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Cloning, Characterization, and Developmental Expression of a Putative Farnesoic Acid *O*-Methyl Transferase in the Female Edible Crab *Cancer pagurus*

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Abstract. Farnesoic acid methyl transferase (FAMTase) catalyzes methylation of farnesoic acid to yield the crustacean juvenoid, methyl farnesoate (MF). A full-length cDNA encoding a 275 amino acid putative FAMTase has been isolated from the mandibular organ of the female edible crab (*Cancer pagurus*) by reverse transcriptase-polymerase chain reaction in conjunction with cDNA library screening. A high degree of sequence identity was found between this and other putative crustacean FAMTases. Conceptual translation and protein sequence analysis suggested that phosphorylation could occur at multiple sites in the FAMTase. This finding is consistent with the recent observation that endogenous FAMTase activity in mandibular organ extracts can be regulated by phosphorylation *in vitro*. We demonstrated that the recombinant FAMTase could be expressed as a LacZ-fusion protein in *Escherichia coli* and have undertaken its partial purification from inclusion bodies. In an established assay system, the recombinant FAMTase lacked activity.

Northern blotting demonstrated widespread expression of an approximately 1250-nucleotide FAMTase transcript in female *C. pagurus* tissues. Levels of FAMTase transcripts in mandibular organs of female *C. pagurus* were found to fluctuate during vitellogenesis and embryonic development. Throughout the spring of 2002, an HPLC-based method was used to measure hemolymph MF titers in more than 70 female specimens of *C. pagurus*, which segregated into "high MF" and "low MF" groups. The high MF titers, which occurred before or during early vitellogenesis, coincided with, or were preceded by, elevated levels of putative FAMTase mRNA in the mandibular organs.

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Abbreviations: FAMTase, *S*-adenosyl-L-methionine farnesoic acid *O*-methyl transferase; MF, methyl farnesoate; MO-IH, mandibular organ-inhibiting hormone.

Introduction

Methyl farnesoate (MF), a sesquiterpenoid structurally similar to insect juvenile hormone III, is produced and secreted by the mandibular organs of crustaceans (Laufer *et al.*, 1987a, b; Borst *et al.*, 1987; Wainwright *et al.*, 1996a, b, 1998). Just as the juvenile hormones maintain larval characteristics between successive molts in insects (for a review, see Riddiford, 1994), a recent report confirms that MF regulates larval metamorphosis in barnacles (Smith *et al.*, 2000). In adult insects, juvenile hormone has been implicated in the regulation of ovarian development (Davey, 1996; Belles, 1998), and a growing body of evidence suggests an analogous role for MF in crustaceans. In the spider crab *Libinia emarginata* and the edible crab *Cancer pagurus*, increased levels of MF synthesis in the mandibular organs (Laufer *et al.*, 1987a) and elevated levels of MF in the hemolymph (Wainwright *et al.*, 1996a) have been found to occur during vitellogenesis. In experiments where a variety of crustaceans were exposed to artificially enhanced levels of MF, either by the effects of eyestalk ablation (Jo *et al.*, 1999), by direct injection of MF (Reddy and Ramamurthi, 1998, Rodriguez *et al.*, 2002), or by administration of MF through the diet (Laufer *et al.*, 1998), oocyte growth and ovarian development were stimulated. Similarly, a significant increase in mean oocyte diameter was reported when ovary explants from shrimp, *Penaeus van-*

namei (Tsukimura and Kamemoto, 1991) and red swamp crayfish, *Procambarus clarkii* (Rodriguez *et al.,* 2002) were incubated *in vitro* with physiologically relevant concentrations of MF. This combined evidence strongly supports a role for MF in the reproductive development of female crustaceans.

In crustaceans, ovarian development is broadly separated into two distinct phases, a pre-vitellogenic phase and a vitellogenic phase. During the pre-vitellogenic phase, primary oocytes begin to accumulate rough endoplasmic reticulum, and endogenous glycoprotein content increases. At the end of this phase, oocyte development arrests at prophase-I of meiosis, which synchronizes the oocytes at the same stage of development (for a review, see Charniaux-Cotton and Payen, 1988). Synchronization is critical because crustaceans fertilize and spawn all of their oocytes simultaneously. In locusts and shrimp, the meiotic block is released by physiological doses of ecdysteroids (Clédon, 1985; Lanot and Clédon, 1989). After meiosis resumes, germinal vesicle breakdown occurs, and vitellogenesis follows. The vitellogenic phase is characterized by a significant increase in the size of the ovary and an accumulation of yolk protein within the oocytes of the developing ovary tissue. In *C. pagurus,* oocytes are fertilized as they are laid, by sperm that has been stored in the spermathecae since copulation, when the female was soft-bodied after molting. Eggs are brooded under the abdomen attached to ovigerous pleopod hairs until embryogenesis is complete, and the hatched larvae are released as free-swimming zoeae. Egg-laying is believed to occur during the winter, and the larvae hatch 6 months later (Warner, 1977). The precise timing of vitellogenic events in *C. pagurus* has not been fully determined, although vitellogenesis appears to begin during the spring, presumably in response to environmental cues, such as photoperiod and temperature. The exact role of MF in crustacean ovarian development is still unclear, but in *C. pagurus* the hemolymph MF concentration is reported to peak during the earliest stage of vitellogenesis, suggesting its involvement in the control of this process (Wainwright *et al.,* 1996a).

Previously, we investigated the regulation of MF production in the mandibular organs of *C. pagurus,* isolating and characterizing two 78-residue peptides—mandibular organ-inhibiting hormones (MO-IHs)—that down-regulate the production of MF by the mandibular organs (Wainwright *et al.,* 1996b). Furthermore, we demonstrated that the action of MO-IH on mandibular organs ultimately regulates an *S*-adenosyl-L-methionine farnesoic acid *O*-methyl transferase (FAMTase), an enzyme that catalyses the final step of MF biosynthesis (Wainwright *et al.,* 1998). To date, putative FAMTase sequences from three decapod crustaceans have been published in on-line databases. Of these sequences, there is no evidence that the cloned gene products from the spiny lobster *Panulirus interruptus* (GenBank accession number AF249871) or from the clawed lobster *Homarus americanus* (U25846) have FAMTase activity. However, the recombinant FAMTase from the shrimp *Metapenaeus ensis* (AF333042) has recently been reported to catalyze, *in vitro,* the conversion of farnesoic acid to methyl farnesoate (Silva Gunawardene *et al.,* 2002). No brachyuran decapod has been studied to date, but these findings with shrimp suggest that a homologous putative FAMTase from the crab *Cancer pagurus* might also have enzyme activity.

