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SHORT COMMUNICATION

Molecular cloning and characterization of a novel pathogen-induced trypsin-like protease in *Scylla paramamosain* (Estampador 1949)

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Serine protease (SP) is one of the oldest characterized and largest multigene proteolytic families, in which serine serves as the nucleophilic amino acid at the catalytic site. The active serine and two other residues (a histidine and an aspartate) are referred to as the 'catalytic triad' in the catalytic sites of many families of SP including trypsin (S1), alpha-lytic endopeptidases (S2), togavirus endopeptidase (S3), subtilisn (S8), prolyl oligopeptidase (S9) and serine carboxypeptidase (S10) families (Rawlings & Barrett 1993). In the trypsin subfamily, the three residues, serine, histidine and aspartate are, respectively, surrounded by 'GDSGGP', 'TAAHC' and 'DIMLL', which are all highly conserved motifs (Yousef, Elliott, Kopolovic, Serry & Diamandis 2004). Serine protease homologs (SPHs) are similar in amino acid sequence to S1 family SPs, but apparently lack amidase activity because the mutation of one or more of the catalytic residues (Ross, Jiang, Kanost & Wang 2003).

In invertebrates, arthropod SPs and SPHs are probably involved in various immune responses including haemolymph coagulation, melanotic encapsulation, induction of antimicrobial peptide synthesis and activation of cytokines by constituting a complex enzyme system in haemolymph (Ross *et al.* 2003; Piao, Kim, Kim, Park, Lee & Ha 2007). Melanization is an important immune response in many invertebrates and its (prephenoloxidase activating) cascade is regulated by SPs including the proPO-activating enzyme (PPAE), and SPHs (Cerenius, Lee & Söderhäll 2008). PPAE is the final link in the cascade leading to PO activation and keep it under tight control by another proteinase, their cofactors (non-catalytic serine proteinase homologues) and serine proteinase inhibitors. All those proteinases can properly be referred to as prophenoloxidase-activating factors (PPAF) (Buda & Shafer 2005). Many PPAE genes encoding either serine proteases (SPs) or serine protease homologs (SPHs) have been cloned and identified from a variety of arthropod species such as *Holotrichia diomphalia* (Kwon, Kim, Choi, Joo, Cho & Lee 2000; Kim, Baek, Lee, Park, Lee, Söderhäll & Lee 2002), *Manduca sexta* (Jiang, Wang & Kanost 1998; Yu, Jiang, Wang & Kanost 2003; Gupta, Wang & Jiang 2005), the crayfish *Pacifastacus leniusculus* (Wang, Jiang & Kanost 2001), the black tiger shrimp *Penaeus monodon* (Amparyup, Jitvaropas, Pulsook & Tassanakajon 2007; Charoensapsri, Amparyup, Hirono, Aoki & Tassanakajon 2011), Chinese mitten crab *Eriocheir sinensis* (Gai, Qiu, Wang, Song, Mu, Zhao, Zhang & Li 2009), the swimming crab *Portunus trituberculatus* (Cui, Liu, Wu, Luan, Wang, Li & Song 2010) and *Fenneropenaeus indicus* (Vaseeharan, Shanthi & Prabhu 2011). Most of the reported SPs have multi-domains such as *H.diomphalia* PPAF-1 and HP-14. The *H.diomphalia* PPAF-1 consists of clip and chymotrypsin-like domains (Lee, Kwon, Hyun, Choi, Kawabata, Iwanaga & Lee 1998; Piao *et al.* 2007), while HP-14 is an upstream proteinase from the moth *Manduca sexta* containing five low density lipoprotein receptor class A repeats, a Sushi domain, a unique Cys-rich region and a proteinase-catalytic domain (Ji, Wang, Guo, Hartson & Jiang 2004).

