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Profiling of thymosin beta 3 peptide (Pmthymosin3) expression across tissues and developmental stages in *Penaeus monodon*, and its antimicrobial potential via in silico analysis

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Abstract

The exploration of antimicrobial peptides, such as thymosin β peptides, is crucial to reduce antibiotic dependence. Thymosin β peptides play roles in inflammation regulation, wound healing, cell migration, and angiogenesis, with recent attention on their antimicrobial potential. This study focuses on thymosin beta 3 peptide (Pmthymosin3) from *Penaeus monodon*, a 128 amino acid peptide identified through TA cloning of three gene isoforms. In silico analysis explored its antimicrobial properties and expression across tissues and developmental stages. Results show the highest expression in haemolymph, moderate in gills, and negligible in muscle. Developmental stage expression follows a concave-up pattern. Structural analysis reveals an α-helical configuration with scattered coils. Functional analysis highlights antimicrobial properties of Pmthymosin3, suggesting its potential applications in cancer treatment and biofilm mitigation. The physicochemical properties, combined with in silico findings, underscore Pmthymosin3 as a promising candidate for further antimicrobial research.

Keywords Antimicrobial peptides, Aquaculture, Shrimp, Thymosin

Introduction

The escalating growth and global spread of antimicrobial resistance underscore the urgent need to discover new antimicrobial agents. While antibiotics were previously effective in treating a wide range of bacterial infections, the emergence of antimicrobial resistance has reduced the efficacy of these existing antibiotics. Antimicrobial peptides present distinct advantages compared to conventional antibiotics, including a slower emergence of resistance and broad-spectrum activity [1]. The aquaculture industry, a prominent sector extensively utilizing antibiotics, could find a potential solution for various infections through antimicrobial peptide applications. The aquaculture sector especially the black tiger shrimp

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(*Penaeus monodon*) farming is encountering economic setbacks attributed to diseases caused by bacteria, for example, acute hepatopancreatic necrosis disease (AHPND) by *Vibrio parahaemolyticus* [2], bacterial white tail disease (BWTD) by *V. harveyi* [3], early mortality syndrome (EMS) by *V. alginolyticus* [4], and other non-bacterial challenges. Antimicrobial peptides (AMPs) play a crucial role as a dynamic and varied set of bioactive small proteins, constituting a vital initial defence mechanism against pathogens. Their mechanism involves not only the disruption of bacterial cell membranes but also through modulation of the immune response, thus collectively contributing to the inactivation of harmful pathogens [5]. A diverse array of antimicrobial peptides has been identified within penaeid shrimps, exhibiting various structures and versatile functions. Among these are penaeidins, proteins with the whey acidic protein (WAP) domain like crustins, antilipopolysaccharide factors (ALFs), hemocyanin, lysozymes, histones-derived peptides, stylicins, and more [6–12]. Despite the primary focus on exploring their antibacterial properties, these peptides have demonstrated potential in non-infectious functionalities reviewed by Vincent et al. [13]. The review highlights the myriad functions exhibited by antimicrobial peptides derived from shrimps.

Thymosins, originally derived from the thymus organ, are peptides categorized as thymosin α, β, and γ based on isoelectric points. Among these, β thymosins have been extensively studied across various species due to their antimicrobial properties. It consists of around 40–44 amino acids and it has an approximate molecular mass of 5 kDa and an isoelectric point falling within the range of pH 5.0–7.0. β thymosins function as actin-binding proteins, showcasing versatile roles such as promoting angiogenesis, expediting the healing of corneal scarring and skin wounds, regulating the immune system, and participating in cancer development [14]. The diverse functions of thymosin peptides have been investigated in both invertebrates and vertebrates. The primary emphasis in the study of invertebrate thymosins lies in unraveling their antimicrobial properties, while in vertebrates, thymosins are acknowledged for their therapeutic potential. β Thymosin has been identified in numerous invertebrates, each exhibiting distinct functions. The expression levels of two thymosins were observed to increase upon challenge with *Listonella anguillarum* in the Chinese mitten crab *Eriocheir sinensis* [15]. In freshwater crayfish, *Pacifastacus leniusculus*, β Thymosin is noted for its role in regulating hemocyte homeostasis [16]. Thymosin Pcthy-4 has demonstrated the ability to reduce white spot syndrome virus (WSSV) replication and facilitate its phagocytosis in the red swamp crayfish, *Procambarus clarkia* [17]. Furthermore, in *P. monodon*, β Thymosin has been reported to exert a negative impact on ovarian development [18]. Recently, the thymosin beta-3 isoform of *Marsupenaeus japonicus*, was found to be upregulated in hemocytes in response to challenges from *Staphylococcus aureus* and *Vibrio anguillarum*. Moreover, the peptide displayed noted bacterial clearance and activity towards gram-positive and as well as gram-negative bacteria [19].

In the current study, three thymosin isoforms were recognized from tiger shrimp, *P. monodon*. Thymosin beta 3 isoform (Pmthymosin3) was specifically chosen for in-depth investigation to thoroughly explore its potential as an antimicrobial peptide. The expression pattern of Pmthymosin3 was analysed in various developmental stages of *P. monodon* by qRT-PCR. Furthermore, expression profiling was conducted in various tissues of black tiger shrimp. Prior to implementing experimental techniques to assess the antimicrobial properties of the peptide, in silico analysis was performed to facilitate the preclinical and clinical development of innovative antimicrobial peptides in shrimp culture. The findings suggest the pivotal role of Pmthymosin3 in shrimp immunity, indicating its potential as a subject for additional research as a novel antimicrobial drug in shrimp farming.

Materials and Methods

Cloning of Pmthymosin3 gene

Total RNA was isolated from the haemocytes of healthy adult black tiger shrimp by TRI reagent kit (Sigma, USA) according to the kit protocols. The quality and quantity of isolated RNA were evaluated by 0.8% agarose gel electrophoresis and spectrophotometer, respectively. Subsequently, the first strand of cDNA was reverse transcribed. Primers PmThyF1 and PmThyR1 were designed using Primer 3 tool and used for β thymosin gene amplification (Table 1). The PCR amplification process adhered to the subsequent conditions: 95 °C for 3 min; 35 cycles each with denaturation at 94 °C for 15 s; annealing at 55 °C for 30 s and extension at 72 °C for 30 s; and a final extension to follow up for 10 min at 72 °C. Three fragments were obtained and the subsequent sequencing of the fragments retrieved partial sequences of thymosin isoforms such as Thybeta2.pm, Thybeta3.pm and Thybeta4.pm. The PCR products were integrated into the pGEMT (pGEM-T Easy TA cloning Kit, Promega) to retrieve the full length cDNA sequence of Thybeta3.pm designated as Pmthymosin3 thereafter.