In this report, we describe the isolation and characterization of a full-length cDNA encoding a 275-residue putative FAMTase from mandibular organs of female specimens of *C. pagurus* (GenBank accession number AY337487). The recombinant FAMTase was heterologously expressed, and enzyme activity was investigated in an established assay system (Wainwright *et al.,* 1998). The distribution of putative FAMTase expression is presented for a range of *C. pagurus* tissues, throughout the course of ovarian development and during embryogenesis. We also present further details regarding the previously reported peak of hemolymph MF (Wainwright *et al.,* 1996a), which occurs prior to or during the earliest stage of vitellogenesis.

Materials and Methods

Animals

Adult females of *Cancer pagurus* (Linnaeus), the edible crab, were obtained weekly from local fishermen and maintained in a recirculating seawater system at ambient light and temperature. These wild-caught animals constituted a non-synchronized population; therefore, crabs at different stages of ovarian development could be encountered at any given time. Crabs were dissected after cold-anesthesia, and the stage of ovarian development was determined according to established criteria (Wainwright, 1995; Wainwright *et al.,* 1996a). In brief, crabs were assigned a stage of ovarian development from 0 to 4. Stage 1 to 4 ovaries are vitellogenic, with steadily increasing organ size, oocyte diameter, and quantity of accumulated yolk protein (orange color). Stage 0 crabs, with cream-colored undeveloped ovarian tissue, are classed as either "pre-vitellogenic," if the hemolymph color is gray, or "vitellogenic," if the hemolymph color is orange; the orange color indicates the presence of circulating yolk protein.

Two egg-carrying females were caught during the winter and maintained in individual tanks at the Marine Biological Laboratories at Port Erin, Isle of Man. Embryonic offspring were collected from the egg-bearing pleopod hairs in the abdominal brooding pouch using flat-ended forceps. Hatched larvae were collected by filtration of the tank water. Embryos, larvae, and dissected adult tissues were stored in Trizol reagent (Life Technologies, Inc.) at −80 °C prior to analysis.

Isolation of a cDNA fragment of FAMTase using a nested PCR approach

The mandibular organs were dissected from three female *C. pagurus* specimens (ovary stage 0, hemolymph orange), and total RNA was isolated using Trizol reagent. Nested degenerate PCR primers were designed based on conserved regions within the crustacean FAMTase sequences published online.

First-strand cDNA synthesis was performed using 1 μg total RNA, as described in the SMART cDNA Library Construction Kit User Manual (version #PR92334) (Clontech Laboratories, Inc.). Most of this material was used to synthesize a mandibular organ cDNA library (see below). One-tenth of the first-strand synthesis reaction was used as cDNA template for PCR, with DMTS1 sense (5′-GCNCAYGAYGCNCAYGTNGC) and DMTAS1 antisense (5′-GGYTCNGGRTCNGTCCAYTC) primers (Fig. 1), and with the following temperature profile: 94 °C for 2 min, followed by 25 cycles of 94 °C for 1 min, 53 °C for 2 min, 72 °C for 1 min, and a final extension step of 7 min. PCR was carried out in a 20-μl reaction volume containing 0.5 units Taq DNA polymerase (Roche) in the manufacturer's reaction buffer (with 1.5 m*M* $MgCl_2$) in the presence of 0.5 μM PCR primers (MWG Biotech) and 0.25 m*M* each dNTP (Promega). Nested PCR was performed with DMTS2 sense (5′-CNCCNGAYATHYTNWSNGARGAR) and DMTAS2 antisense (5′-YTTNCKYTCYTCYTCRCARC) primers (Fig. 1), using 5 μl of the first PCR reaction as a template. The following thermal cycle was employed: 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min for 25 cycles. Other PCR conditions and reaction mixture constituents were as described above. Agarose gel electrophoresis of the

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1     taagttgttggtgcttctcctgtctgtacacacccccacacaccacacaccgagatcatggctgatgagattcccgccct   80
                                                          M  A  D  E  I  P  A  L

81    tggcacggacgagaacaaggagtaccgcttcagggagcttgatggcaagacccttcgattccaggtcaaaacagcgcacg  160
        G  T  D  E  N  K  E  Y  R  F  R  E  L  D  G  K  T  L  R  F  Q  V  K  T  A  H
      DMTS1
161   attgtcatgtggcattcacgtcagccggtgaagagacagaccccatagtggaggtgttcattgggggatgggagggcgct  240
      D  C  H  V  A  F  T  S  A  G  E  E  T  D  P  I  V  E  V  F  I  G  G  W  E  G  A
                                                                DMTS2
241   gcctcggccatcaggttcaagaaagctgatgatctagtgaaggtggatacgccagacattcttagcgagggagagtaccg  320
       A  S  A  I  R  F  K  K  A  D  D  L  V  K  V  D  (T)  P  D  I  L  (S)  E  G  E  (Y)  R
            GSP3                GSP2                GSP1
321   tgaattctggattgctgtggaccacgacgaaataagagtaggcaagggcggagagtgggagccgctcatgcaggcgccca  400
        E  F  W  I  A  V  D  H  D  E  I  R  V  G  K  G  G  E  W  E  P  L  M  Q  A  P

401   tcccagagcccttccctatcacccactacggctactccacaggctggggtgctgttggctggtggaagttcatgaacgac  480
      I  P  E  P  F  P  I  T  H  Y  G  Y  S  T  G  W  G  A  V  G  W  W  K  F  M  N  D

481   agggtcctaaacactgaagactgcctcacctacaacttcgagcctgcctacggtgacaccttctccttcagcgtcgcctg  560
       R  V  L  N  T  E  D  C  L  T  Y  N  F  E  P  A  (Y)  G  D  T  F  S  F  S  V  A  C

561   cagtaacgatgctcatttggctcttacctctggcgccgaagagaccacgccaatgtacgagatcttcattggtgggtggg  640
        S  N  D  A  H  L  A  L  T  S  G  A  E  E  T  T  P  M  Y  E  I  F  I  G  G  W

641   agaaccaacactccgccatccgcctcaataagggtgacgacatggccaaagtagagactccggacgcactgtgttgtgag  720
      E  N  Q  H  S  A  I  R  L  N  K  G  D  D  N  A  K  V  E  T  P  D  A  L  C  C  E
      DMTAS2
721   gaggagaggaagttcttcgtgtccttcaggaacggccatatcaaggtgggctacaaggacactgatcccttcctgcagtg  800
       E  E  R  K  F  F  V  (S)  F  R  N  G  H  I  K  V  G  (Y)  K  D  T  D  P  F  L  Q  W
      DMTAS1
801   gactgaccctgagccctggaaggtaacccatgtgggatactgtacgggatggggcgctaccggcaagtggaagcttgata  880
        T  D  P  E  P  W  K  V  T  H  V  G  Y  C  T  G  W  G  A  T  G  K  W  K  L  D

