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Short communication

Cloning and characterization of a sialic acid binding lectins (SABL) from Manila clam *Venerupis philippinarum*

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ABSTRACT

Sialic acid binding lectin (SABL) is a member of immunoglobulin-like lectins family that are thought to promote cell–cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition. In the present study, the full-length cDNA of SABL was identified from Manila clam *Venerupis philippinarum* (denoted as VpSABL) by cDNA library and RACE approaches. The cDNA of VpSABL consisted of a 5′terminal untranslated region (UTR) of 62 bp, a 3′ UTR of 354 bp with a poly (A) tail, and an open reading frame (ORF) of 588 bp encoding a polypeptide of 195 amino acids with a typical C1q domain in the C-terminus. Multiple alignment analysis indicated that the deduced amino acid of VpSABL shared higher positive to other SABLs and C1q-contained proteins and should be adopted typical 10 β-strand jelly-roll folding topology common to all C1q-TNF family. Spatial expression analysis indicated that mRNA transcript of VpSABL was predominantly detectable in tissues of mantle, hepatopancreas and gill, and to a lesser degree in the tissues of muscle and haemocytes. After challenged by *Vibrio anguillarum*, the mRNA level of VpSABL in overall haemocytes population was recorded by quantitative real-time RT-PCR. VpSABL mRNA was down-regulated in the first 24 h post-infection. Then, the expression level increased to the peak at 72 h and recovered to the original level at 96 h. All these results indicated that VpSABL was involved in the immune response against microbe infection and might be contributed to the recognition of bacterial pathogens.

1. Introduction

Clams, without the benefit of an adaptive immune system, possess an immensely strong innate immune response to counteract the continuous challenge of infection in marine environment [1]. Lectin, the pattern recognition protein (PRP) recognizing and binding to terminal sugars on glycoproteins and glycolipids, is one of the major components in these host-defence systems [2]. It has been isolated and characterized from viruses [3], bacteria [4], fungi [5,6], plants [7,8], and animals [2,9,10]. Among the lectin of invertebrates origin, sialic acid binding lectins (SABL) show attractive attention for their potentially roles in glycobiology or cancer research such as detection, localization, and isolation of sialoglycoconjugates [11]. SABL from American horseshoe crab *Limulus polyphemus* (Limulin) has been successfully commercially available as a cytochemical probe for detection of sialoglycoconjugates in a variety of systems [12].

Invertebrates SABL could also mediate the innate immune response by inducing bacterial agglutination or activation of phagocytes through binding to sialic acid on foreign cells (opsonin activity) [13], for pathogenic microorganisms are known to have evolved the capacity to synthesize or capture sialic acids from their hosts and incorporate these into their own glycoconjugates [14], The SABL from *Cepaea hortensis* could agglutinate human erythrocytes in the presence of the divalent cations calcium, manganese or magnesium and also could interact with all strains of the group B streptococci (*Streptococcus agalactiae*) containing type-specific polysaccharides [15].

As an abundant source for SABL, some species in Mollusca has been reported to contain the protein, such as snails, abalone and Pacific oyster. However, little information is available about the molecular features and immune response against pathogen infection in commercially cultured clam *Venerupis philippinarum*. The main objectives of this study are: (1) to clone the full-length cDNA of SABL from *V. philippinarum* (VpSABL), (2) to examine the tissue expression

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Table 1
Primers used in the study.

Primer	Sequence (5′–3′)	Sequence information
P1	CAACTACGACTGCGGTTCTATGA	3′ RACE primer
P2	ACCGTATCATAACCGTTTCCT	5′ RACE primer
P3	GTTGGACTGGCGTATGTGGCTAT	Full-length verified primer
P4	GGCCACGCGTCGACTAGTACT17	Full-length verified primer
P5	AGATTTGAGCGAGGCACAGACT	Real time primer
P6	ATACCGATGACCGTATCATAACC	Real time primer
P7	CTCCCTTGAGAAGAGCTACGA	Real time actin primer
P8	GATACCAGCAGATTCCATACCC	Real time actin primer
T3	AATTAACCCTCACTAAAGGG	Vector primer
T7	GTAATACGACTCACTATAGGGC	Vector primer
M13-47	CGCCAGGGTT TTCCCAGTCACGAC	Sequencing primer
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing primer

profile of VpSABL in healthy clam, (3) to investigate the expression profile of VpSABL after being infected by *Vibrio* pathogen.

