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cDNA Cloning and Expression of Translationally Controlled Tumour Protein (TCTP) Isolated from Mud Crab *Scylla paramamosain* in *Escherichia coli*

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ABSTRACT

Translationally controlled tumour proteins (TCTP) cDNA were cloned from the haemolymph of *Scylla paramamosain* by reverse transcription PCR using primers derived from *Penaeus monodon*. Nucleotide sequence analysis revealed 507 bp open reading frame (ORF) encoding 168 amino acid residues with a predicted molecular mass and pI of approximately 19.2 kDa and 4.5, respectively. Interestingly, mud crab-TCTP shows 99 % homology with *Penaeus monodon* translationally controlled tumour proteins (Pm-TCTP) and *Fenneropenaeus merguiensis* TCTP. This gene was also expressed in pQE40 vector as histidine tagged fusion proteins. The recombinant protein has a molecular mass of approximately 25 kDa. From the biological function study of Pm-TCTP, It is proposed that mud crab-TCTP might function as anti-apoptosis like Pm-TCTP does.

Keywords: *Scylla paramamosain*, TCTP, biodefense molecule, RT-PCR

INTRODUCTION

A family of TCTPs (translationally controlled tumour proteins) were initially described as growth-related proteins in mouse Ehrlisch ascites tumour cells and erythroleukemia cells [1-3]. Subsequently TCTPs were found to be present in many cell types and throughout the entire animal and plant kingdom [3-7]. This protein is also referred to as P23, P21, and Q23 [8]. Characterization studies have revealed that TCTPs are calcium binding [9], heat stable proteins [10]. In addition, they can induce histamine release from basophils only in the presence of a special type of IgE called IgE^{+} [11-12]. Besides inducing histamine release, this protein also enhances chemotaxis, IL-8 production by human eosinophils [11], and IL-4 secretion from human basophils [8]. TCTPs can also bind to tubulin and induce intracellular signaling and affect cognition function in neurodegenerative disorders such as Down's syndrome and Alzheimer's disease [8]. Recently, a human TCTP named fortilin was found to be a novel anti-apoptotic protein involved in cell survival and apoptosis regulation [13]. Furthermore, it also found that fortilin specially interacts with MCL-1 (Myeloid cell leukemia 1 protein or anti-cell death protein) and has anti-apoptotic effects preventing cells from undergoing apoptosis [14].

In crustaceans, TCTPs has been isolated from the *Penaeus monodon* haemolymph called Pm-TCTP. It was reported that the amount of TCTP messaging is severely down-regulated in moribund white spot syndrome virus (WSSV) infected shrimps but only a slight difference in normal and early WSSV-infected shrimp. As a result, TCTP in shrimp might be involved in the WSSV infection response [15]. Furthermore, Pm-TCTP or shrimp fortilin was reported to protect cells under toxic conditions from death, like human fortilin [16].

In the present study, we attempted to investigate translationally controlled tumour proteins from the haemolymph of mud crab *Scylla paramamosain* and compare its sequence with other TCTPs. The recombinant clone was constructed and expressed in *Escherichia coli* in order to study biochemical functions.

MATERIALS AND METHODS

Isolation of Total RNA

Haemolymph of mud crabs *Scylla paramamosain* (500 µl) was withdrawn by inserting a syringe into the sinus at the base of the right chelate leg into a microcentrifuge tube containing 500 µl of Trizol reagent (Invitrogen, CA, USA). The sample solution was homogenized by vigorous vortex mixing. The homogenized samples were incubated for 5 min at 30 °C to permit the complete dissociation of nucleoprotein complexes and then 0.1 ml of chloroform was added. The sample tube was shaken vigorously by hand for 15 sec and incubated for 2 - 3 min at room temperature. The sample was centrifuged at 12,000×*g* for 15 min at 4 °C. Subsequently, the aqueous phase was removed to a fresh tube and mixed with 0.25 ml of isopropyl alcohol to precipitate RNA. The sample was kept at 30 °C for 20 min and then centrifuged at 12,000xg for 15 min at 4 °C for RNA precipitation. The pellet was washed with 0.5 ml of 75 % ethanol followed by centrifugation at 7,500×*g* for 5 min at 4 °C. The RNA pellet was dried under vacuum, resuspended in 50 µl of RNase free water and incubated at 60 °C for 10 min in order to increase its solubility. The concentration of the total RNA was determined by measuring the absorbance at 260 nm.