881   tctaagtgaacaaaaggtggaggtggtgatgtgatgtttgtcagtcatgcatcacactcaccaccactcgtcacactatc  960
      I  *

961   accacccctgctgccatgctatccactaccattggtacgtaataacgcttctatccttatctttgtcttcagtttataat 1040

1041  aaagcttccaaagcctgagaagccctatgagggtggagcgttgcgcacacacctgttgctgcattaacctttaaatgtcc 1120

1121  tcttacatggaattaaaagtgggagttatttttcgtactctttgtagcttcacgtcaataaaacctgaaaactag(a)30 1225
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Figure 1. Sequence of cDNA from the mandibular organ encoding the putative FAMTase. The full-length cDNA was isolated by PCR and nucleotide sequence determined (see Methods). The cDNA sequence shows an 825-bp open reading frame, which encodes a 275 amino acid protein. The stop codon is indicated by an asterisk, and a polyadenylation signal (AATAAA) is enclosed in a box. Circles indicate potential phosphorylation sites in the mature protein. Arrows indicate positions of primers, which are identified (see Methods). A single *Eco RI* site at 322 bp is underlined.

nested PCR products revealed a band of about 450 bp, which was cloned into the pGEM T-easy TA cloning vector according to the manufacturer's instructions (Promega). Sequencing of the 450-bp cloned insert showed it to be very similar to the existing crustacean FAMTase sequences in databases. This cDNA fragment was used as a probe to isolate a full-length cDNA encoding *C. pagurus* FAMTase.

Isolation of full-length FAMTase cDNA from a mandibular organ cDNA library

From the remainder of the first-strand synthesis reaction (see above), a unidirectional, mandibular organ cDNA library was constructed in bacteriophage λTriplEx2. The titer of the primary, unamplified library was 0.97×10^6 pfu/ml with >95% positive recombinants.

To isolate clones containing full-length cDNAs encoding *C. pagurus* FAMTase, plaque hybridization screening was carried out using the 450-bp FAMTase fragment end-labeled with [α-^{32}P]$^-$dCTP using the Ready-To-Go DNA Labeling Kit (Amersham Pharmacia) as a probe. Plaque lifts were carried out with BioTrace NT nitrocellulose blotting membrane (Gelman Sciences), and hybridizations and washes were carried out according to manufacturer's guidelines. A feature of the λTriplEx2 vector is that it contains an embedded bacterial plasmid DNA, pTriplEx2. Excision and circularization of the plasmid DNA from the linear bacteriophage DNA is readily achieved by a process involving *in vivo* excision using Cre recombinase-mediated site-specific recombination at two *loxP* sites flanking the embedded plasmid. This is carried out by incubating λTriplEx2 at 31 °C in the presence of *E. coli* BM25.8 (Clontech Laboratories, Inc.). The resultant plasmid can be used to express LacZ fusion protein variants of the encoded proteins under the regulation of a LacZ promoter. Positive clones from the library screening were converted into plasmid clones and analyzed by restriction enzyme digestion with *Eco*RI and *Sal*I.

5′-Rapid amplification of cDNA ends (5′-RACE)

To obtain the 5′-end of the FAMTase cDNA clone, 5′-RACE was carried out using total RNA from mandibular organs. A 5′-RACE system version 2.0 (Life Technologies) was used to amplify the 5′ terminus of the message for sequencing. Briefly, a gene-specific primer (GSP1, 5′-ACTCTCCGCCCTTGCC) was hybridized to the mRNA, and cDNA was synthesized using Superscript II reverse transcriptase. The RNA was then degraded with RNase mix (RNase H and RNase T1), and the cDNA was purified using a GlassMax spin cartridge supplied with the kit. A poly(dC) tail was added to the 3′-terminus of the purified cDNA using dCTP and terminal deoxynucleotidyl transferase, and the cDNA region corresponding to the 5′-end of the mRNA was amplified by two successive rounds of PCR using additional gene-specific primers (GSP2, 5′-TACTCTTATTTCGTCGTGGTCC; GSP3, 5′-ACAGCAATCCAGAATTCACGG), together with anchor primers supplied by the manufacturer. The second-round PCR product of about 380 bp was cloned into pGEM-T Easy Vector, and the nucleotide sequences of several clones were determined.

Expression of FAMTase protein

Expression of protein encoded by cDNA within pTriplEx2 plasmid was carried out, as follows, in *E. coli* TOP10 F′. Bacteria containing the pTriplEX2 plasmid were grown to a density of OD_{600} 0.5 to 0.6, at 37 °C, in LB medium supplemented with 50 μg/ml of ampicillin. Expression of LacZ fusion protein was achieved by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 m*M* (Brent, 1994).

SDS PAGE analysis of proteins

Extracts of proteins from *E. coli* were prepared to yield both soluble protein fractions and insoluble inclusion-body fractions, according to a published method (Brent, 1994). In both cases, *E. coli* cells were harvested by centrifugation (3000 × *g*, 10 min, 4 °C), washed in PBS buffer, and centrifuged (3000 × *g*, 10 min, 4 °C). The pellet was resuspended in HEMGN buffer (100 m*M* KCl, 25 m*M* HEPES [pH 7.6], 0.1 m*M* EDTA [pH 8.0], 10% [v/v] glycerol, 0.1% [v/v] Triton X-100) containing protease inhibitors (1 m*M* dithiothreitol, 0.1 m*M* phenylmethylsulfonyl fluoride, 0.1 m*M* sodium metabisulfite and protease inhibitor cocktail [Sigma, #P8340] added at 10 μl per 100 ml of original culture volume) and lysozyme (0.5 mg/ml), and lysed by sonication. After centrifugation (27,000 × *g*, 15 min, 4 °C) to separate soluble (supernatant) and insoluble fractions (pellet), the pellet was extracted into HEMGN buffer containing 8 *M* guanidinium-HCl, and centrifuged (87,000 × *g*, 30 min, 4 °C). The supernatant was dialyzed once against HEMGN buffer containing 1 *M* guanidinium-HCl and protease inhibitors (see above), then twice against HEMGN buffer containing protease inhibitors. The dialysate was centrifuged (12,000 × *g*, 5 min, 4 °C) to yield 8 *M* guanidinium-HCl soluble (supernatant) and insoluble (pellet) protein extracts (see Fig. 3). Portions of all extracts were analyzed by electrophoresis on a 10% polyacrylamide gel. Protein bands were visualized by staining with colloidal Coomassie blue G250.