2. Materials and methods

2.1. Clams and bacterial challenge

The clams *V. philippinarum* (7.5–11 g in weight) were purchased from Qingdao, Shandong Province, China. The clams were acclimated for a week before commencement of the experiment. The temperature was held at 20–22 °C throughout the whole experiment. The salinity for the supplied seawater was kept at 30‰. The performance of *V. anguillarum* challenge experiment was according to our previous work [16,17]. There were five replicates for each challenge point and control group.

2.2. Cloning of the full-length cDNA of VpSABL by cDNA library and RACE

The cDNA library was constructed and sequenced as previously described by Ref. [16]. BLAST analysis of all the 3226 EST sequences revealed that one EST of 508 bp was highly similar to the previously identified SABL. Therefore, the EST sequence was selected for further cloning of the full-length cDNA of SABL from *V. philippinarum*. Two gene specific primers, sense primer P1 and reverse primer P2 (Table 1), were designed based on the EST to clone the full-sequence cDNA of VpSABL. PCR amplification to clone the 3′ end of VpSABL was carried out using sense primer P1 and reverse primer T7, while sense primer T3 and reverse primer P2 were used to get the 5′ end of VpSABL. The full-length cDNAs of VpSABL was verified their validity with primer sets of P3 and P4.

2.3. Sequence analysis of VpSABL

The VpSABL gene sequence was analyzed using the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Sequence alignment of VpSABL was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment show program (http://www.biosoft.net/sms/index.html).

2.4. Tissue-specific expression of VpSABL mRNA transcripts

Total RNA from haemocytes, gill, mantle, muscle and hepatopancreas was isolated using the TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed according to Promega M-MLV RT Usage information with the RQ1 RNase-Free DNase (Promega)-treated total RNA (1 μg) as template and oligo (dT) primer. The reactions were incubated at 42 °C for 1 h, terminated by heating at 95 °C for 5 min.

The expression of VpSABL transcript in different tissues was measured by quantitative real-time RT-PCR in Applied Biosystem 7500 fast Real-time PCR System. Gene specific primers P5 and P6 (Table 1) were designed to amplify products of 228 bp. The product was purified and sequenced to verify the PCR specificity. Two clam β-actin primers, P7 and P8 (Table 1) were used to amplify a 121 bp fragment as internal control to verify the successful reverse transcription and to calibrate the cDNA template. The reaction component, thermal profile, and the data analysis were conducted as previously described [17]. All data were given in terms of relative mRNA expression as means ± S.D.