Reverse transcription (RT)-PCR

RT-PCR was performed using primers (5'- and 3'-) designed from shrimp-TCTP and synthesized by Life Technologies. Oligonucleotides used as PCR primers are as follows: TCTP1 sense primer: 5′-CGGGATCCATGAAGGTCTTCAAG-3′ and TCTP2 antisense primer: 5′-GCGTCGACTTATAGCTTCTCCTCG-3′. The total RNA (1 µg) from mud crab haemolymph was used as the template in a 50 µl one step RT-PCR reaction mixture according to the manufacturer's instructions (QIAGEN OneStep RT-PCR, Catalog # 210210, QIAGEN GmbH). The reaction was held at 50 °C for 30 min followed by an initial PCR activation step at 95 °C for 15 min followed by 30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min. The RT-PCR products were analyzed on a 1.8 % agarose gel and visualized by an ethidium bromide stain under ultraviolet light. The obtained PCR fragments were cloned into pGEM-TEasy (Promega) and sequenced using the ABI prism 377 apparatus.

DNA sequencing and data analysis

The DNA sequence was analyzed using BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (PE Applied Biosystems, Catalog # P/N4303150) and an Applied Biosystems 377 sequencer (Perkin-Elmer, Norwalk, CT, USA). Gene database searches were performed through the National Center for Biotechnology Information using the BLAST network service. The multiple alignments and phylogenetic tree of TCTP amino acids sequence was performed by using the Clustral X and Phylip programs

Construction of mud crab-TCTP expression cassette

The open reading frame (ORF) of mud crab-TCTP was amplified by PCR. For convenience of cloning, a *Bam*HI restriction site was added to the 5′-end of the forward PCR primer and a *Sal*I restriction site was added to the 3′-end of the reverse primer after the stop codon. PCR primer sequences were TCTP-F: 5′-CGGGATCCATGAAGGTCTTCAAG-3′ and TCTP-R: 5′-GCGTCGACTTATAGCTTCTCCTC-3′, where restriction sites are underlined. PCR parameters were an initial heating to 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min. The purified PCR product obtained was digested with restriction enzymes and cloned in-frame with 6×Histidine tags into the *Bam*HI and *Sal*I restriction sites of the pQE40 (QIAGEN GmbH). The resulting plasmid for expression of mud crab TCTP was termed pQE-TCTP. Expression studies were undertaken for the *Escherichia coli* strain M15 (pRep4), which contains an IPTG-inducible T5 polymerase.

Growth and Induction of *E.coli*

The *Escherichia coli* strain M15 (pRep4) harboring pQE-TCTP was inculated in LB medium containing 100 μg/μl ampicillin and 25 μg/μl kanamycin. Cultures were incubated at 37 °C until OD_{600} reached 0.3 - 0.5 whereupon protein expression was induced by addition of 0.1 mM IPTG. After incubation for an additional 3 h, cells were harvested by centrifugation for 2 min at 12,000×*g*. SDS-PAGE was carried out under reducing conditions with 12.5 % running gels and the protein bands were colored by coomassie blue staining.

RESULTS AND DISCUSSION

The open reading frame (ORF) of mud crab-TCTP was amplified by using a primer pair of TCTP designed based on the Pm-TCTP sequence. It contains 507 bp beginning with a methionine codon and ending with a TAA termination codon (**Figure 1**). The theoretical 168 amino acid polypeptide has a calculated molecular mass of 19.2 kDa and predicted pI = 4.5. Based on the deduced amino acids, the possible glycosylation sites were found at positions 34 and 48 predicted by NetNGlyc software (ExPASy). The *Scylla paramamosain* TCTP cDNA sequence and deduced amino acid sequence has been submitted to the NCBI GenBank as accession number ABG50531. Sequence analysis with the BLAST algorithm of this deduced amino acid sequence has highest similarity to the *Penaeus monodon* translationally controlled tumour protein (Pm-TCTP) and *Fenneropenaeus merguiensis* TCTP at 99 % (2e - 90) and 99 % (4e - 90), respectively. According to the three-dimension structure of $p23^{fyp}$ (the TCTP from *Schizosacchoromyces pombe*) and sequence analysis, it was shown that TCTP structures are composed of four β-sheets and three main helices connected in a complex topology [17]. The TCTP family including mud crab-TCTP has two primary regions of high sequence homology termed TCTP1 and TCTP2 [18]. Like all others, mud crab-TCTP also has a homologous microtubule binding domain [8] and a calcium binding domain [9] (**Figure 2**). Moreover, TCTPs revealed the existence of a newly conserved domain (positions 1 - 16) at the N-terminus which includes CKII phosphorylation site and GTPase binding surface [18].