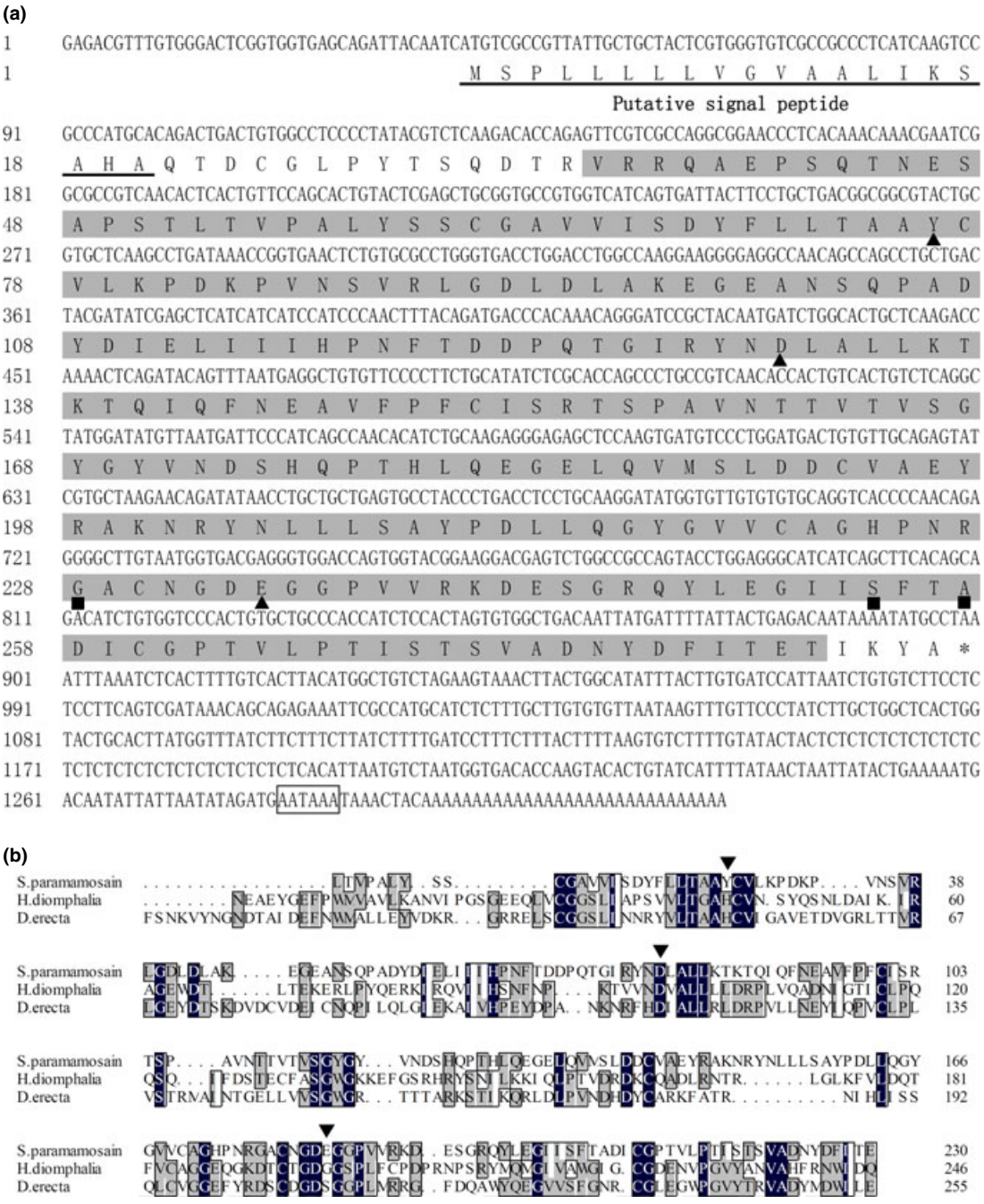

Figure 1 Nucleotide sequence (above) and deduced amino acid sequence of the ORF (below) of TLP cDNA (a), multiple alignment of the Tryp_SPc domains of TLP and other SPHs (b). Nucleotides are numbered from the first base at the 5′end. Amino acids are numbered from the initiating methionine. The predicted signal peptide and structural modules are underlined and marked. The polyadenylation signal AATAAA is boxed at the C-terminal part. The catalytic triads (Tyr^{76}, Asp^{131} and Glu^{234}) at the active sites are indicated with "▲" (or "▼") and the amino acid residues forming the substrate specificity pocket are labelled with black squares. The asterisk (*) indicates the stop codon. The sequence is updated with full-length cDNA (GenBank accession number FJ774773.1). Proteins shown in the alignment are *Scylla paramamosain* (*S. paramamosain*), *Drosophila erecta* (*D. erecta*) and *Holotrichia diomphalia* (*H. diomphalia*).