2.5. Temporal expression profile of VpSABL mRNA in haemocytes post V. anguillarum challenge

Haemocytes were selected as candidate tissue for investigating the temporal expression profile of VpSABL challenged by *Vibrio*

```
1   GTTGGGACTAAAAGTATTTGTTGTTGTTGGACTGGCGTATGTGGCTATATTGACAACCGGAG
63  ATGATCAGCCTGATCGATTTGCGTACCATGCACCTTCTGGACAAGATGGTCCGTATGGAA
        M  I  S  L  I  D  L  R  T  M  H  L  L  D  K  M  V  R  M  E
123 ATGAAGATGGAACGAATGGAAGAAGATTTGAGCGAGGCACAGACTAATGTCAACGTGTTT
        M  K  M  E  R  M  E  E  D  L  S  E  A  Q  T  N  V  N  V  F
183 ATAAAGAAACAAACTAAAATACTAGAAAACAAAACAGCCGAACTCGAACTATTAGAAGGA
        I  K  K  Q  T  K  I  L  E  N  K  T  A  E  L  E  L  L  E  G
243 AGAGTCGATCTACCACTAATTGCTTTCAATGCATATAGTCCTATCGACACGAGCCCGGAT
        R  V  D  L  P  L  I  A  F  N  A  Y  S  P  I  D  T  S  P  D
303 ACAAACGAGATCATTATATGGCAAAGTACTCATTTAAATGAAGGAAACGGTTATGATACG
        T  N  E  I  I  I  W  Q  S  T  H  L  N  E  G  N  G  Y  D  T
363 GTCATCGGTATATTCAAGGCACCAGTTTCTGGTCTCTACTACTTTGCTGTACATGTATGT
        V  I  G  I  F  K  A  P  V  S  G  L  Y  Y  F  A  V  H  V  C
423 AACTACAGCAGCCAAGTTTTCCAGTACGCCATTGTACTTGAAAATAACAACATTGCCACG
        N  Y  S  S  Q  V  F  Q  Y  A  I  V  L  E  N  N  N  I  A  T
483 TCGTACAAGTACGATAACAACAACTACGACTGCGGTTCTATGAGTACTTTCACCAAGGTG
        S  Y  K  Y  D  N  N  N  Y  D  C  G  S  M  S  T  F  T  K  V
543 GCAGCCGGACAAAGGGTATGGGTGAGATGTACTAGCGGAAGTACGAGCGCTCTGTTGTGG
        A  A  G  Q  R  V  W  V  R  C  T  S  G  S  T  S  A  L  L  W
603 GAGAGTAGCGGTAGAAGCTCATTCATTGGTGCTCTCATTCATACATAAACAAACATTGAT
        E  S  S  G  R  S  S  F  I  G  A  L  I  H  T  *
663 TAAAAAATGGCTGGCTGTGTAATGGAAAAAAATGGCTGACTTTGACTACCAAACATGTTT
723 CCCTTTGTTATTTTTCATATTAAAGCGAAATGTTGTTAGTTTAAGGTAAAATATCTAAAT
783 AAAAGCATATACAATGTAATTCGTAAAAAACACACACAGAATTTGATTAATTTGATTGAC
843 GTACATGTATTACAGCAAGGTCGCCTGTAATCATTGCATATTTCAATATCAAGGTAGAAA
903 GAGGGGGAACAGGAGTCCTTCATGATAGTTTATATTGAATTGCATTTGTATATCTCAGTC
963 AACCCAT AATAAA AGTATAACAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide sequence (above) and its deduced amino acid sequence (below) of VpSABL. Nucleotides were numbered from the first base at the 5′ end. The canonical polyadenylation signal sequence AATAAA was italics. The asterisk indicated the stop codon.

pathogen. The results were subjected to One-way Analysis of Variance (ANOVA) followed by Tukey HSD test to determine differences in the mean values between treatment and control groups. Significance was concluded at $P < 0.05$. Statistical analysis was performed using SPSS 11.5 for Windows.

3. Results and discussion