FAMTase assays

Broken-cell extracts of *E. coli* were assayed for FAMTase activity using assay conditions previously published (Wainwright *et al.*, 1998). Briefly, extracts (200 μl) were dialyzed against a hypotonic HEPES buffer (0.037 *M* HEPES, 0.3 *M* sucrose, 0.01 *M* KF, pH 7.4) before the

addition of 2.4 μM [12-^{3}H] farnesoic acid and 250 μM *S*-adenosyl-L-methionine, in a final reaction volume of 50 μl. Incubations were carried out at 37 °C for 1 h and were terminated by the addition of 150 μl of acetonitrile. The reaction products were analyzed by reversed-phase HPLC with on-line radioactivity monitoring, as described previously (Wainwright *et al.*, 1998).

Expression of FAMTase in C. pagurus *tissues*

Total RNA from a variety of tissues (see Results) was isolated using Trizol reagent (Life Technologies). For Northern blotting, about 10 μg of total RNA from individual tissues was electrophoresed on a formaldehyde/1% agarose gel for 3 h at 75 V. The RNA was blotted onto Electran nylon membrane (BDH) with 20 × SSC (SSC is 0.15 *M* NaCl/0.15 *M* sodium citrate) and RNA cross-linked to the membrane by UV radiation. The FAMTase probe was prepared, as described above, and hybridization was carried out in QuickHyb solution (Stratagene) for 1.5 h at 68 °C. After hybridization, the blot was washed three times, at room temperature, for 10 min each, in 2 × SSC containing 0.1% SDS, and twice, at 45 °C, for 10 min in 0.1 × SSC containing 0.1% SDS. Autoradiographs were exposed at −70 °C.

To compare relative levels of expression of FAMTase in samples, a mouse 18S rRNA probe (Takeuchi *et al.*, 2000) was co-hybridized under the same conditions. This procedure provided an internal calibration for each sample and allowed for differences among lanes in the loading of RNA. In preliminary control experiments, when Northern blotting was carried out with individual probes alone, using identical hybridization and wash conditions, the hybridization patterns showed single bands of appropriate sizes for each probe (18S rRNA, 2000 nt; FAMTase mRNA, 1250 nt). Under the conditions used, therefore, the 18S rRNA and FAMTase mRNA probes did not cross-hybridize with their respective target transcripts. So we co-hybridized the blots in subsequent experiments. Densitometric analyses were carried out using Quantity One software (Bio-Rad) and amounts of FAMTase mRNA normalized against those of 18S rRNA.

Preparation of hemolymph methyl farnesoate extracts for HPLC

Hemolymph samples were taken from adult female specimens of *C. pagurus* with a hypodermic syringe; the arthrodial membrane at the base of a walking leg was punctured, and 2 ml of hemolymph was extracted. Methyl farnesoate isomers were extracted into hexane using a triphasic procedure (Borst and Tsukimura, 1991) incorporating modifications according to Wainwright *et al.* (1996a). Briefly, hemolymph samples (2 ml) were added to tubes containing 2 ml NaCl 4% (w/v) in H_2O, 5 ml acetonitrile (Merck, far UV HiPerSolv grade), and 100 ng *cis, trans*-MF isomer as an internal standard. The mixture was partitioned against 2 ml *n*-hexane (Merck, HiPerSolv grade), achieving phase separation by centrifugation at 500 × *g* for 10 min at 20 °C. The hexane layer (top) was removed and 300 μl subjected directly to HPLC analysis.

HPLC quantification of methyl farnesoate

Levels of *all-trans*-MF contained in hemolymph hexane extracts were determined by adsorption HPLC on a Varian Pro Star chromatography workstation, with a modification of the method previously described by Borst *et al.* (2002). Separation of MF isomers contained in hexane extracts (300 μl) from hemolymph was achieved on a Rainin MicroSorb MV silica adsorption column (5 μm, 250 × 4.6 mm internal diameter) using isocratic elution at 2 ml/min for 45 min in 0.4% diethyl ether in *n*-hexane (Merck, HiPerSolv grade) that had been dried overnight after the addition of 50 g of molecular sieve 4 Å (bead), 8–12 mesh (Sigma, #M1760), per 2.5 l of solvent. Eluted compounds were detected by UV absorbance at 229 nm. Peak areas were calculated using Star workstation software (Varian). *All-trans*-MF content of hemolymph samples was calculated by comparison of the *all-trans*-MF peak area to the *cis, trans*-MF peak area (internal standard 100 ng).

Results

Isolation, characterization, and expression of FAMTase from C. pagurus *mandibular organ*

Isolation and characterization of FAMTase. To obtain full-length cDNA encoding FAMTase, nested PCR was followed by isolation of full-length cDNA from a mandibular organ cDNA library. Previously published putative FAMTase sequences provided information for the design of degenerate oligonucleotide primers for the isolation of a *ca.* 450-bp fragment of cDNA that encoded a putative FAMTase. Subsequent sequence analysis suggested that this was, in fact, a putative FAMTase cDNA fragment of 442 bp. This partial cDNA was used to screen a mandibular organ cDNA library. Initially, approximately 8×10^4 recombinant bacteriophage were grown in two 15-cm petri dishes and screened. This initial screen identified four positive clones that were subsequently isolated and purified. All four phage clones were converted to their corresponding plasmid clones as described, and the inserts were analyzed by restriction enzyme digestion with *Sal*I and *Eco*RI; the products were then separated electrophoretically on a 1% agarose gel. Of the clones analyzed, two distinct types were apparent: one type, on digestion, produced two products (900 and 300 bp) originating from the cloned insert cDNA, while the other type produced only a 900-bp insert. Sequencing and BLAST searching revealed the former type,

Group 1, to be putative FAMTase sequence clones. The complete sequence of the putative FAMTase clone was obtained (see Fig. 1). The clone was 1216 bp in length, and conceptual translation indicated an open reading frame of 825 bp encoding a 275 amino acid protein with a predicted molecular weight of 31114. The 5′-UTR was 48 bp long. The 3′-UTR was 336 bp long and contained a polyadenylation signal, AATAAA, 13 bp upstream of the poly(A) tail. 5′-RACE demonstrated that the isolated and sequenced clone was 9 bp shorter than the full length of the 5′-UTR. A single *Eco*RI restriction enzyme cutting site occurs at 322 bp.

ClustalW alignment of the isolated putative FAMTase from *C. pagurus,* with sequences identified in other crustacean species, demonstrated a high degree of sequence identity (Fig. 2). Analysis of the protein sequence with SignalP ver. 1.1 software (http://www.cbs.dtu.dk/services/SignalP/) suggested that the protein does not contain a signal peptide cleavage site. Further analysis of the protein sequence, with NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and ScanProsite (http://ca.expasy.org/tools/scanprosite/), for potential posttranslational modifications shows multiple high-scoring (score > 0.8) sites for possible phosphorylation at serine, threonine, and tyrosine side chains within the molecule (Fig. 2).