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        10        20        30        40        50        60
         |         |         |         |         |         |
ATGAAGGTCTTCAAGGATATGCTGACCGGTGATGAGATGTTCACTGACACCTATAAGTAT
 M  K  V  F  K  D  M  L  T  G  D  E  M  F  T  D  T  Y  K  Y

        70        80        90       100       110       120
         |         |         |         |         |         |
GAGGAGGTGGATGATGCCTTCTACATGGTAATTGGAAAAAATATTACTGTTACTGAAGAT
 E  E  V  D  D  A  F  Y  M  V  I  G  K  N  I  T  V  T  E  D

     130       140       150       160       170       180
       |         |         |         |         |         |
AACATTGAGCTGGAGGGAGCCAATCCATCAGCTGAAGAGGCAGATGAAGGCACTGACACT
 N  I  E  L  E  G  A  N  P  S  A  E  E  A  D  E  G  T  D  T

     190       200       210       220       230       240
       |         |         |         |         |         |
ACTAGTCAGTCTGGTGTTGATGTAGTTATATATATGCGTCTGCAGGAAACCGGCTTCCAA
 T  S  Q  S  G  V  D  V  V  I  Y  M  R  L  Q  E  T  G  F  Q

     250       260       270       280       290       300
       |         |         |         |         |         |
GTCAAGAAGGATTATCTTGCATACATGAAAGAATACCTAAGGAATGTAAAGGCAAAGTTG
 V  K  K  D  Y  L  A  Y  M  K  E  Y  L  R  N  V  K  A  K  L

       310       320       330       340       350       360
         |         |         |         |         |         |
GAAGGCACGCCTGAAGCTTCAAAGTTAACATCTATCCAGAAGCCTCTGACAGACCTTTTG
 E  G  T  P  E  A  S  K  L  T  S  I  Q  K  P  L  T  D  L  L

       370       380       390       400       410       420
         |         |         |         |         |         |
AAGAAGTTCAAGGACTTGCAATTCTTCACTGGAGAATCAATGGACCCTGATGGCATGGTT
 K  K  F  K  D  L  Q  F  F  T  G  E  S  M  D  P  D  G  M  V

       430       440       450       460       470       480
         |         |         |         |         |         |
GTTCTCATGGATTGCAAAGACATTGATGGAGAAGAGCGGCCAGTCTTGTACTTCCCAAAA
 V  L  M  D  C  K  D  I  D  G  E  E  R  P  V  L  Y  F  P  K

       490       500
         |         |
TACGGTCTAACAGAGGAGAAGCTATAA
 Y  G  L  T  E  E  K  L  *
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Figure 1 Nucleotide sequence (above) and deduced amino acid sequences of the ORF (below) of mud crab-TCTP cDNA. Nucleotides are numbered from the first base at the 5′ end. The possible glycosylation sites are underlined. The sequence was submitted to GenBank with accession number ABG50531.

In this study, are compared to the multiple alignment and phylogenetic relationship of the mud crab-TCTP to sequences of other TCTPs (**Figures 2** and **3**). The data shows that the amino acid sequence of TCTPs is highly conserved in eukaryotic organisms as reported by Gnanasekar and colleagues in 2002 [19]. Their conserved structure suggests a crucial cellular role but the precise function of the family remains elusive [19-20]. From phylogenetic analysis, TCTPs are classified into 6 groups: plants & hydra (*Pisum sativum*, *Medicago sativa*, *Glycine max*, *Oryza sativa*, *Nicotina tabacum*, *Cucumis melo*, *Arabidopsis thaliana* and *Hydra vulgaris*), worms (*Brugia malayi*, *Wuchereria bancrofti* and *Caenorhabditis elegans*), shrimps (*Penaeus monodon* and *Fenneropenaeus merguiensis*), insects (*Drosophila melanogaster*, *Anopheles gambiae* and *Tigriopus japonicus*), yeasts (*Schizosacchoromyces pombe*, *Sacchromyces cerevisiae*, *Schisotoma mansoni* and *Schisotoma japonicum*) and animals (*Gallus gallus*, *Mus musculus, Sus scrofa*, *Oryctolagus cuniculus* and *Homo sapiens*) (**Figure 3**). The results revealed that mud crab-TCTP (T1) is classified into the same group as *Penaeus monodon* and *Fenneropenaeus merguiensis*, called decapod crustacean. It might be because *Scylla* sp., *Fenneropenaeus merguiensis* and *Penaeus monodon* are in the Class Crustacea and Order Decapoda and this gene might be conserved in this group of organism as well.