As a multivalent carbohydrate-binding protein with the agglutination activity towards erythrocytes, bacteria and other cell, lectin appeared to play a critical role in the host-defence mechanisms of bivalve mollusk both by recognizing and binding to pathogenic microorganisms and by opsonizing for phagocytic haemocytes [18]. For lectin from the economically cultured Manila clam, the agglutination activity of serum and cell lysate was originally found to be significant increase after *Perkinsus* infection [19], revealing that lectin was also an important component in clam host immunity. After that, around five types lectins had been investigated in the species and reported its isolation, induction by infection with protozoan parasite, and EST analysis, such as MCL-1, MCL-3, and MCL-4 [18,20]. MCL-4 was demonstrated to serve as an opsonin through recognition of terminal GalNAc/Gal residues on the

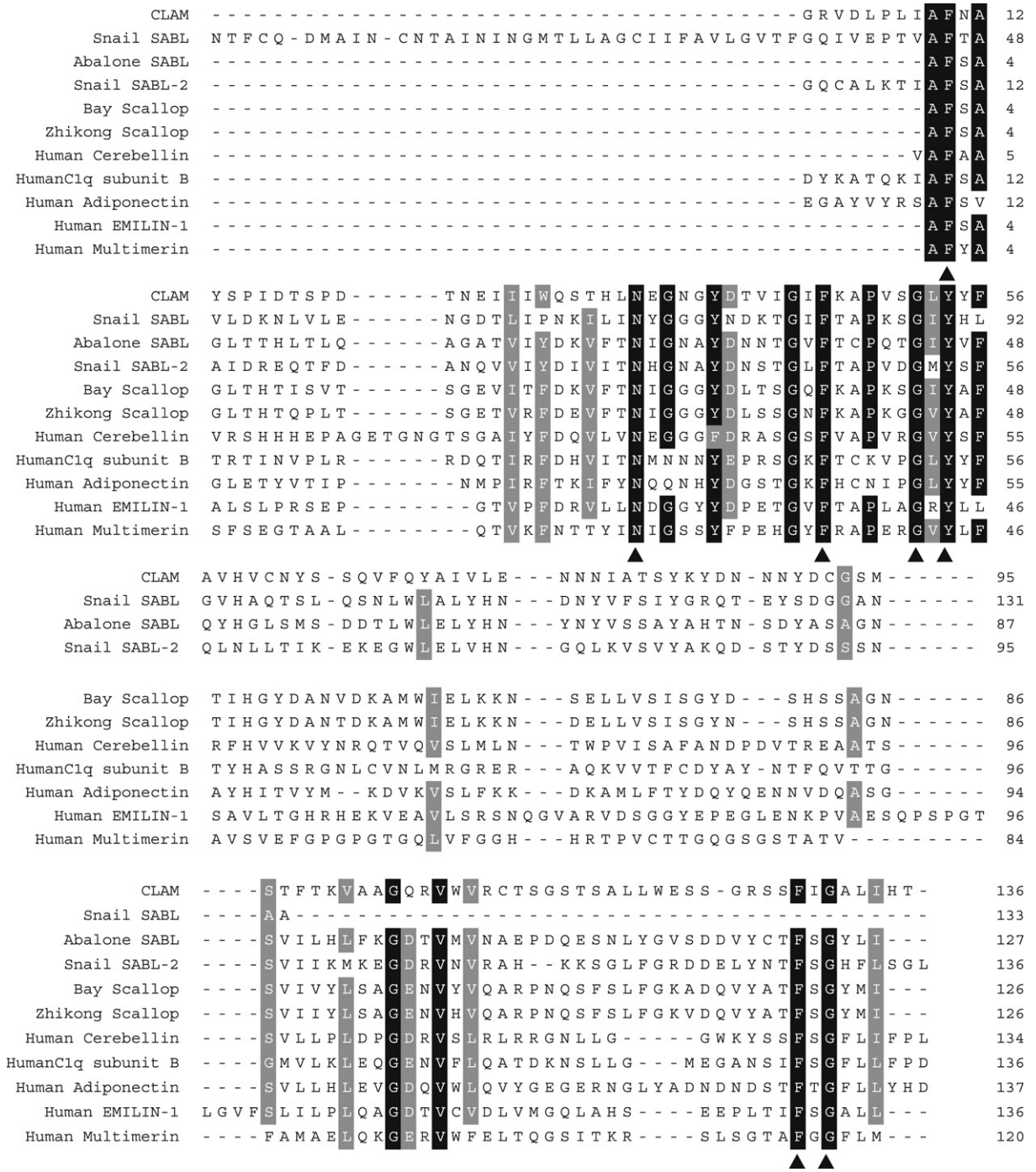

Fig. 2. Multiple alignment of VpSABL with other known SABLs and C1q-contained proteins. Identical amino acids were in white letters with black background, and grey background indicated high levels of amino acid similarity.

parasites [21]. As a ubiquitous lectin in Arthropoda, Mollusca, and Urochordata, sialic acid binding lectin (SABL) attracted much attention for its high affinity to its ligand compared to other counterparts [22]. Although clam SABL had been also identified in previous study [20], the biological property of SABL was rarely investigated to our knowledge.

3.1. cDNA cloning and sequence analysis of VpSABL

Sequencing of clam cDNA library with T3 primer yielded 3226 EST sequences. Blastx analysis revealed that a 508 bp fragment was similar to C1q-contained SABL identified previously. Based on the sequence of this EST, two gene specific primers (P1 and P2) were designed to clone the full-length cDNA of VpSABL, and two fragments of 578 bp and 455 bp were amplified by 3′ RACE and 5′ RACE respectively. A 1004 bp nucleotide sequence representing the complete cDNA sequence of VpSABL was obtained by overlapping the two fragments mentioned above with the EST sequence. The sequence of VpSABL was deposited in GenBank under accession no. GQ384402. The complete sequence of VpSABL cDNA and the deduced amino acids were showed in Fig. 1.

The complete sequence of VpSABL cDNA consisted of a 5′ terminal untranslated region (UTR) of 62 bp, a 3′ UTR of 354 bp with a canonical polyadenylation signal site and a poly (A) tail (Fig. 1), and an open reading frame (ORF) of 588 bp encoding a polypeptide of 195 amino acids. The predicted molecular weight of VpSABL was 22.02 kDa, which was slightly larger than that of molluscan SABLs from snail *Helix pomatia* (16.82 kDa, ABF00124), snail *Cepaea hortensis* (17.34 kDa, CAD83837) and abalone *Haliotis discus discus* (17.87 kDa, ABO26662). The slightly significant difference in molecular mass might be attributed to its different evolution manners in different organisms, which should be further elucidated through genomic sequence analysis.