Sequence analysis using bioinformatics tools

The sequence translation of the nucleotides was conducted with ExPASy Translate tool. The prediction of the isoelectric point was carried out utilizing the Compute pI/Mw in ExPASy tool. The MEGA 11.0 and GeneDoc

Table 1 Primer sequences used for gene cloning and gene expression analysis

Name of the primers	Primer sequence	Annealing temperature
βactin[a] (housekeeping gene)	F – 5′ CCACGAGACCACCTACAA C 3′	60 °C
	R – 3′ AGCGAGGGCAGTGATTTC 5′	
PmThy[a]	F – 5′TCGAACCAGAATCACCAC CA 3′	55 °C
	R – 3′AGGCGGAAGCAAGGTGAA AT 5′	
EF-1α[b]	F – 5′TGGCTGTGAACAAGATGG ACA 3′	60 °C
	R – 3′TTGTAGCCCACCTTCTTG ACG 5′	
Pm-Thy-RT[b]	F – 5′TGCCAAACAGGGAGGACG TG3′	68 °C
	R – 3′ GCGGAGTGCCTGCTGACC CT5′	

[a] Primers used for gene cloning experiment

[b] Primers used for gene expression experiment

softwares were deployed for sequence alignment. The characteristic beta actin-binding domains of thymosins were depicted using the SMART program (Simple Modular Architecture Research Tool). Utilizing protein sequences of thymosin isoforms from *P. monodon* and other species, a phylogenetic tree comprising 1000 replicates was constructed using MEGA 11.0 software.

Molecular and functional characterisation of Pmthymosin3 by In silico tools

The processing of the nucleotide sequence was carried out through the application of GeneTool software, leading to the acquisition of the corresponding sequence of amino acids via translation with ExPASy translate tool. Diverse isoforms of thymosin beta from different organisms were sourced from the GenBank database hosted by NCBI. To examine the relationships between these sequences, the ClustalW algorithm was employed to execute a multiple sequence alignment. Subsequently, the MEGA 11.0 software was used to generate a phylogenetic tree, using the method of Neighbor-Joining.

For the assessment of physicochemical characteristics of the protein, the ExPASy ProtParam tool was employed. Aside from the isoelectric point, instability, and aliphatic indices prediction, this tool also calculated the molecular weight, the extinction coefficient, and GRAVY score of the peptide. Boman index, a measure indicating the potential of the peptide for protein-binding was predicted using APD3 tool. Additionally, the Wimley-White whole-residue hydrophobicity, representing the free energy of transfer of the peptide to POPC interface from water, was also predicted using the APD3 tool.

The examination of domain architecture utilized the SMART server. Protein motifs were identified through a MOTIF search using the genome motif tool. Evolutionary relationships were determined by querying the Pfam and SuperFamily databases. For protein folding pattern recognition, the server PFP-FunDSeqE, was employed. Phosphorylation sites in the peptide for amino acids serine, threonine, and tyrosine in the peptide were assessed using the NetPhos 3.1 server. Helical wheel analysis was conducted by submitting the amino acid sequence to the heliquest server. The Kyte-Doolittle plot in the ExPASy-ProtScale server was utilized for the prediction of the peptide's hydrophobicity. The analysis of the protein's accessible surface area was calculated using the ExPASy NetSurfP tool. The PSIPRED was used for secondary structure prediction of peptides and analysis. The three-dimensional structure prediction employed homology analysis through SWISS-MODEL, and the Ramachandran plot constructed using SWISS-MODEL was utilized for the evaluation of stereo-chemical quality of the predicted model. To comprehend the cellular role of a peptide it is crucial to examine its sub-cellular localization. In the instance of Pmthymosin3, the DeepLoc-1.0 analysis tool was employed for predicting its subcellular localization. Also, the Pmthymosin3 active peptide was investigated for potential cleavage sites using the Peptide Cutter server. To forecast the probability of protein expression in diverse expression systems, the Codon Adaptation Index (CAI) calculator was utilized.

The evaluation of the antimicrobial attributes of Pmthymosin3 involved the utilization of various online tools. The CAMP (Collection of Antimicrobial Peptides) database and iAMP-2L were employed for predicting antimicrobial activity. For the assessment of its various activities, different tools were utilized. The antifungal activity was assessed using the AntiFP, for antiviral, the AVPdb, for antiparasitic, the ParaPep, and for anticancer, the AntiCP tools were used. Predictions for the antibiofilm activity of the peptide were made using the dPABB tool. The AntiAngioPred tool was utilized to analyze the anti-angiogenicity of Pmthymosin3, while the determination of its cell-penetrating ability was analyzed using CellPPD tool. To assess the DNA-binding potential of the peptide, the DPbind tool was utilized. The evaluation of the toxicity of the peptide was performed with ToxinPred tool.

Pmthymosin3, tissue and ontogenetic distribution

Six healthy live adults of *P. monodon* were obtained from a shrimp farm situated in Moolanpally, Pizhala, Kochi, India. Various tissues (haemolymph, gill, intestine,

muscle, hepatopancreas, and heart) of *P. monodon* were used to examine the distribution of thymosin β 3. For studying the ontogenetic distribution of thymosin β 3, healthy and live larval stages of *P. monodon* were routinely collected according to the days of development and feeding habits from the Government Regional Shrimp Hatchery, Azhikode, Kerala, India. The collected stages included Nauplii-N5, Zoea-Z1, Mysis-M1, and Post Larvae (Stages—4,10,13,19 & 25). All the specimens were preserved at −80 °C.

Gene expression analysis

Pm-Thy-RT and one constitutive gene (EF-1α) was used for qRT-PCR (Bio-Rad laboratories, USA). For quantification, 10 µl reactions were employed containing 5 µl TB Green Premix dye, 0.2 µl ROX Reference Dye, 1.8 µl milli Q, 2 µl cDNA (1:50 dilution) and 1 µl F: R primer mix (500 nM each primer). The cycling conditions of qRT-PCR were set on the instrument as per the instructions of TB Green Premix Ex Taq II (TI: RNase H Plus) (Takara, Japan). The sequence details and annealing temperature of Pm-Thy-RT and EF-1α are mentioned in Table 1. The fluorescence from 65 to 95 ˚C was monitored after the amplification to determine the melting curves of PCR products with increments of 0.5 ˚C temperature. The constitutive gene of EF-1α was used to normalize the cycle threshold (Ct) values of Pm-Thy-RT using the formula $2^{-\Delta\Delta Ct}$ [20].

Statistical analysis

The significant differences in thymosin β 3 gene expression patterns between the tissues and developmental stages were analysed in triplicates by one-way ANOVA using SPSS 17.0 followed by Tukey's post-hoc test (SPSS Inc., Chicago, IL, USA) with a significance level set at $p < 0.05$.

Results

Thymosin isoforms sequence analysis

The PmThyF1 and PmThyR1 primers designed to ascertain the presence of thymosin in black tiger shrimp yielded three isoforms of thymosins. Three distinguishable bands were observed and the subsequent sequencing by Agrigenome, Kochi, India identified them as three isoforms of thymosins: Thybeta2.pm, Thybeta3.pm and Thybeta4.pm designated according to their motif numbers. All three isoforms showed isoelectric points that falls between the range of pH 5.0 to pH 7.0 making them thymosins of the family β thymosin. The partial sequence of Thybeta2.pm, Thybeta3.pm and Thybeta4.pm encoded 83, 127 and 159 amino acids, and holds thymosin beta-actin-binding motifs 2, 3 and 4 respectively (Fig. 1a). The thymosin isoforms beta-actin-binding motifs depicted in Fig. 1A, showed high identities in their sequences. In order to examine the evolutionary connection between beta thymosin isoforms of *P. monodon* and of others, a phylogenetic tree was constructed through the method of Neighbor-Joining (NJ). The analysis revealed that these isoforms constituted a distinct clade, demonstrating the closest phylogenetic affinity to *P. japonicus* (Fig. 1b).