Mud crab-TCTP was subcloned into pQE40 and expressed as histidine-tagged fusion proteins in IPTG-inducible T5 polymerase system. In order to minimize the effect the fusion protein might have on the biological functions, the DHFR (dihydrofolate reductase) fusion protein from the pQE40 expression vector was removed by cloning at *Bam*HI and *Sal*I sites. Therefore, the recombinant protein only has a histidine tag at the N-terminal part. Mud crab-TCTP is expressed in soluble form and has a molecular mass of approximately of 25 kDa with the histidine tag on SDS-PAGE gel (**Figure 4**). This protein will be purified by using Ni-NTA resin and characterized in further experiments to investigate its biological functions. A previous study showed that TCTPs are highly regulated in response to a wide range of extracellular stimuli and cellular conditions e.g. growth signals and cytokines, starvation, heat shock, heavy metals, calcium stress or proapoptoic/cytotoxic signals [20]. The biological function study of *Penaeus monodon* TCTP (Pm-TCTP) reported that this gene is involved in anti-apoptosis or preventing cell death under toxic conditions [15-16]. From the amino acid and nucleotide sequence study, we propose that mud crab-TCTP might have the same functions as *Penaeus monodon* does, although further studies will need to be undertaken to confirm this hypothesis.

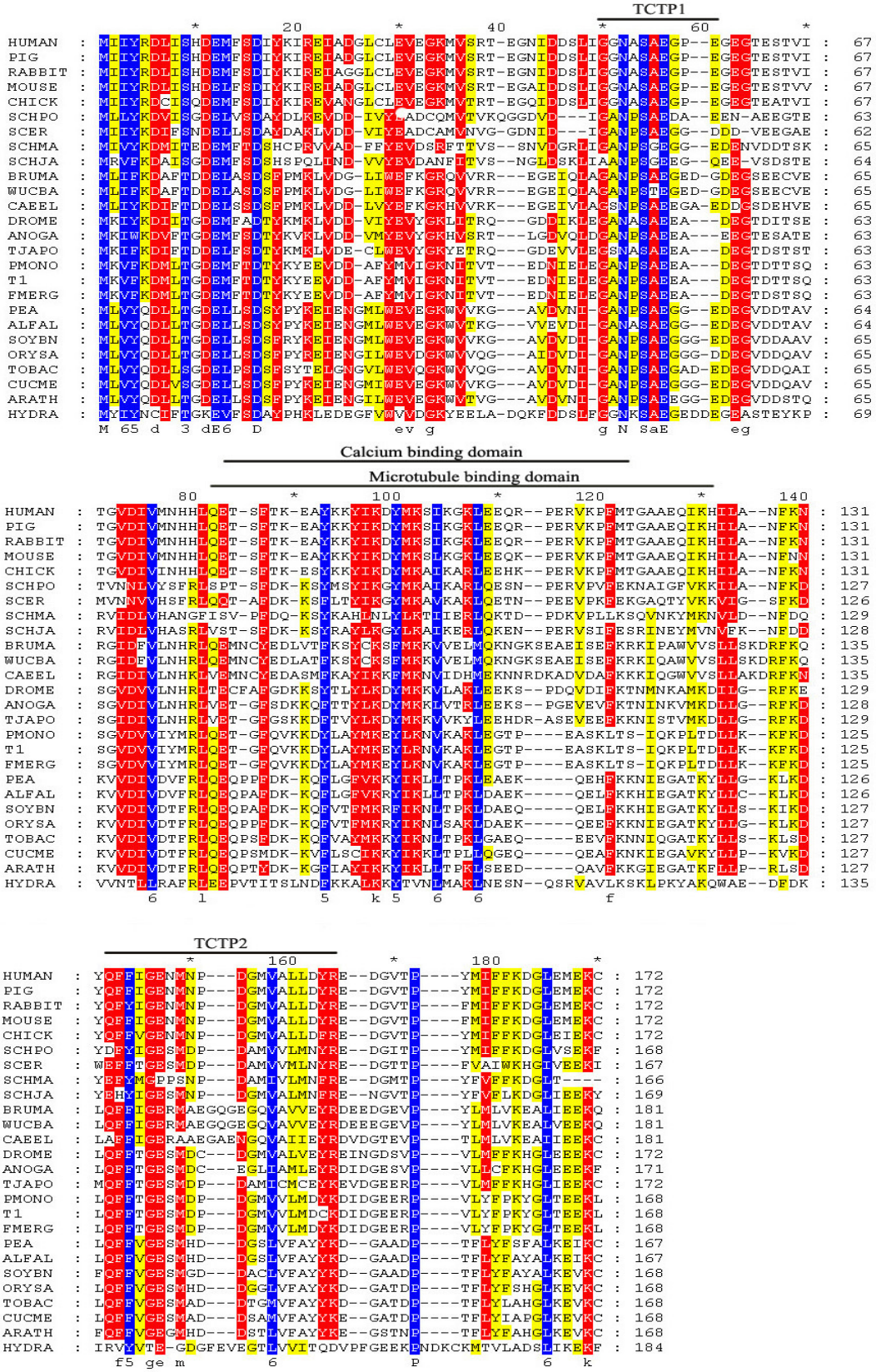
TCTP1
Calcium binding domain
Microtubule binding domain
TCTP2