The crab *S. paramamosain* is an important commercial crustacean species in China and Pacific areas. In the past decade, this economic species frequently suffered from outbreaks of diseases that had caused obvious decrease in production and severe economic losses; however, there were few effective ways of preventing these puzzles due to limited understanding of the innate immunity defence of *S. paramamosain*. In our previous study, a partial cDNA sequence (GenBank no. FJ774773.1) of trypsin-like serine protease homologs gene was screened from the haemocyte subtractive suppression hybridization (SSH) library of *S. paramamosain* and its mRNA expression was strongly up-regulated with LPS-challenge (Chen, Liu, Bo, Ren & Wang 2010). In the study, the full-length cDNA sequence of the new trypsin-like protease, designated TLP, was completely determined by performing a 3′ and 5′ RACE with the gene-specific primers TLPR1 (5′-aacctgctgctgagtgcc tacc-3′) and TLPF1(5′-ggtccaccctcgtcaccattac-3′) respectively. The identified full-length cDNA of the TLP sequence comprises 1325 bp. The coding region of the TLP cDNA starts 39 bp downstream from the 5′ end and is 861 bp long followed by 425 bp 3′ untranslated region (UTR). The cDNA does not contain any Kozak consensus sequences or Kozak consensus-like sequences upstream of the initiation codon ATG. TLP has an 861 bp ORF encoding a predicted protein of 286 amino acid residues (Fig. 1a). The deduced protein includes a predicted signal peptide and one tryp-like domain. The predicted cutting site of the signal peptide is located between Ala^{20} and Gln^{21}. The putative whole protein molecular weight is approximately 31.2 kDa with an estimated isoelectric point of 4.58 (Fig. 1a). Sequence analysis revealed that the Tryp_SPc domain lacks two catalytic residues, with the substitution of His and Ser in the active site triad to Tyr^{76} and Glu^{234}, which were conserved in the TAAHC and GDSGGP regions of serine proteases. **A**mino acid sequence database searching using the BlastP algorithm (http://www.ncbi.nlm.gov/BLAST/) revealed that the identified TLP Tryp_SPc domain that had highest identity to a *Drosophila erecta* SPHs (GG14174) was only 33% and second to *Holotrichia diomphalia* prophenoloxidase-activating factor (CAC12665.1) with 31% identity (Fig. 1b). In addition, the result of nucleotide sequence search using ENA program (http://www.ebi.ac.uk/ena/) showed that TLP shared high similarity (over 80% identity) and a common replacement of His by Tyr^{76} among several examined crab SPHs (*Carcinus maenas*, GenBank no. GT562940; *Callinectes sapidus*, GenBank no. CV527144; *Portunus trituberculatus*, GenBank no. GT562940). Those suggested that the TLP and other related SPHs from crabs belong to a new subfamily of the trypsin-like protease (Ross *et al.* 2003).

The trypsin-like serine protease (Tryp_SPc) family is ubiquitous in eukaryotic animals and plays diverse roles in human processes of food digestion, haemostasis, immune defence response and the nervous system (Wang *et al.*, 2008). Some Tryp_SPc proteases even contribute to the digestion of the blood meal in the mosquito (Wu *et al.*, 2009). However, the function of trypsin-like serine protease homologs in crustacean is little known by far. To investigate its function, we firstly detect the expression patterns of TLP mRNA transcripts in various tissues and different development stages of *S. paramamosain* by semi-quantitative RT-PCR. The results indicated that the TLP gene expression level in haemocytes and gills was higher than that in heart, hepatopancreas, stomach and nerve. Meanwhile, the mRNA of TLP was detected at all developmental stages of *S. paramamosain* and a higher level of expression was observed in embryo developmental stages (I, III and V), zoea (I), juvenile and adult crab, but lower in embryo (II) and megalops (Fig. 2). Previous studies reported that arthropod SPs and SPHs were predominantly expressed in haemocytes (Lin, Hu, Ho & Song 2006; Vaseeharan, Lin, Ko & Chen 2006; Liu,