In silico: Molecular and functional characterisation of Pmthymosin3

The entire complementary DNA (cDNA) sequence of Pmthymosin3 in *P. monodon* comprises a 387-base pair segment of nucleotides encoding 128 amino acids, forming a protein with a predicted molecular weight of 14.268 kDa and 5.45 theoretical isoelectric point (pI). The amino acid composition of Pmthymosin3 includes three standard thymosin domains (THY) and a strongly preserved actin-binding motif, LKKTET unique to the family of Tβ protein (Fig. 2a). The relationship of the sequence with previously reported thymosin beta family amino acid sequences was revealed by ClustalW multiple alignments (Fig. 2b). Two cluster groups were revealed in the bootstrap distance tree built using the method of Neighbor-Joining (NJ) in MEGA11 for the phylogenetic analysis of the Pmthymosin3 sequences (Fig. 2c). The first one has two subgroups of β thymosin isoforms from different shrimp varieties such as *Penaeus japonicus, Penaeus vannamei* etc. and other subgroup contains thymosin beta isoforms from crayfish, cockroach, etc. The other cluster includes thymosin beta isoforms from species other than shrimp, including *Scylla paramamosain, Armadillidium vulgare, Chionoecetes opilio* etc.

ProtParam prediction analysis predicted a molecular weight of 14.268 kDa, a theoretical isoelectric point (pI) of 5.45, and a net charge of −4 for the peptide. The net negative charge of the 128-mer Pmthymosin3 was contributed by 24 negatively charged residues of Asp + Glu and positively charged 19 Arg + Lys residues. Pmthymosin3 showed amino acid abundance with Ala (10.9%), Glu (14.8%), lys (13.3%). The lack of Trp, Tyr or Cys in the considered region makes the protein invisible under UV spectrophotometry. The aliphatic index was calculated as 74.06, which gives an indication towards the peptide's thermal stability. The half-life of the peptide was identified as 30 h with respect to mammalian reticulocytes, > 20 h with yeast (in vivo) and > 10 h for *E. coli* (in vivo). The peptide was recognised as unstable with an instability index of 47.47. The peptide GRAVY was recorded with −0.933. The prediction using APD3 showed that Pmthymosin3 has a chance to be an antimicrobial peptide with a potential for Protein-binding (Boman index) of 2.27 kcal/mol with 66.73 of Wimley whole residue hydrophobicity. It was also predicted

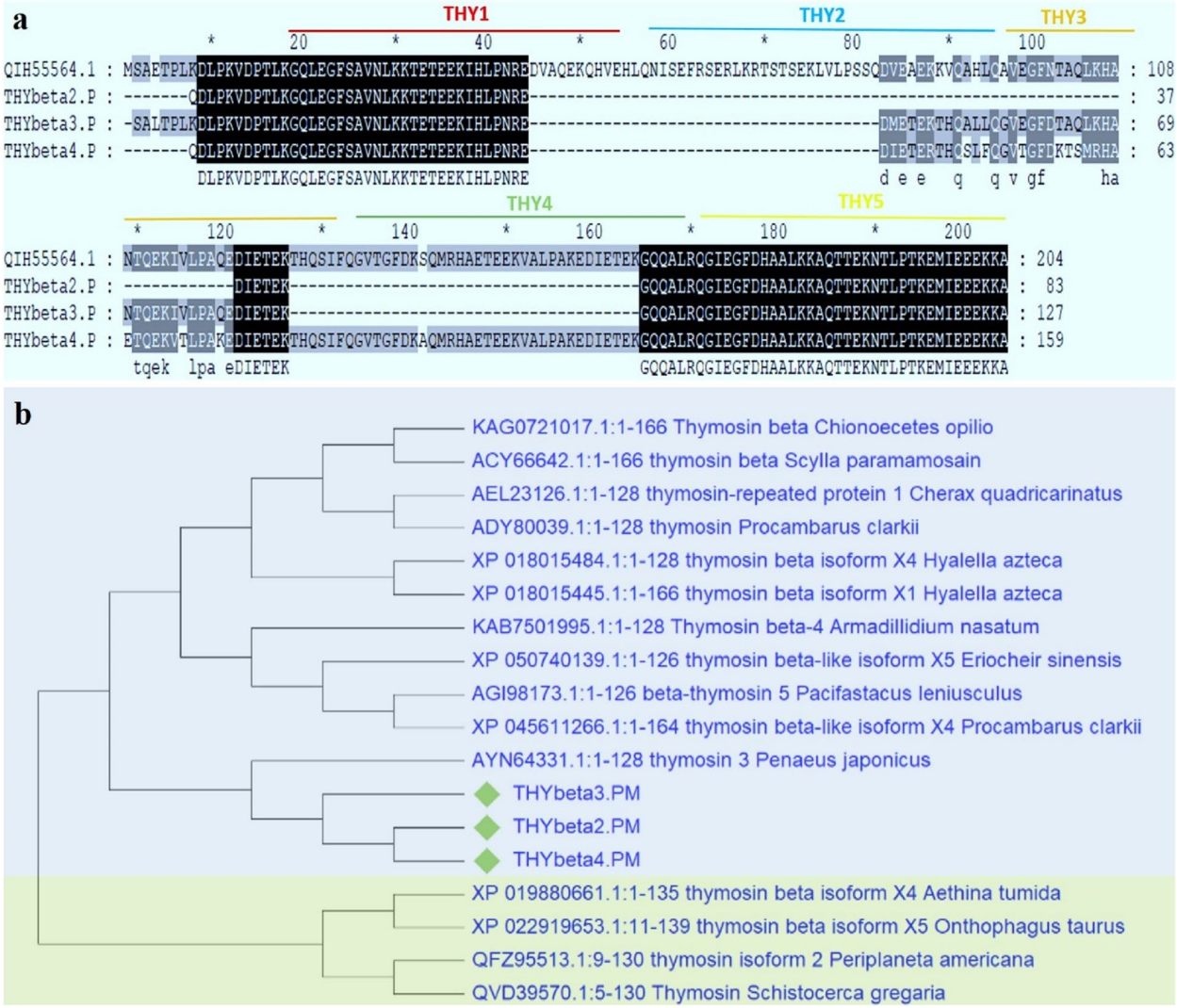

Fig. 1 Analysis of three isoforms of thymosin: **a** Multiple sequence alignment of Thybeta2.pm, Thybeta3.pm and Thybeta4.pm aminoacids with Thymosin beta 5 from *Penaeus monodon* (GenBank ID: QIH55564.1) using GeneDoc depicting the domains. The lines with different colours indicate different beta-actin-binding motifs present in Thymosin beta 5. **b** Phylogenetic tree analysis of thymosin isoforms: a neighbor-joining tree was built using MEGA 11 software, with 1000 bootstrap replication with the thymosin protein sequences collected from GenBank

that the peptide could form alpha helices and may hold a minimum of 24 residues on the same hydrophobic surface. The peptide was expected to be devoid of any signal peptide sequence with SignalP 5.0 server prediction. The presence of three thymosin domains and extra non-featured (scores less than required threshold) two domains; i.e. K homology RNA binding (KH domain) and calmodulin-binding protein (CaM) domain (Fig. 3) was predicted using MOTIF server motif search and SMART domain prediction.