Figure 2 Multiple alignment (ClustalX) of the amino acid sequence of TCTPs: sequences were from mud crab-TCTP (T1, ABG50531), *Penaeus monodon* (PMONO, AY13685) *Fenneropenaeus merguiensis* (FMERG, AY700595), *Homo sapiens* (HUMAN, NP_003286), *Sus scrofa* (PIG, AAL68965), *Oryctolagus cuniculus* (RABBIT, P43348), *Mus musculus* (MOUSE, P14701), *Gallus gallus* (CHICK, P43347), *Schizosacchoromyces pombe* (SCHPO, Z69944), *Sacchromyces cerevisiae* (SCER, P35691), *Schisotoma mansoni* (SCHMA, AF358139), *Schisotoma japonicum* (SCHJA, AAB42079), *Brugia malayi* (BRUMA, U80971), *Wuchereria bancrofti* (WUCBA, AY039808), *Caenorhabditis elegans* (CAEEL, NP_492767), *Pisum sativum* (PEA, AAB19090), *Medicago sativa* (ALFALFA, X98618), *Glycine max* (SOYBN, AF421558), *Oryza sativa* (ORYSA, D12626), *Nicotina tabacum* (TOBAC, AF107842), *Cucumis melo* (CUCME, AF230211), *Arabidopsis thaliana* (ARATH, P31265), *Hydra vulgaris* (HYDRA, Q94587), *Drosophila melanogaster* (DROME, AFF54603), *Anopheles gambiae* (ANOGA, EAA08161) and *Tigriopus japonicus* (TJAPO, AY496449). A dash represents a gap in the indicated proteins. Positions where the chemical character of residues is conserved in 100 %, 80 % and 60 % of sequences are highlighted in blue, red and yellow respectively.