Expression and activity of FAMTase protein

To determine whether the protein encoded by the isolated FAMTase cDNA clone was indeed an active FAMTase, expression of the protein in *E. coli* was carried out as described (Methods). Just prior to the addition of IPTG to

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C.pagurus          MA-DEIPALGTDENKEYRFRELDGKTLRFQVKTAHDCHVAFTSAGEETDPIVEVFIGGWE
H.americanus       MGDDNWASYGTDENKEYRFRDISGKTLHFQVKTAHDAHVALTSGAEETDPMVEIFIGGWE
M.ensis            MA-DNWPAYGTDENKEYRFRIIKGKTLRFQVKAAHDAHIALTSGEEETDPMLEIFIGGWE
P.interruptus      MGDDNWPSYGTDENKEYRFRDIGGKCLRFKVKTAHDAHVALTSGAEETDPIVEVFIGAWE
                   *.* : .: *********** : ** *:*:**:***.*:*:**. *****::*:***.**

C.pagurus          GAASAIRFKKADDLVKVDTPDILSEGEYREFWIAVDHDEIRVGKGGEWEPLMQAPIPEPF
H.americanus       GAASAVRFKKGEDLVKVDTPDILSEEEYREFWIAFDHDEIRVGKGGEGEPFMQCPIPEPF
M.ensis            GAASAIRFKKADDLTKVDTPDILNAEEYREFWIAFDHDNVRVGKGGEWEPFMSATVPEPF
P.interruptus      GAASAIRFKKADDLAKVDTPDILNEEEYREFWITFDNDEVRVGKAGDWEPFMMSPSQSHS
                   *****:****.:**.********.  *******:.*:*::****.*: **:* ..  .

C.pagurus          PITHYGYSTGWGAVGWWKFMNDRVLNTEDCLTYNFEPAYGDTFSFSVACSNDAHLALTSG
H.americanus       GITHYGYSTGWGAVGWWQFHAEKSYNTEDCLTYNFIPVYGDTLEFSVSCSNDAHVALTSA
M.ensis            EITHYGYSTGWGATGWWQFHSEMHFQTEDCLTYNFVPVYGDTFSFSVACSNDAHLALTSG
P.interruptus      KSPTMAIPLAGVLSAGGSFIMR-DFHTEDSQAYKFEPVYGDSLTFSVSCGHDAHLALTSG
                     .  . . .   .  .*      :***. :*:* *.***:: ***:*.:***:****.

C.pagurus          AEETTPMYEIFIGGWENQHSAIRLNK-----GDDMAKVETPDALCCEEERKFFVSFRNGH
H.americanus       AEETTPMYELLLGGWENQHSAIRLNK-----GDDMIKVDTPDILCCEEERKFWVSFKNGH
M.ensis            PEETTPMYEVFIGGWENQHSAIRLSKEGRSSGEDMIKVDTPDIVCCEEERKFTSSFKDGH
P.interruptus      PEETTPMYEVFIGGWENQHSAIRLNK-----GDDMIKVDSPDIVCSEEERKFWLSFKNGR
                   .********:::************.*     *:** **::** :*.******  **::*:

C.pagurus          IKVGYKDTDPFLQWTDPEPWKVTHVGYCTGWGATGKWKLDI
H.americanus       IRVGYKDTDPFMEWTDPEPWKITHIGYCTGWGATGKWKFEY
M.ensis            IKVGYQDSDPFMEWTDPEPWKITHVGYCTGWGASGKWKFEF
P.interruptus      IRVGYKDSDPFMEWTDPEPWKVTHVGYTTAWGAAGKWMLEI
                   *:***:*:***::********:**:** *.***:*** ::
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Figure 2. Amino acid sequence alignment of putative FAMTases from four crustaceans. ClustalW alignment of FAMTases from *Cancer pagurus* (this report, GenBank accession number AY337487), *Homarus americanus* (U25846), *Metapenaeus ensis* (Silva Gunawardene *et al.,* 2001, AF333042), and *Panulirus interruptus* (AF249871). Identical amino acids at a particular position, in all sequences, are denoted by an asterisk. Colons denote alignment of amino acids with strong similarities; periods indicate aligned residues with weaker similarities, according to their physicochemical properties. Hyphens denote gaps introduced to maximize the sequence alignment. Circles indicate potential phosphorylation sites in the mature protein. EMBL ClustalW default alignment settings were used (www.ebi.ac.uk/clustalw/).

the cultures, a 1-ml sample (time = 0 h) was taken and protein extracts prepared as described (see Methods). Samples (1 ml) of bacterial culture were taken at 1, 2 and 5 h post-IPTG addition. Both soluble and insoluble extracts of the bacteria were prepared and analyzed by SDS-PAGE. Analysis of the samples taken 5 h after the addition of IPTG clearly shows that recombinant protein expression is induced by IPTG, and that the recombinant protein is produced predominantly in the insoluble inclusion body fraction (Fig. 3). Molecular weight analysis demonstrated the induction of a protein of approximately 40 kDa. This size is entirely consistent with the expected size of the FAMTase-LacZ fusion protein.

To determine whether the recombinant fusion protein exhibited any FAMTase activity, the protein extracts were assayed for their ability to convert farnesoic acid into methyl farnesoate using conditions previously described (see above; Wainwright *et al.,* 1998). The broken cell extracts exhibited no detectable FAMTase activity (results not shown).

Expression of FAMTase during ovarian development and embryogenesis

Expression of FAMTase in tissues and developmental stages. To determine the tissue distribution and developmental expression of the putative FAMTase, Northern blotting was carried out using the 450-bp clone as a probe. The probe detected a single band of approximately 1250 nt in a number of tissues, including muscle, eyes, mandibular organs, epidermis, gills, heart, ovary, hepatopancreas and gut (Fig. 4). The extremely low signals detected by Northern blotting in Y-organs and sub-epidermal adipose tissue precluded estimation of relative FAMTase expression levels in these tissues; and in hemolymph RNA, the FAMTase transcript was undetectable by Northern blotting.

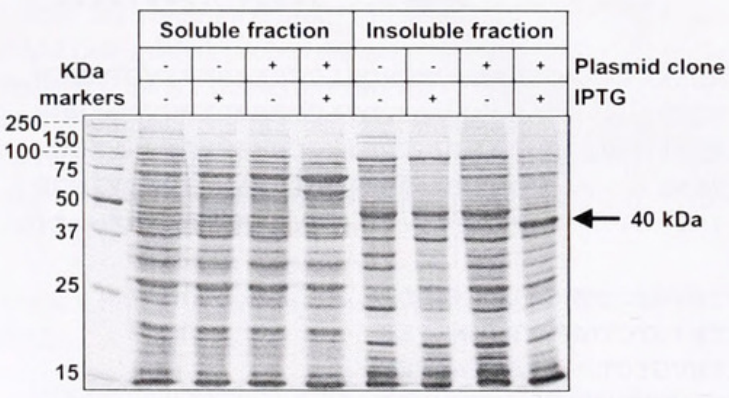

Figure 3. Expression of recombinant putative FAMTase. pTriplEx2 plasmids containing cDNA inserts encoding full-length FAMTase were grown in *E. coli* TOP10 F′. Protein expression was induced by addition of IPTG (see Methods). Extracts of *E. coli* prepared 5 h after induction with IPTG were analyzed for expression of recombinant protein by SDS-PAGE analysis (see Methods). Soluble and insoluble post-dialysis fractions are shown. The absence (−) or presence (+) of either IPTG (to induce recombinant protein expression when plasmid is present) or plasmid clone in the original *E. coli* culture conditions is indicated in the table above the gel image.