Pmthymosin3 was indicated to fall under the thymosin family by Pfam server and SuperFamily server analysis.

(See figure on next page.)

Fig. 2 Pmthymosin3 peptide molecular characterization. **a** Nucleotide and inferred amino acid sequence of Thymosin beta 3 peptide of *Penaeus monodon* mRNA transcripts, Pmthymosin3 (GenBank ID: ON494591). The corresponding single-letter amino acid code (highlighted) of nucleotide sequences is shown below; grey coloured part highlights the THY domains; the underlined region denotes the conserved (LKKTET) actin binding domain; **b** Pmthymosin3 multiple alignment using amino acid sequences of related species. **c** Pmthymosin3 phylogenetic tree reconstructed using the method of neighbor-joining with sequences from different species

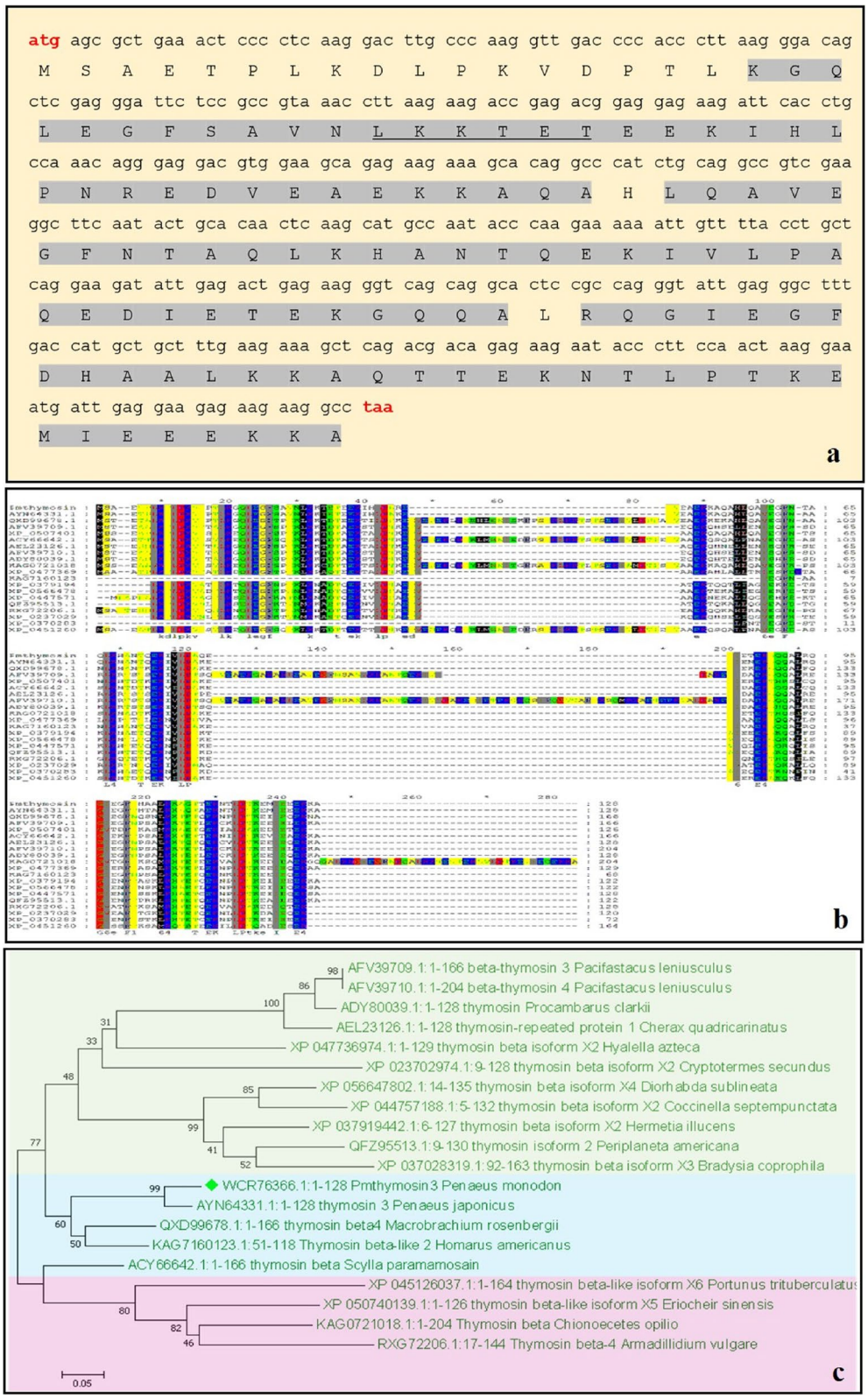

Fig. 2 (See legend on previous page.)

Fig. 3 SMART diagram above represents a summary of the domains found in Pmthymosin3. Three thymosin domains and two overlapping regions: K homology RNA binding (KH domain) and calmodulin-binding protein (CaM) domain

Four helical up-and-down bundles were recognized using fold pattern analysis by PFP-FunDSeqE tool. In the predictions made using the NetPhos 3 tool, a serine phosphorylation site was identified in the second position, while seven potential threonine phosphorylation sites were indicated at positions 5, 16, 32, 72, 86, 110, and 111. Pmthymosin3 was identified as a linear molecule with alpha helices having residues of hydrophilicity and hydrophobicity (PVVA) concentrated at two opposite poles using helical wheel server analysis (Fig. 4). The Kyte-Doolittle hydrophobicity plot of Pmthymosin3 provided confirmation of several findings. Firstly, it indicated the existence of a short region of hydrophobic amino acids, specifically the sequence GFSAVNL, spanning positions 23 to 27 (Fig. 5). Additionally, the plot revealed the existence of scattered hydrophobic residues throughout the protein sequence. NetSurfP analysis revealed that the peptide exhibited a relative surface accessibility indicating exclusively exposed amino acid residues when assessed at a 25% threshold level. The predicted secondary structure of the peptide consisted of random helices and coils. Additionally, the analysis indicated the presence of disordered residues at both ends of the peptide (Fig. 6).

PSIPRED server analysis identified Pmthymosin3 as having an α-helical structure having random coils (Fig. 7). The occurrence of alternating α-helical and random coil regions having a proline hinge stretch was revealed using SWISS MODEL homology modelling for spatial 3D structure prediction of Pmthymosin3 (Fig. 8). The N and C terminal regions reveal coiled structures, indicating that Pmthymosin3 adopts a helix-hinge-helix architecture. The Ramachandran plot (Fig. 9) revealed 99.21% residues falls in the Ramachandran favoured areas and 0.0% as Ramachandran outliers. The DeepLoc server predicted the subcellular localization of Pmthymosin3 as the cytoplasm, and it was also predicted to be soluble. These predictions indicate that the peptide likely functions within the cytoplasm and can freely interact with other soluble components present in the cell.