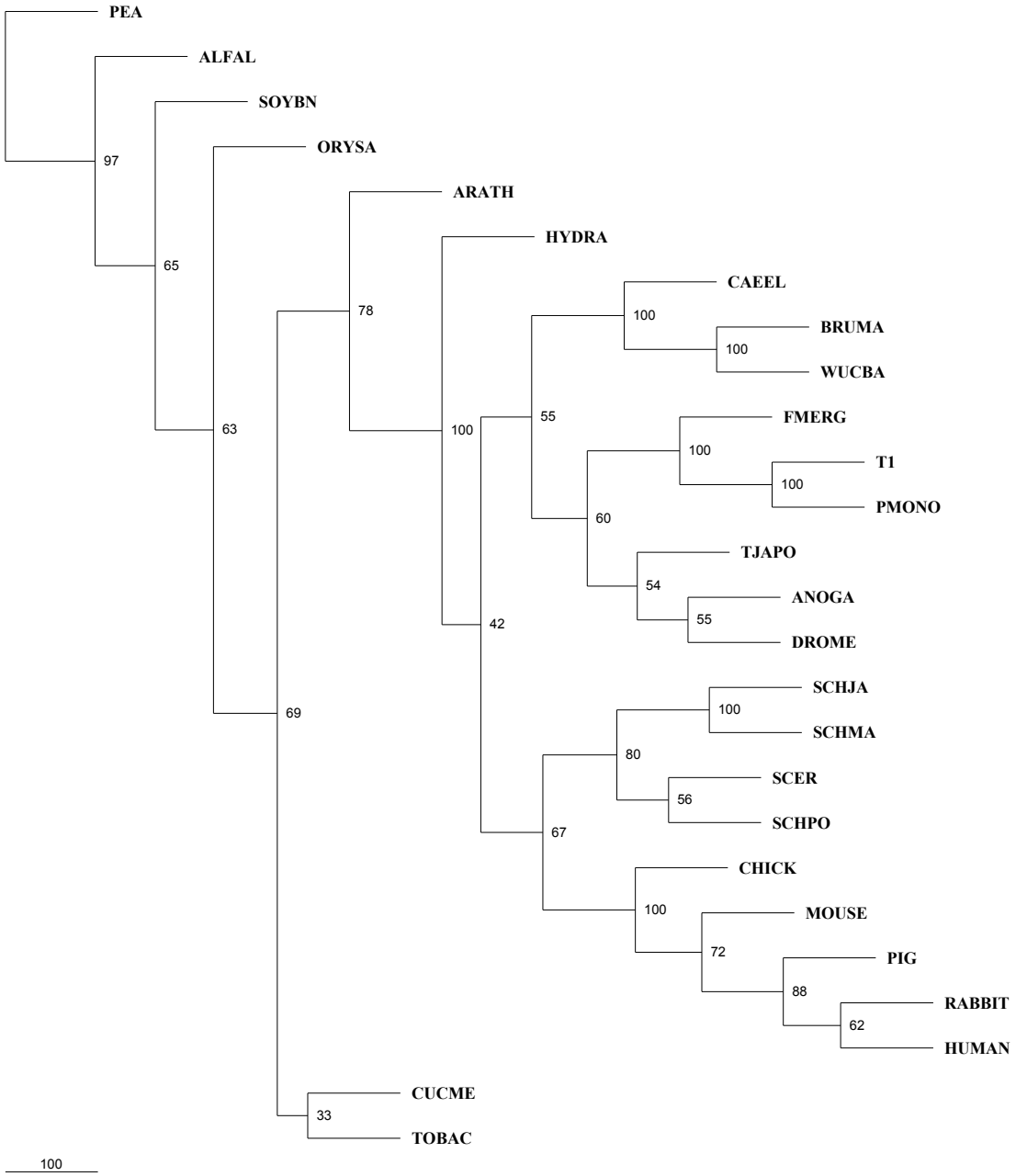

Figure 3 Phylogenetic tree of the amino acid sequences of TCTPs.
Mud crab-TCTP (T1, ABG50531), *Penaeus monodon* (PMONO, AY13685) *Fenneropenaeus merguiensis* (FMERG, AY700595), *Homo sapiens* (HUMAN, NP_003286), *Sus scrofa* (PIG, AAL68965), *Oryctolagus cuniculus* (RABBIT, P43348), *Mus musculus* (MOUSE, P14701), *Gallus gallus* (CHICK, P43347),

Schizosacchoromyces pombe (SCHPO, Z69944), *Sacchromyces cerevisiae* (SCER, P35691), *Schisotoma mansoni* (SCHMA, AF358139), *Schisotoma japonicum* (SCHJA, AAB42079), *Brugia malayi* (BRUMA, U80971), *Wuchereria bancrofti* (WUCBA, AY039808), *Caenorhabditis elegans* (CAEEL, NP_492767), *Pisum sativum* (PEA, AAB19090), *Medicago sativa* (ALFALFA, X98618), *Glycine max* (SOYBN, AF421558), *Oryza sativa* (ORYSA, D12626), *Nicotina tabacum* (TOBAC, AF107842), *Cucumis melo* (CUCME, AF230211), *Arabidopsis thaliana* (ARATH, P31265), *Hydra vulgaris* (HYDRA, Q94587), *Drosophila melanogaster* (DROME, AFF54603), *Anopheles gambiae* (ANOGA, EAA08161), *Tigriopus japonicus* (TJAPO, AY496449).