To determine the developmental profile of expression of FAMTase in mandibular organs, RNA was extracted from the mandibular organs of female crabs at different stages of ovarian development, and Northern blotting was carried out as described. A representative blot (Fig. 5a) demonstrates a distinct variation in the level of expression of FAMTase in the mandibular organ during ovarian development. Following densitometric analysis of the Northern blot autoradiograms, the ratio of FAMTase to 18S rRNA was determined, and the results were displayed graphically (Fig. 5b). Expression of FAMTase from the mandibular organ is significantly higher before the onset of vitellogenesis than after vitellogenesis has begun (unpaired t test, $P = 0.03$). During the mid and late stages of ovarian development, the levels of mandibular organ FAMTase expression appeared to fluctuate, but definitive trends could not be identified at these stages.

Female methyl farnesoate hemolymph titers. As part of an investigation aimed at further characterizing events that occur during the transition from the pre-vitellogenic to vitellogenic phases of ovarian development, we measured the hemolymph methyl farnesoate (MF) titers of over 100 female specimens of *C. pagurus* throughout the spring of 2002. In stage 0 crabs ($n = 70$), hemolymph MF titers segregated into two groups: "low" (93%) and "high" (7%) MF, with a cut-off titer of about 150 ng/ml between the groups (Fig. 6). It was noted that although one high MF crab was at an early stage of vitellogenesis (stage 0, orange hemolymph), four of the five high MF crabs fell into the stage 0 pre-vitellogenic category (stage 0, gray hemolymph).

FAMTase expression during embryonic and larval development. As an extension of our investigations, samples of developing embryos, up to hatching, were collected and analyzed for expression of FAMTase transcripts. The results (Fig. 7) show that, in both groups of embryos sampled from each of the individual brooding females, levels of FAMTase transcript increased noticeably during development, and fell to near the basal level just before hatching.

Discussion

Here we report the isolation and characterization of a putative farnesoic acid methyl transferase cDNA from mandibular organs of the edible crab *Cancer pagurus.* Using a combination of a nested PCR-based approach and screening of a mandibular organ cDNA library, a 1216-bp cDNA was isolated that encodes an approximately 31-kDa protein molecule (Fig. 1). The putative FAMTase of *C. pagurus* exhibits a high degree of sequence similarity with those

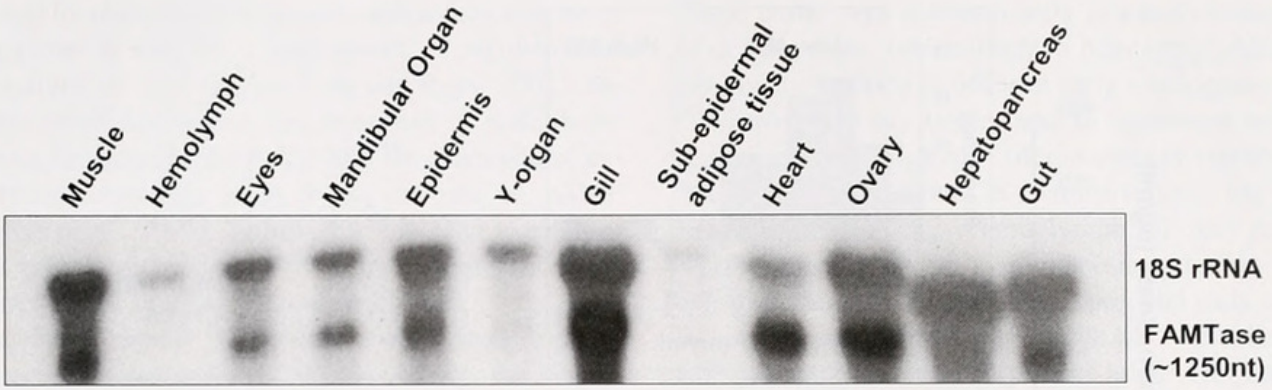

Figure 4. Northern blot analysis. Approximately 10 μg of total RNA from a variety of *Cancer pagurus* tissues and two organ equivalents of Y-organ RNA was electrophoresed, blotted onto a nylon membrane, co-hybridized at 68 °C with a ^{32}P-labeled-FAMTase probe and a mouse 18S rRNA probe, and washed at 45 °C (see Methods). The Northern blot shows the tissue distribution and size of the *C. pagurus* FAMTase transcript.