The Codon Adaptation Index (CAI) functions as a predictor of the likely efficacy of gene expression in a

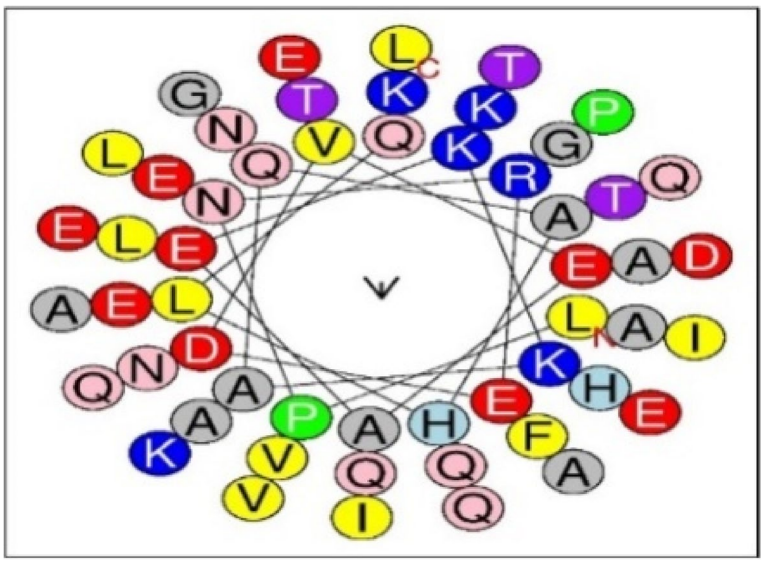

Fig. 4 The online tool Heliquest was employed to illustrate the arrangement of hydrophobic and hydrophilic amino acids on opposite phases of the peptide

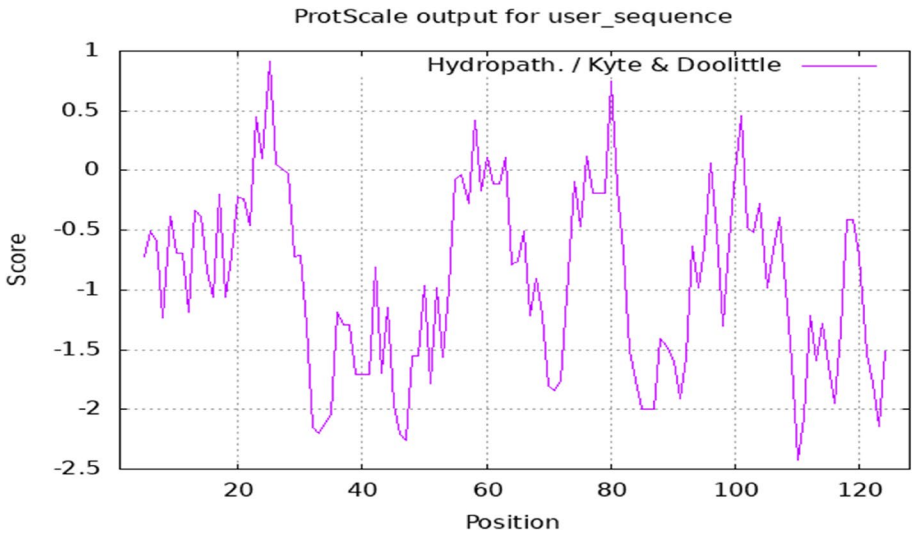

Fig. 5 Hydrophobicity of Pmthymosin3 was shown using the Kyte-Doolittle plot. Positive peaks in the plot represent the predicted hydrophobic regions within the protein sequence

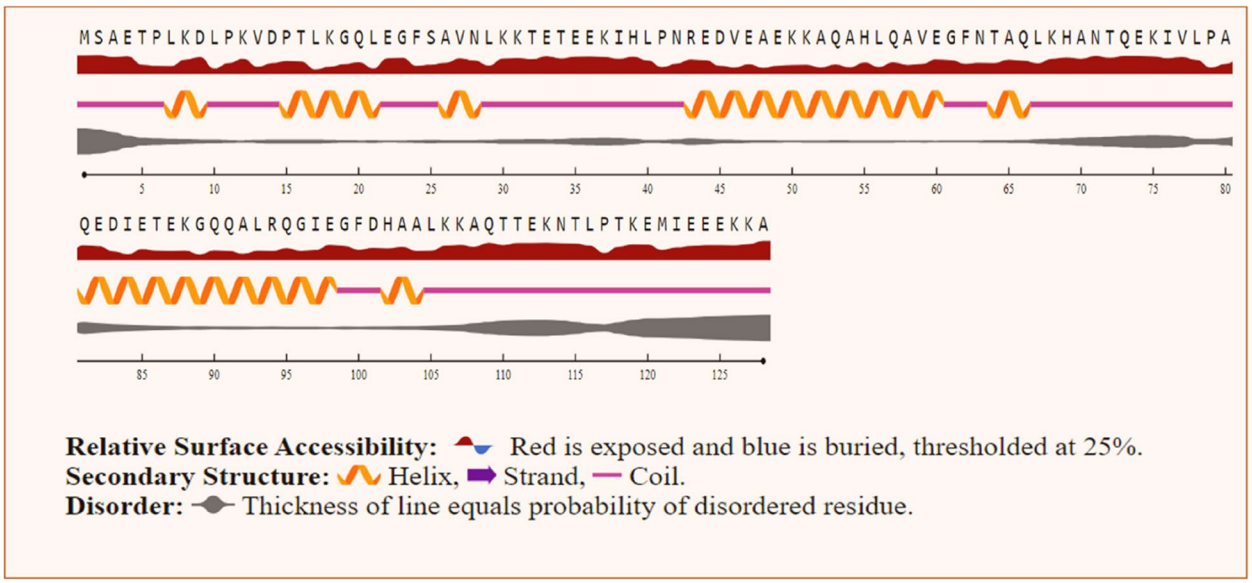

Fig. 6 NetSurfP server prediction of protein surface accessibility

different biological system. The projected CAI values for the peptide in expression systems of *Escherichia coli*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Spodoptera frugiperda* with expected success rates of 0.55, 0.72, 0.64, and 0.81 respectively. These results collectively imply that there is promising potential for the successful expression of Pmthymosin3 in all of these systems, opening avenues for further exploration. 69 cleavage sites were predicted for Proteinase K in Pmthymosin3, followed by 32 cleavage sites in thermolysin using the PeptideCutter tool ExPASy. 19 cleavages were found to be mediated by Trypsin at cleavage sites with Lys (K) and Arginine (R) residues. The peptide was predicted not to be cut with enzymes such as caspase, granzymeB, and thrombin.

