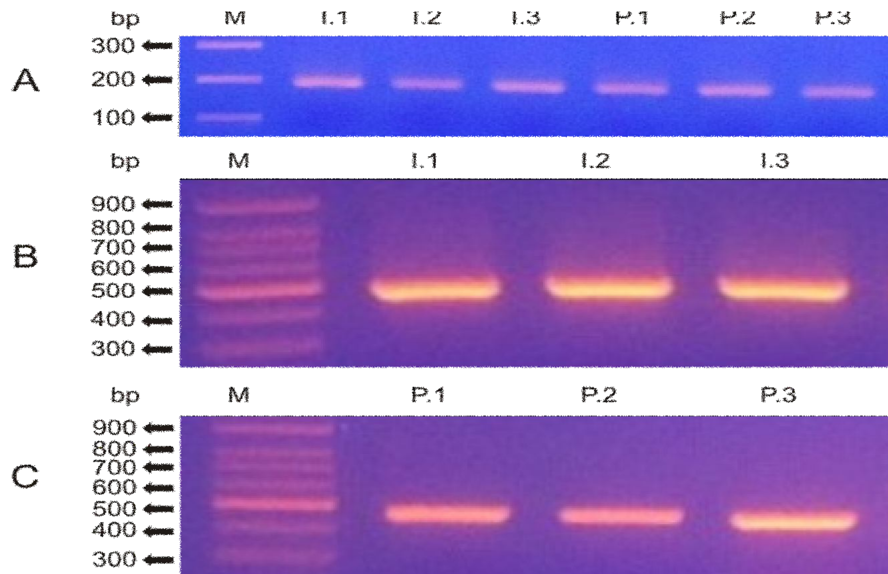

Figure 1. Amplification of FAmeT RNA of MO in *Scylla olivacea*. A: amplification β-actin, B: FAmeT RNA during intermolt, C: FAmeT RNA during premolt; M: marker 100 bp, I.1–I.3: MO during Intermolt, P.1–P.3: MO during premolt.

Analysis of RNA sequence. Sequencing process was conducted partial, but some relevant information of FAmeT RNA sequence was obtained. Kuballa et al (2007) and Yang et al (2012) divides the sequence of FAmeT RNA of mud crabs into 3 types i.e. short, intermediate and long. Yang et al (2012) also revealed that the differences in the three forms of FAmeT RNA lies in several nucleotide bases in the coding region. The intermediate isoform have 6 bp deletion and the short isoform have 15 bp deletion when compared with the long isoform. The existence of diverse forms of the FAmeT RNA refers to the possibility of control mechanisms to regulate the synthesis of MF through regulation of expression form of the enzyme that catalyzes the activation of the final stage of conversion farnesoic acid (FA) into MF (Kuballa et al 2007).

Sequences of FAmeT RNA of *S. olivacea* in this study could not be determined the sequence type, whether included in the form of short, intermediate or long. The sequence of FAmeT RNA obtained is not a complete sequence, but only to the extent sequences flanked by a pair of specific primers FAmeTQ along 450 bases in mid position of sequences of FAmeT RNA in *S. serrata* and *S. paramamosain*.

Results of the alignment analysis with several crustacean types showed similarity value of sequences of *S. olivacea* RNA with two types of mud crab *S. paramamosain* and *S. serrata* by 99% (Table 1). Different results are shown in crab *P. trituberculatus* that is equal to 95% and 94% of *P. pelagicus*. This value indicates that the sequence of FAmeT RNA between *Scylla sp*. with *Portunus sp*. has a different sequence but not significant wiyh only 4–5%.

Beside the alignment analysis, sequences of FAmeT RNA of *S. olivacea* was also analyzed using Bioedit to see the difference of sequences of FAmeT RNA between the three species of mud crabs (Figure 2) and detail differ base showed in Table 2. There are several different nucleotide bases when compared with nucleotide bases of FAmeT RNA in *S. serrata* and *S. paramamosain*.

Table 1
Alignment analysis of oligonucleotide sequences of *Scylla olivacea* FAmeT RNA compared by several species of crustacean

Sequences of FAmeT RNA	*Gen bank access code*	*Similarity of S. olivacea (%)*
Short Scylla paramamosain	HQ587050.2	99
Intermediate Scylla paramamosain	HM352791.2	99
Long Scylla paramamosain	HQ587049.2	99
Short Scylla serrata	DQ187989.1	99
Intermediate Scylla Serrata	DQ187990.1	99
Long Scylla serrata	DQ187991.1	99
Short Portunus pelagicus	DQ085280.1	94
Intermediate Portunus pelagicus	DQ085281.1	94
Long Portunus pelagicus	DQ085282.1	94
Portunus trituberculatus	KC192659.1	95