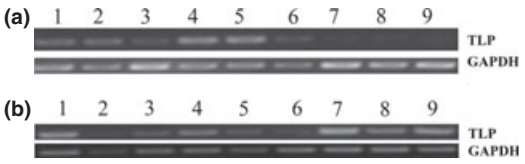

Figure 2 Tissue distribution and developmental expression profile of TLP mRNA in *S. paramamosain*. (a) Tissue distribution analyses by performing semi-quantitative RT-PCR in the tested tissues. Lane 1: eyestalk, 2: heart, 3: stomach, 4: gills, 5: haemocyte, 6: hepatopancreas, 7: nerve, 8: midgut gland, 9: body wall. (b) Developmental expression profile of TLP mRNA in various stages using semi-quantitative RT-PCR analysis. Lane 1: embryo I, 2: embryo II, 3: embryo III, 4: embryo V, 5: zoea I, 6: megalops, 7: haemocyte from juvenile (10 ± 2 g), 8: haemocyte from juvenile (35 ± 5 g), 9: haemocytes from adult crab. The gene of GAPDH is used as an internal control.

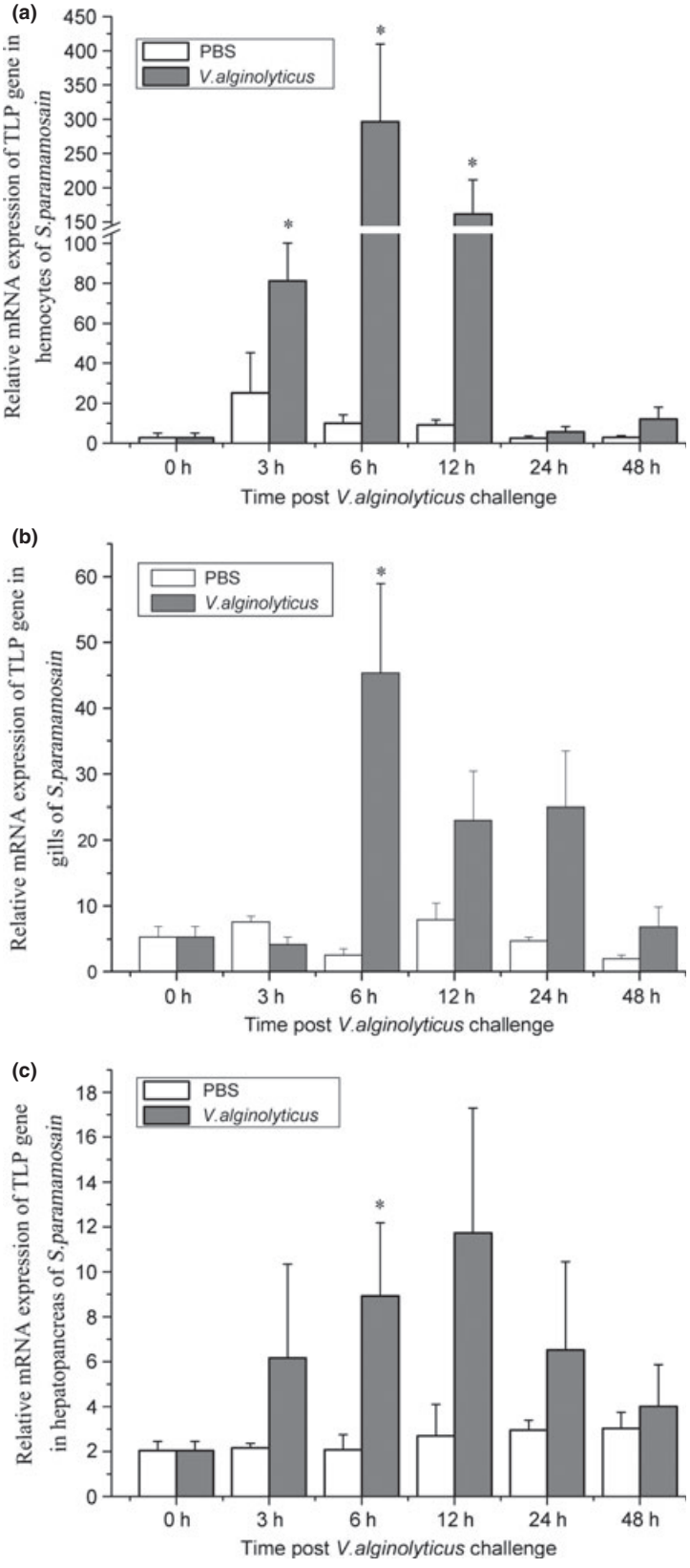

Figure 3 Quantitative real-time RT-PCR analysis of TLP mRNA expression in haemocytes (a), gills (b) and hepatopancreas (c) of *S. paramamosain* injected with PBS (control) and *V.alginolyticus* respectively. Bars indicated mean ± SE ($n = 3$). '*' indicates significant difference at $P < 0.05$.

Chen, Zhang & Wang 2010; Charoensapsri *et al.* 2011; Vaseeharan *et al.* 2011), and also in gills (Sriphaijit, Flegel & Senapin 2007; Liu *et al.* 2010), by which it was thought their roles in host immune defence. Similarly in this study, the mRNA transcripts of TLP were demonstrated in most tested tissues and higher expressed in the haemocytes and gills than that in heart, hepatopancreas, stomach and nerve. Therefore, it was supposed that the TLP might act as an important protease with potential immune function as other known SPHs. Besides, the high level of TLP mRNA expression was observed in eyestalks, suggesting that these proteases might be involved in activity related to other unknown physiological process similar to the *Sp*-SPH from the same species, or like the PtSPH from the swimming crab *Portunus trituberculatus* (Cui *et al.* 2010; Liu *et al.* 2010). The distinct expression pattern was also reported in the studies of EsSPH and EscSP on which the mRNA transcripts of EsSPH were highly increased in the hepatopancreas, whereas EscSP gene was highest expressed in the muscle of Chinese mitten crab *E. sinesis* (Gai *et al.