Blastp analysis indicated that the deduced amino acid sequence of VpSABL shared high homology with other reported C1q-contained proteins, such as 49% positive to sialic acid binding lectin from *H. pomatia* (ABF00124), 47% to type VIII collagen from *Homo sapiens* (AAA62822), 46% to complement C1q tumour necrosis factor-related protein 3 precursor from *Osmerus mordax* (ACO09509) and to C1q-like from *Neoditrema ransonnetii* (BAI40067). The higher positive to other C1q-contained protein and SABL indicated VpSABL should be a novel member of this protein family.

SMART program analysis revealed that VpSABL contained a typical C1q domain from the 60th to the 195th amino acid residues in C-terminus. Multiple alignment of C1q domain showed that C1q domain of VpSABL adopted a typical 10 β-strand jelly-roll folding topology common to all C1q-TNF family (Fig. 2). Eight invariant amino acid residues in human C1q-contained protein were almost totally conserved in VpSABL with exception of F142, in which a synonymous mutation occurred by tryptophan (W) replacement. This mutation was also detected in SABL from snail and abalone. No collagen-like region with Gly-X-Y repeats were scanned in the deduce amino acid of VpSABL, indicating that the protein could hardly form trimers or multimerize those homotrimeric or heterotrimeric subunits to a bouquet structure as C1q did. These phenomena had been validated in other C1q-contained protein in other organisms [15,23]. In snail, purified sialic acid binding lectin exterted its agglutination function through assembling three subunit together. While, the recombinant product had no activity although it could be reacted specifically with native lectin [15].

3.2. Tissues distribution of VpSABL

Semi-quantitative RT-PCR was employed to quantify the expression of VpSABL mRNA in different tissues of Manila clam

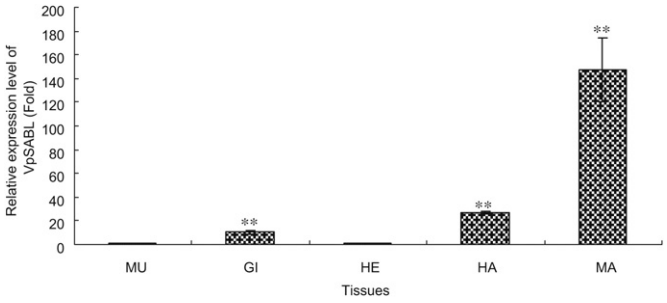

Fig. 3. Tissues expression level of VpSABL measured by quantitative real-time PCR. Each symbol and vertical bar represents the mean ± S.D ($n = 3$).

V. philippinarum. In healthy clams, the VpSABL transcript was found to be constitutively expressed in a wide range of tissues with different expression levels. The expression of VpSABL transcript was predominantly detectable in tissues of mantle, hepatopancreas and gill, and to a lesser degree in the tissues of muscle and haemocytes (Fig. 3). The results were consistent with the temporal expression patterns in other C1q-contained protein. In scallop, CfC1qDC transcript was found to be constitutively expressed in a wide range of tissues. It could be mainly