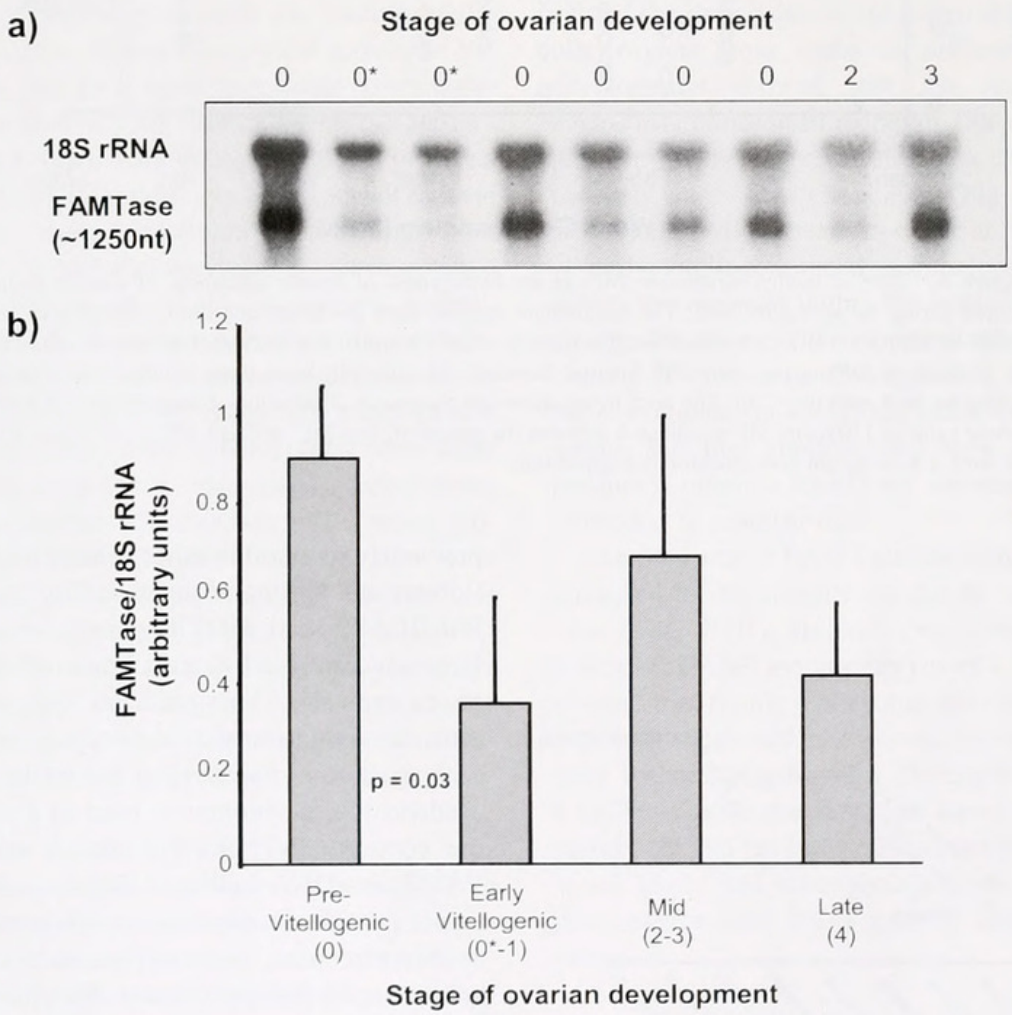

Figure 5. Profile of expression of putative FAMTase mRNA in mandibular organ throughout ovarian development in *Cancer pagurus*. (a) A typical Northern blot analysis of RNA isolated from mandibular organs from female crabs at different stages of ovarian development (0* indicates stage 0 vitellogenic animals). Stage 4 (late vitellogenic stage) not shown. (b) Autoradiograms developed from Northern blotting experiments were analyzed by computerized densitometry. Images were acquired with a GS710 scanning densitometer (Bio-Rad) and analyzed with Quantity One software (Bio-Rad). The relative expression of FAMTase was normalized to the 18S rRNA signal to take account of unequal loading between samples in different lanes. Results are grouped as follows: pre-vitellogenic (stage 0), early vitellogenic (stage 0* vitellogenic and stage 1), mid-stage vitellogenic (stages 2 and 3), and late vitellogenic (stage 4) animals. All values are mean ± standard error of the mean for n = 3–6 samples.

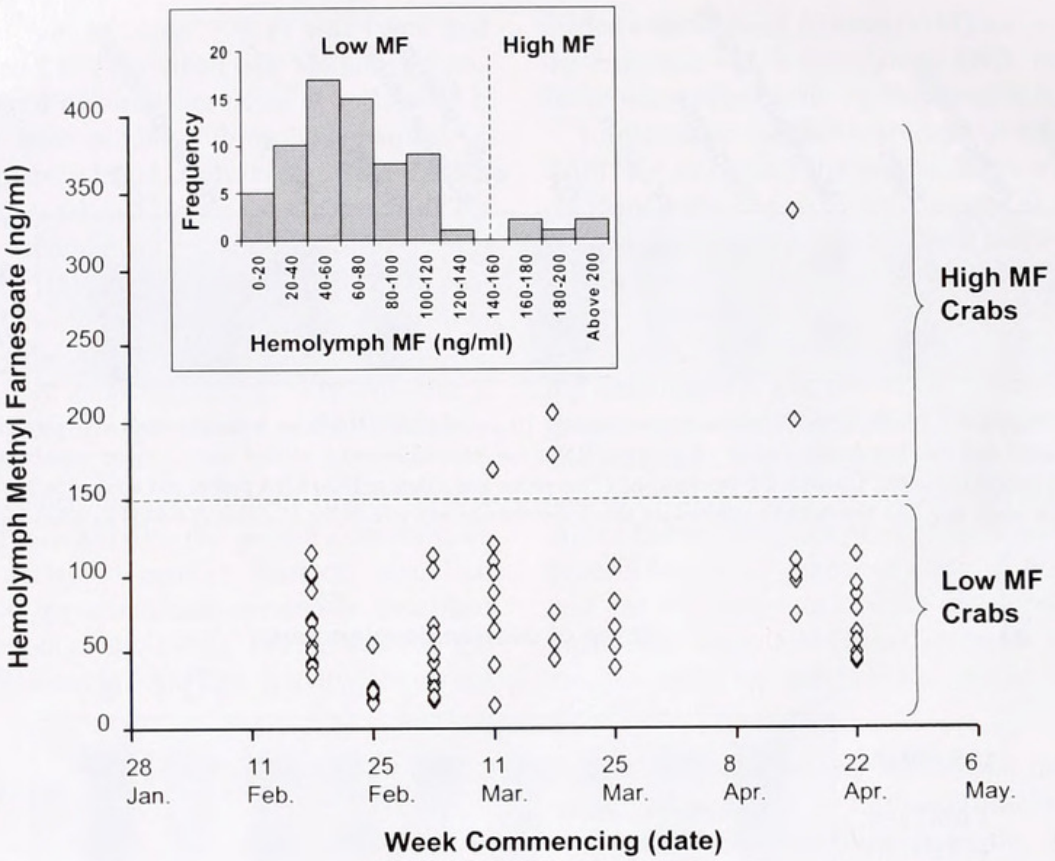

Figure 6. Titer of methyl farnesoate (MF) in the hemolymph of female specimens of *Cancer pagurus* measured during the spring of 2002. The hemolymph samples were processed and the hexane extracts were analyzed by adsorption HPLC, as described. The quantity of *all-trans*-MF was calculated by comparison of peak areas to those of 100-ng *cis, trans*-MF internal standard. *All-trans*-MF titers were plotted against date of sampling for each crab (n = 70). The inset figure shows the frequency distribution of hemolymph MF titer. A threshold value of 150 ng/ml MF is indicated, between the groups of "low MF" or "high MF" crabs. Note: These crabs were a wild-caught non-synchronized population.