CAMP analysis confirmed that peptide Pmthymosin3 as an antimicrobial peptide, having a 0.75 AMP probability. AntiCP, using an average SVM score of 0.74, identified Pmthymosin3 as an anticancer peptide. According to AntiAngioPred, the peptide was predicted to be an antiangiogenic peptide with a 0.23 SVM score,

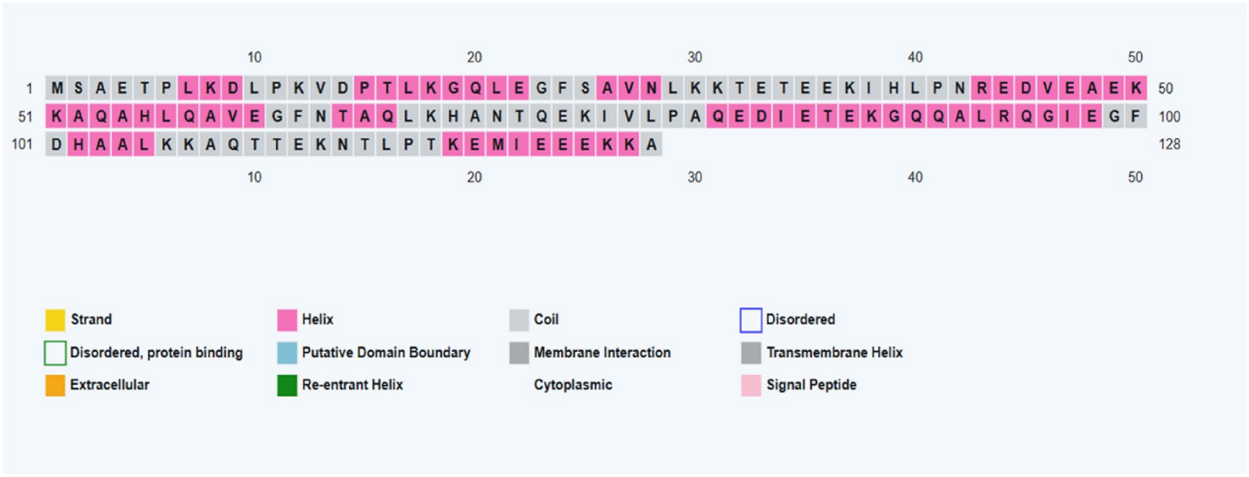

Fig. 7 Pmthymosin3 secondary structure predicted using PSIPRED server. The pink-coloured box indicates the α-helix region and the white-coloured box indicates the coiled region. α-helical structure having random coils was indicated for the structure of Pmthymosin3

Fig. 8 SWISS MODEL homology modelling prediction for the 3D structure of Pmthymosin3

suggesting its cancer progression inhibition potential. Notably, Pmthymosin3 showed no activity against fungus, virus, or parasites. The dPABBs software, utilizing the SVM method, analyzed and identified the antibiofilm property of the peptide and its mutants. Pmthymosin3, a cell-penetrating peptide, exhibits an inclination for DNA binding. The analysis indicates that of the residues within the 128-mer peptide 25% possess a binding potential range between 0.540 and 1, implying the presence of DNA binding properties in the peptide. Pmthymosin3 was classified as a non-toxic peptide by ToxinPred server. Thus, Pmthymosin3 could be a potentially impactful compound for drug development with these functional characterisations. (The links to the software used in this study are provided in Additional File A1).

Tissue wise expression profile of Pmthymosin3

The Pmthymosin3 expression profile was assessed tissue wise through qRT-PCR analysis, employing an internal control EF-1α. Findings revealed widespread expression of Pmthymosin3 across all examined tissues except for muscle tissues, albeit with varying levels (Fig. 10a). This suggests a potential multifunctional role of Pmthymosin3 in *P. monodon* tissues. The relative expression was highest in hemolymph, succeeded by the intestine, gill, heart, and the least by hepatopancreas.

Expression pattern of Pmthymosin3 across developmental stages

Pmthymosin3 mRNA occurred in all the initial developmental stages of *P. monodon*, ranging from Nauplii-N5, Zoea-Z1, Mysis-M1 to Post Larvae Stages (4, 10, 13, 19, & 25) (Fig. 10b). Notably, Pmthymosin3 mRNA exhibited significant expression in Nauplius, Zoea, and Mysis stages, with relatively high levels observed initially and a sharp decline to very low values in Post Larvae (PL) stages. Subsequently, there was a slight resurgence of expression in PL19 and PL25 stages. Notably, the mRNA expression of Pmthymosin3 demonstrated significant differences among the various early developmental stages ($p < 0.05$).

Discussion and Conclusion

This study aimed to characterize the thymosin beta 3 peptide from *P. monodon*. Among three isoforms of beta-thymosins identified from *P. monodon* thymosin beta 3 (Pmthymosin3) was cloned and characterized to elucidate the role of thymosin in shrimp immunity. To our

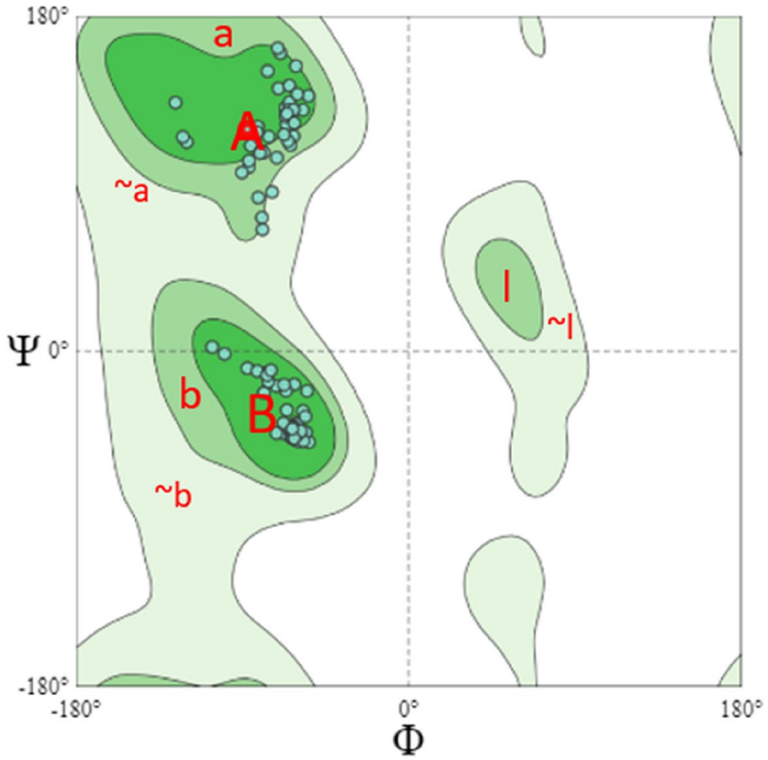

Fig. 9 Analysis of the Ramachandran plot: Examining the phi-psi graph of the anticipated 3D structure of Pmthymosin3 generated by SWISS MODEL through analysis of the Ramachandran plot reveals distinct regions. Areas A and B align with the most favoured regions, denoted by a, b, and l, indicating additional permissible areas. The regions marked as ~ a, ~ b, and ~ l represent generously allowed regions. Any segment falling outside these defined categories is identified as part of the disallowed region

understanding, this is the first report on the characterization of thymosin beta 3 peptide from *P. monodon*.