detected in the kidney, mantle, adductor muscle and gill, and to a less degree in the haemocytes [23]. While, bay scallop C1qDC had the highest expression in hepatopancreas compared to other tissues [24]. The highest expression level of VpSABL mRNA in mantle implied that VpSABL might have a significant contribution in preventing microbial reproduction, because mantle was constantly being flushed with seawater that contains pathogen and pollutants.

3.3. VpSABL expression pattern in response to bacteria challenge

It had been reported that over 20 pathogenic microorganisms were known to have the capacity to synthesize or capture sialic acids from their hosts and incorporate these into their own glycoconjugates for successful infection [14]. Therefore, SABL should be finely regulated by pathogenic bacterial challenge. In present study, the temporal expression of VpSABL mRNA in haemocytes after *Vibrio anguillarum* challenge was conducted by qT-PCR with β-actin as internal control (Fig. 4). There was slightly down-regulated in the first 24 h. After that, the expression of VpSABL transcript was obviously up-regulated. At 48 h after challenge, there was a 2.6-fold increase compared with the blank group. At 72 h post-injection, the mRNA expression reached the maximum level and was 4.4-fold higher than that of blank clams. In the end, the expression of VpSABL transcript

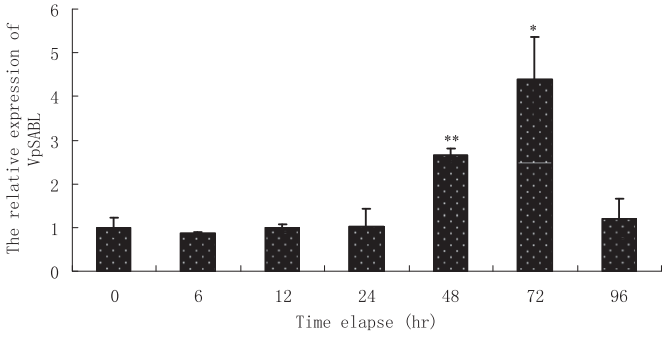

Fig. 4. Time-course expression level of VpSABL transcript in haemocytes after *Vibrio anguillarum* infection measured by quantitative real-time PCR at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. Each symbol and vertical bar represents the mean ± S.D ($n = 5$).

was down-regulated greatly and dropped back to the original level at 96 h post-injection. An unpaired, two-tailed *t*-test with the blank and challenged groups showed a significant difference in VpSABL mRNA expression at 48 h ($P < 0.01$) and 72 h ($P < 0.05$) post-injection.

4. Conclusion and remarks

As a charge pattern recognition molecular, C1q-contained protein were demonstrated to be bind the surface of many Gram-negative bacteria or fungi mediated by LPS and glucan. In scallops, the C1q-contained protein had been validated to be induced by *V. Anguillarum* or *Pichia pastoris* and to specifically bind to LPS from *E.coli*0111:B4 [23,24]. The present results informed us that VpSABL could function as a pattern recognition receptor in innate immunity of clam and perhaps be involved in the recognition of invading Gram-negative pathogen. Further work should be emphasized on the specifically binding activity of the recombinant VpSABL to fully elucidate its role in clam defence system.

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