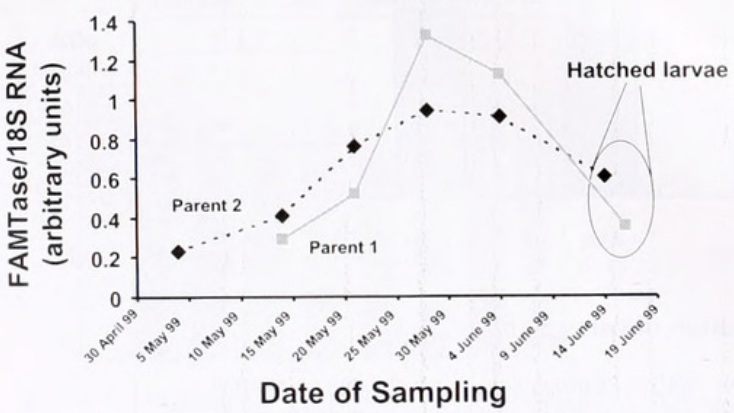

Figure 7. Expression of putative FAMTase in developing embryos and larvae of *Cancer pagurus*. Approximately 10 μg of total RNA from whole embryos and larvae was electrophoresed, blotted onto a nylon membrane, co-hybridized at 68 °C with a ^{32}P-labeled-FAMTase probe and a mouse 18S rRNA probe, and washed at 45 °C (see Methods). After densitometric analysis and normalization of the signal relative to that of rRNA, the expression levels were plotted against the time of year (date of sampling).

previously reported in other species of decapod crustaceans (lobster and shrimp sequences; Fig. 2). However, BLAST, PSI-BLAST, and MOTIF searches based on the putative farnesoic acid methyl transferases of *C. pagurus* or other crustaceans show no significant sequence similarity to any other *O*-methyl transferase enzymes, although, in common with most other members of the methyl transferase family, *S*-adenosyl-L-methionine is used as a cofactor. Analysis of the conceptually translated amino acid sequence of the FAMTase cDNA indicates that the native protein lacks a signal peptide. Further analysis suggests that a number of serine, threonine, and tyrosine side chains are potential substrates for phosphorylation. No other significant consensus sequences for other types of posttranslational modifications were found.

The apparent lack of signal peptide indicates that the putative FAMTase expressed in mandibular organ tissue is not secreted into the circulating hemolymph and may well be regulated by reversible phosphorylation of specific serine, threonine, or tyrosine residue side chains. In previous work, we have demonstrated that FAMTase activity in cytosolic extracts from *C. pagurus* mandibular organs can

be regulated by phosphorylation/dephosphorylation *in vitro,* suggesting that it may be a mechanism of regulation of enzyme activity *in vivo* (Wainwright and Rees, 2001). Indeed, other work has shown that treatment of mandibular organs with the peptide hormone MO-IH (mandibular organ-inhibiting hormone) leads to an increase in cAMP (Wainwright *et al.,* 1999). In turn, this may lead to changes in the phosphorylation state of FAMTase, thereby modulating the production of methyl farnesoate (MF).

To determine whether the protein characterized from the mandibular organ possesses FAMTase activity, the recombinant protein was expressed in *E. coli* as a LacZ fusion protein of approximately 40 kDa. On analysis, an approximately 40-kDa protein appeared in the inclusion body subcellular fractions; however, the inclusion body fractions had no detectable FAMTase activity. This observation has three possible explanations. First, the presence of the LacZ tag and linker sequence may interfere with the folding of the nascent protein and, thus, with the biological activity of the enzyme. Second, it may have been impossible to resolubilize the inclusion bodies *in vitro.* In this event, the activity of the FAMTase may have been disrupted. Third, previous attempts to stabilize FAMTase activity in extracts of cytosol during storage—and, thus, to facilitate traditional methods of enzyme purification and characterization—have resulted in almost complete loss of activity (G. Wainwright, unpubl. obs.). This sensitivity of FAMTase activity to its environment may also explain the lack of observed activity in the recombinantly expressed protein, under the experimental conditions described here. Similar difficulties have been encountered in expressing a functional recombinant FAMTase from the shrimp *Metapenaeus ensis,* when procedures largely equivalent to those described here were followed. In the *M. ensis* study, biological activity was eventually detected in a partially purified bacterial extract when a high-level expression system was employed (Silva Gunawardene *et al.,* 2002). It was surmised that only a tiny proportion of recombinant FAMTase may have been expressed in a correctly folded, active conformation in bacteria.

The distribution of the putative FAMTase among a variety of tissues was demonstrated by Northern blotting, which revealed a single transcript prevalent in muscle, eyes, mandibular organs, epidermis, gills, heart, ovary, hepatopancreas, and gut. Hemolymph did not exhibit a signal detectable by this method. Expression of the putative FAMTase transcript in a range of non-mandibular organ tissues in *C. pagurus,* described here, resembles that in *M. ensis* (Silva Gunawardene *et al.,* 2001, 2002), suggesting that FAMTase is quite broadly distributed in crustacean tissues. Perhaps the substrate specificity of the enzyme is not strict.

HPLC analysis of MF levels in the hemolymph of previtellogenic and early vitellogenic crabs revealed a small number of individuals possessing "high MF" titers (Fig. 6). These crabs were predominantly at a pre-vitellogenic stage of development. Interestingly, a hemolymph MF peak was previously reported to occur in early vitellogenic specimens (Wainwright *et al.,* 1996a) and, in agreement with this, we also observed a "high MF" titer in an early vitellogenic crab. These data suggest that, in contrast to what has previously been observed, elevated hemolymph MF may occur at different times during pre-vitellogenesis and early vitellogenesis in *C. pagurus.* As only 7% of pre- and early vitellogenic specimens were found to have high MF, we believe that the MF peak may be relatively short-lived. The transience of this peak (or peaks) would reduce the likelihood of it being observed in a wild-caught, non-synchronized population, and has presumably hindered its characterization to date.

We investigated, experimentally, whether the FAMTase also changes in expression in relation to ovarian development and the circulating levels of MF. Northern blot analysis of the expression of the putative FAMTase in mandibular organs from crabs at different stages of ovarian development showed that the relative expression of FAMTase, normalized using an 18S rRNA probe, was significantly higher in pre-vitellogenic crabs than in animals in the early stages of vitellogenesis (Fig. 5b). Since the peak in hemolymph MF appears to occur at some time during pre- or early vitellogenesis, it is apparent that FAMTase transcripts are elevated within the mandibular organ before, or during, the stages of development when the peaks in hemolymph MF titer occur. Consequently, the observed temporal pattern of peaks in FAMTase mRNA expression and hemolymph MF titer supports the view that our cloned gene product is indeed a FAMTase, although additional evidence is needed to confirm this.

The presence of FAMTase transcripts has also been demonstrated in the juvenile shrimp *M. ensis* (Silva Gunawardene *et al.,* 2001). Similarly, we detected the expression of a putative FAMTase in embryos of *C. pagurus.* The results showed that during embryonic development of *C. pagurus,* a peak in expression is observed during late embryogenesis, prior to hatching (Fig. 7). The significance of the role of FAMTase in biochemical and developmental processes in embryonic and juvenile crustaceans is as yet unclear, but it seems likely that, through regulation of production of MF, this enzyme will affect growth and developmental processes.

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