* 2009; Qin, Chen, Qin, Zhao, Zhang, Wu & Li 2010). The discrepancy in gene expression pattern of SPHs in tissues implies that the expression of these proteases is tissue- and species-specific in crustaceans. Moreover, the results on the expression of TLP in different developmental stages revealed that the TLP gene could be not only expressed early in embryos I but also in the haemocytes of juvenile crabs, suggesting that this protease might exert immune reaction throughout the development stage of crabs.

The presumption on TLP involving immune roles was further evidenced by the challenge experiments in the study. The mRNA expression of TLP after *Vibrio alginolyticus* challenge was evaluated using quantitative real-time PCR. A partial fragment of GAPDH gene was amplified via primers GF (5′-ctcca ctggtgccgctaaggctgta-3′) and GR (5′-caagtcaggtcaa ccacggacacat-3′) and served as an internal control for normalization. The TLP expression was remarkably up-regulated at 3, 6 and 12 h in haemocytes, and reached a maximum 30-fold increase over control values at 6 h after *V.alginolyticus* challenge. In addition, its mRNA transcripts were also significantly increased at 6 h in gills, and at 3 h in hepatopancreas respectively (Fig. 3). It is reported that haemocytes and other cell types from the gills and hepatopancreas were involved in cellular and humoral defences in crustaceans (Rowley & Powell 2007). These data led us to hypothesize that the induction of TLP gene might be served as an acute-phase defence molecule against pathogen infection in *S. paramamosain*.

Conclusions

In conclusion, a new trypsin-like serine protease named TLP was identified in *S. paramamosain*. The full-length cDNA sequence and the gene expression pattern of TLP were determined. Its mRNA transcripts were significantly increased with *V. alginolyticus* challenge, suggesting its immune response against bacterial infection. However, this study is an initial step to understand the function of TLP in immune system of *S. paramamosain.*

Acknowledgments

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