Thymosin beta family isoforms are typically categorized based on their beta actin binding domains. In most cases, invertebrates possess multiple types of thymosin isoforms. Notably, four beta thymosin isoforms have been recently discovered in Kuruma shrimp [19], while up to nine thymosin isoforms have been investigated in the red swamp crayfish *P. clarkia* [17]. On the other hand, in vertebrates, thymosins possess only one domain, as reported by Van et al. (1996), which is a thymosin beta actin-binding motif that binds to actin monomers. Consequently, the disparity in the thymosin domain number implies that thymosins may have a more intricate role in invertebrates compared to vertebrates. Three isoforms found in *P. monodon* feature 2, 3, and 4 beta actin-binding motifs of thymosin, and due to their substantial sequence similarity in these domains, they can be classified into beta thymosin family [21]. The presence of a strongly conserved actin-binding motif, LKKTET, in all three isoforms suggests that there might be a conserved function associated with it [22]. Pmthymosin3 peptide holds three THY domains with the presence of conserved LKKTET domain. The multiple alignment and phylogenetic analysis of the peptide confirmed its significant similarity with its nearest ortholog *Penaeus japonicus*.

In silico and computational methods for molecular and functional characterization of peptide prediction offer a quick, readily available, and cost-effective approach for forecasting thousands of bioactive compounds [23]. Recent in silico studies have provided valuable insights into the binding affinities of specific proteins with promoters and their regulatory roles in gene expression, highlighting their critical involvement in various physiological processes [24]. Analysis conducted in silico using the SMART and ProtParam tools confirmed that the peptide Pmthymosin3 possesses three THY domains and falls within the isoelectric point range typical of beta thymosins. The lack of a signal peptide suggests that it's improbable for thymosin β 3 to be a secreted peptide [25]. Pmthymosin3 is predicted to hold the characteristics of an anionic antimicrobial peptide by ADP3 tool. The anionic character of the peptide suggests that its mechanism of action does not involve interactions with negatively charged bacterial membranes, unlike cationic AMPs. In Gram-negative bacteria, the AMP sensor system, specifically the PhoP/PhoQ system, modulates lipopolysaccharide modification to reduce sensitivity to

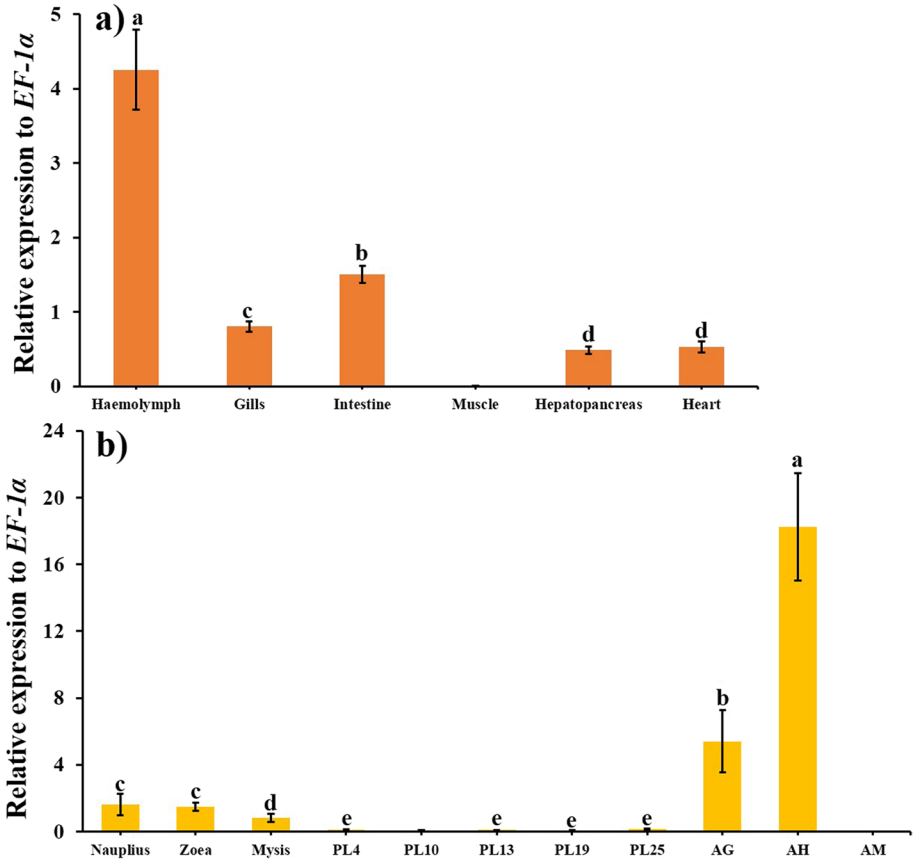

Fig. 10 Expression profiling of Pmthymosin3. Relative mRNA expression levels of Pmthymosin3 across different conditions. Data are presented as mean ± SD (error bars) from triplicate samples denoted by different alphabets for significantly different values at ($p < 0.05$) determined using Tukey's post-hoc test: **a** Tissue wise distribution of Pmthymosin3 in *P. monodon*. **b** Expression profiling of Pmthymosin3 in various developmental stages and in adult *P. monodon* (AG- Adult gill, AH- Adult haemolymph, AM- Adult muscle)

cationic AMPs [26]. The mechanism of action of anionic peptides overcomes the resistance mechanism of Gram-negative bacteria. Dermcidin (DCD), an anionic peptide continually produced in the eccrine sweat glands, has been observed to engage with negatively charged bacterial membranes through ion channel formation. This process is facilitated by the Zn^{2+} dependent creation of oligomeric complexes within the bacterial membrane [27]. The C=O and N–H groups with their hydrogen bonds in the peptide backbone, stabilizes the crucial secondary structure constituted in the α-helix. Two-dimensional projections, referred to as helical wheels, can be used to illustrate α-helical peptides and to understand the amphipathic nature of alpha helices, which is important for various biological processes, including membrane protein interactions and the design of peptides with specific properties [28]. The Kyte-Doolittle scale is employed for examination of protein sequences, aiming to pinpoint potential transmembrane regions. Through scrutinizing a protein sequence and aggregating the hydrophobicity values of neighbouring amino acids, one can anticipate regions likely to be incorporated into the lipid bilayer. This knowledge proves beneficial in comprehending the structure and functionality of membrane proteins. Membrane proteins are pivotal in processes like signal transduction, the conveyance of molecules across membranes, and the recognition of cells [29]. The primary purpose of the Netsurfp server is to provide information about the solvent accessibility and secondary structure of amino acids within a protein. It also provides predictions related to the disorder or flexibility of certain regions within a protein. Intrinsically disordered regions that lack a well-defined three-dimensional structure can play important roles in protein function [30]. Ramachandran-plot assess the conformational stability and allowed regions of protein backbone torsion angles, aiding in the analysis and validation of protein structures. The protein Pmthymosin3 exhibits a distribution of residues in the Ramachandran plot, with 99.21% of the residues falling within the most favoured region and the rest within the additional