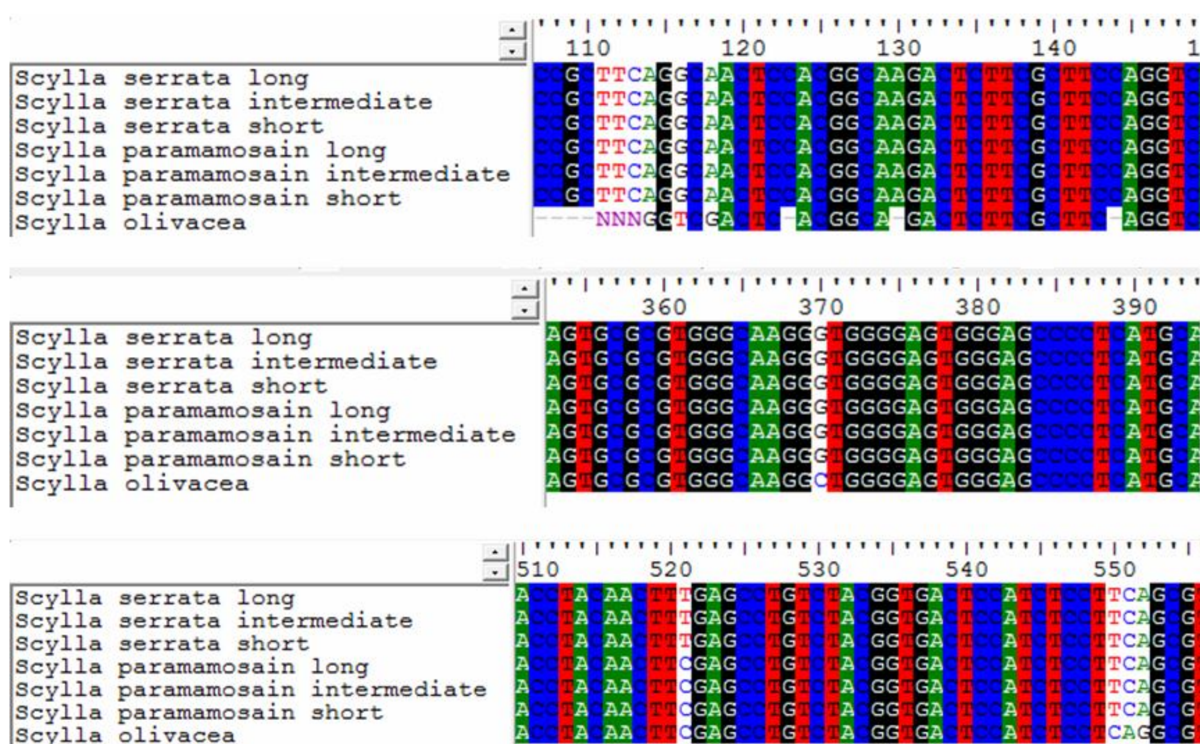

Figure 2. The alignment of the FAmeT RNA sequences of *Scylla olivacea* with *Scylla serrata* and *Scylla paramamosain*. Colored sequences are conserve area that have similar sequence nucleotide.

Table 2
Disparity of nucleotide bases of FAmeT RNA in *Scylla olivacea*, *Scylla serrata* and *Scylla paramamosain*

Base sequence	*S. olivacea*	*S. serrata*	*S. paramamosain*
114	G	A	A
116	T	G	G
118	G	A	A
370	C	G	G
521	C	T	C
550	C	T	T
551	A	C	C
552	G	A	A

Discussion. In present study, we found concentration of expression of FAmeT RNA was increased gradually from intermolt to premolt stages. These finding did not show significant difference and unconformity with hypotheses that expression of FAmeT RNA will increase significantly in premolt stage. Differ from it, the concentration of MF in haemolymph of crabs *Libinia emarginata* showed significant enhancement i.e. 1 ng/mL at 23 days before ecdysis, then increased 1.5 ng/mL at 7 days toward ecdysis (Laufer et al 2002). Thus in *L. emarginata* that ablation the eyestalk has content of MF in haemolymph increased during premolt stage (Laufer et al 1997). It was likely the existence of other factor which has a role in synthesis collaboration (Laufer et al 1997).

The pattern of RNA expression in MO was not high, this may related with the existence of distribution of FAmeT RNA in several organs such eyestalk, hepatopancreas, and ovarium (*Metapenaeus ensis*) (Gunawardene 2002), muscle, haemolymph, eyes, epidermis, Y organ, gills, ovarium, hepatopancreas, intestines (*Cancer pagurus*) (Ruddell et al 2003), head, thorax, stomach, brain, and ovarium (*Nilaparvata lugens*) (Liu et al 2010), brain, thoracic ganglia, eyestalk, stomach, ovarium, hepatopancreas, and gills (*S. paramamosain*) (Yang et al 2012). While in shrimps, expression of FAmeT RNA in MO showed lower than in other organs, this case may happened because of MF biosynthesis catalized by FAmeT enzyme in several tissues (Gunawardene 2002).

Peptide signal which regulated protein translocation was not found in FAmeT RNA of MO (Ruddell et al 2003). It was indicated that FAmeT RNA expressed in MO was not excreted and uncirculated to haemolymph and it would be possible regulated through phosphorilation. Therefore, Yang et al (2012) expected that FAmeT also contributed in methylation in several bioactive molecules and not only catalyzed MF biosynthesis during crustaceans development and reproduction. Moreover reported that distribution of RNA expression in different tissues of mud crabs refers to probability of involvement of FAmeT in other physiological role.

The study concerned to FAmeT RNA in *S. olivacea* is not clearly known. However, through experiment by alignment analysis we found high resemblance among *S. olivacea*, *S. serrata* and *S. paramamosain* (99%). While in swimming crabs i.e. *P. trituberculatus* (95%) and *P. pelagicus* (94%). These were indicated that FAmeT RNA sequence in genus of *Scylla* and also *Portunus* had high resemblance. The results suggest possibility that among crabs there is equal sequence form, roles, expression pattern, and distribution of FAmeT RNA.

Coclusions. The FAmeT of *S. olivacea* is an enzyme which plays a role for converting farnesoic acid into methyl farnesoate. In present study, FAmeT gene expression level in premolt was higher than in intermolt. The FAmeT RNA sequence was identical with *S. serrata* and *S. paramamosain.* This study showed the possibility that crabs may have similarity in FAmeT RNA.

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