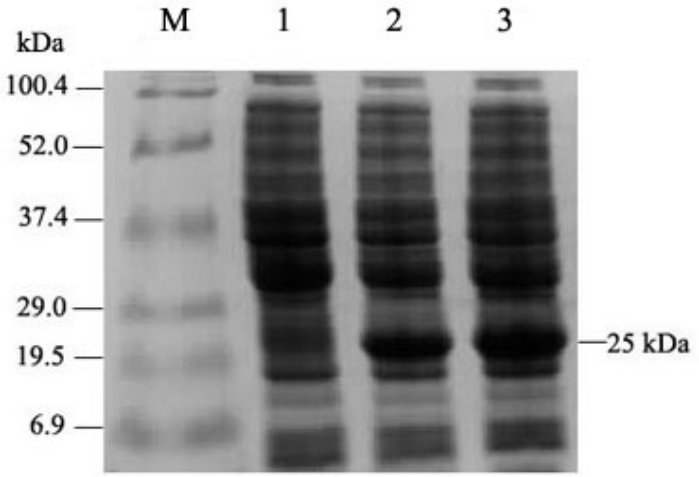

Figure 4 Expression of recombinant mud crab-TCTP in *E. coli*.
M: Low molecular weight standard marker, Lane 1: Cleared lysate from non-induced *E. coli* M15 harboring pQE-TCTP, Lane 2 - 3: Cleared lysate from *E. coli* M15 harboring pQE-TCTP induced with 0.05 and 0.1 mM IPTG, respectively

CONCLUSIONS

Mud crab-TCTP was isolated from the haemolymph of *Scylla paramamosain* by RT-PCR using primers derived from *Penaeus monodon* and expressed in IPTG-inducible T5 polymerase system. The obtained recombinant protein has a molecular mass of approximately 25 kDa with histidine tags. The biological functions of this protein will be characterized in the future.

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บทคัดย่อ

ภูวดล บางรักษ์

การโคลน cDNA และการแสดงออกของ Translationally Controlled Tumour Protein (TCTP) ใน *Escherichia coli* จากเลือดของปูทะเล *Scylla paramamosain*

การโคลน cDNA ของ translationally controlled tumour protein (TCTP) จากเลือดของปูทะเล *Scylla paramamosain* เริ่มจากการเพิ่มจำนวนด้วยวิธี reverse transcription polymerase chain reaction (RT-PCR) โดยใช้คู่ primer ที่ออกแบบมาจากยีนต้นแบบของกุ้งกุลาดำ *Penaeus monodon* จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของ mud crab-TCTP พบ open reading frame (ORF) ที่สมบูรณ์ขนาด 507 bp ซึ่งสามารถถอดรหัสให้กรดอะมิโน 168 โมเลกุล มีน้ำหนักโมเลกุลและค่า pI ประมาณ 19.2 kDa และ 4.5 ตามลำดับ และเมื่อนำลำดับกรดอะมิโนมาเปรียบเทียบกับยีนที่มีรายงานไว้พบว่ามีความเหมือน กับยีน translationally controlled tumour protein หรือ TCTP ของ *Penaeus monodon* (Pm-TCTP) และ *Fenneropenaeus merguiensis* ถึง 99 % เมื่อทำการโคลนในดีเอ็นเอพาหะ pQE40 โดยเชื่อมต่อชิ้นยีนจาก histidine tagged fusion proteins เพื่อชักนำให้เกิดการแสดงของยีนในแบคทีเรีย *Escherichia coli* พบว่าโปรตีนลูกผสมที่ได้มีขนาด 25 kDa ดังนั้นเมื่อเปรียบเทียบลำดับกรดอะมิโนและนิวคลีโอไทด์ รวมทั้งพิจารณาจากการศึกษาหน้าที่ทางชีวภาพของ Pm-TCTP ที่มีรายงานก่อนหน้านี้ จึงมีความเป็นไปได้ว่า mud crab-TCTP อาจมีหน้าที่ทางชีวภาพเป็น anti-apoptosis เช่นเดียวกันกับ Pm-TCTP

สำนักวิชาวิทยาศาสตร์และหน่วยวิจัยการใช้ประโยชน์จากผลิตภัณฑ์ธรรมชาติ มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80161