allowed region. This information suggests the conformational stability and favourable geometry of the protein's backbone torsion angles, contributing to its overall structural quality. Secondary structure and tertiary structure prediction understand the structural characteristics of proteins, which is essential for various biological and biochemical studies. The secondary and tertiary structure prediction of proteins is essential for comprehending the structural features of proteins, providing essential insights for a range of biological and biochemical investigations. The predicted model can offer insights into the potential functions and active sites of the target protein by extrapolating information from structurally characterized homologs [31]. In recent years, in silico approaches have become integral to drug development, particularly in combating critical diseases like White Spot Syndrome Virus (WSSV) and Hepatopancreatic Microsporidiosis (HPM) [32, 33]. These computational methods facilitate the evaluation of potential drugs for efficacy and molecular interactions before advancing to chemical synthesis, significantly accelerating the discovery process. For instance, in silico analyses have revealed the interactions between WSSV viral proteins VP24 and VP28 with chitin-binding proteins in shrimp intestines. Another example includes assessing antibacterial activity against Gram-positive and Gram-negative bacteria through molecular docking with cell surface components [32, 34]. Such studies emphasize the pivotal role of computational techniques in identifying and optimizing therapeutic strategies to manage infectious diseases effectively. Advancements in Artificial Intelligence (AI) have significantly accelerated drug development by leveraging machine learning, problem-solving algorithms, robotics, and other cutting-edge techniques [35, 36].

qRT-PCR was used to determine the ontogenetic gene expression of Pmthymosin3 mRNA and its expression pattern in various tissues of adult *P. monodon*. The analysis of Pmthymosin3 mRNA transcription across various developmental stages revealed its significant presence during the pre larval phases. It was found in minimal quantities during post-larval stages and reappeared in the adult shrimp. The early expression of specific gene transcripts during initial developmental stages highlights the establishment of early defense mechanisms. The observed early peak of Pmthymosin3 mRNA transcripts suggests a possible role in initial immunity. Notably, thymosin beta 4, a single-domain variant of the thymosin family identified in *P. monodon*, has been reported to play a critical role in antiviral immunity. Additionally, synthetic thymosin beta 4 peptides have demonstrated antibacterial activity against *Aeromonas hydrophila*. While there are evidences to prove the AMP nature of beta thymosins, further in vivo and in vitro experiments are essential to conclude Pmthymosin3 as an AMP [37–39]. Quispe et al. evaluated the expression of immune related genes to come to a conclusion that shrimp immune system develop early in ontogenesis [40]. *P. vannamei* penaeidin was detected in early larvae, ranging from Nauplius V to Postlarva VIII, with its mRNA expression specifically restricted to haemocytes [41]. In penaeid shrimps, the developmental expression of antimicrobial peptides (AMPs) revealed the detection of a penaeidin mRNA isoform (mo-penaeidin) from a single cell to the post-larvae stage. Its peak concentration was observed at Nauplius I in *P. monodon* [42]. Another peptide, Mj-sty from *Marsupenaeus japonicus* exhibited significant variations in different developmental stages, notably in Zoea II, followed by Mysis I, and PL1. During the developmental stages, the expression of Mj-sty was comparatively elevated, signifying their potential involvement in various processes such as digestion, immune response, and molting during metamorphosis [43]. Further investigation on the current peptide is necessary to confirm the underlying mechanisms and to conclude the potential role of this peptide in early immunity.

Profiling the expression of Pmthymosin3 in different tissues of adult *P. monodon* revealed varied expression levels, with the haemolymph showing the highest relative expression, followed by the intestine, gill, heart, and hepatopancreas, while no significant expression was detected in the muscles. This finding is consistent with other studies on thymosin beta isoforms identified in different crustaceans, highlighting the hemolymph as the primary organ of production. Among invertebrates, hemolymph stands out as the chief immune tissue, playing a vital role in detecting and eradicating pathogens through mechanisms like phagocytising pathogens, releasing reactive oxygen species (ROS), and by synthesizing of AMPs [44, 45]. The substantial expression observed in the intestines indicates a chance of potential of the peptide for deployment against WSSV infection. Recent studies on WSSV proteins have shown that the intestines are key binding sites for these proteins, and failure of the virus to bind to these sites results in unsuccessful infection. This suggests that targeting intestinal binding could be an effective strategy for preventing WSSV infection [32]. The widespread expression of Pmthymosin3 throughout various tissues suggests that Pmthymosin3 serves diverse functions in *P. monodon.*

Thymosins have been shown to exhibit both antibacterial and antiviral properties in various studies. However, the current work represents preliminary research aimed at evaluating the potential of the Pmthymosin3 peptide as an antimicrobial peptide (AMP) through in silico analysis and expression profiling across tissues and developmental stages. While these findings provide encouraging

evidence, further challenge experiments are required to validate the antimicrobial properties of Pmthymosin3 conclusively.

In conclusion, examining the expression of Pmthymosin3 in *P. monodon* during non-infective stages has elucidated the role of Pmthymosin3 in natural conditions. However, additional research is required to analyze its fluctuation during different infections and understand how this peptide contributes to innate immunity. Concurrently, through various functional characterizations using in silico analysis, Pmthymosin3 has demonstrated potential as an antimicrobial peptide. To firmly establish its antimicrobial capabilities in shrimp culture, further antimicrobial assays and experimental challenges are imperative.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44315-024-00021-7.

Supplementary Material A1.

Acknowledgements
The authors are grateful to University Grants Commission (UGC) India, for the financial assistance for this research project. Deep gratitude to the Government Regional Shrimp Hatchery in Azhikode, Kerala, India, for supplying *P. monodon* live samples of various developmental stages. We extend heartfelt thanks to RUSA Major Projects T2B in providing necessary technical support. Furthermore, we are thankful to Cochin University of Science and Technology (CUSAT) and Rajagiri College of Social Sciences (RCSS) for providing the necessary laboratory facilities.

Authors' contributions
Sheethu Annie Vincent: Investigation and writing the original draft. K. K. Noorjahan: Investigation . Theivanayagam Maharajan: Investigation and editing the manuscript. Stanislaus Antony Ceasar: Supervision and editing the manuscript. Anne Maria Thomas: Investigation . Kavya Gokul: Investigation . Raniya Samad: Investigation . Pratheesh Mathew: Investigation and editing the manuscript. Swapna P. Antony: Conceptualization, Funding acquisition, editing the manuscript.

Funding
This study was funded by Kerala State Council for Science, Technology and Environment, Govt. of Kerala for the research grant (KSCSTE/926/2022-SRSLS).

Data availability
Sequence data that support the findings of this study have been deposited in the GenBank with the primary accession code GenBank ID: ON494591.

Declarations

Ethics approval and consent to participate
Ethical approval for this study was obtained from the institutional animal ethical committee (Approval Number: 363/Go/Re/S/01/CPCSEA/27). The care and treatment of animals used in this study were done following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment & Forests (Animal Welfare Division), Govt of India, on care and use of animals in scientific research.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 17 October 2024 Accepted: 26 November 2024
Published online: 18 December 2024

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