The antifungal activity of a thaumatin-like protein from oyster *Crassostrea gigas*

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Abstract

In the present study, a thaumatin-like protein (CgTLP) was identified from the oyster *Crassostrea gigas*. The full-length cDNA of CgTLP was of 913 bp with a 5' untranslated regions (UTR) of 98 bp, a 3' UTR of 80 bp, and an open reading frame (ORF) of 735 bp encoding a polypeptide of 244 residues. The CgTLP gene was expressed ubiquitously in mantle, gonad, hemocytes, hepatopancreas, gill, and adductor muscle with the higher expression levels in adductor muscle, hemocytes, and hepatopancreas. Immunofluorescence assay indicated that CgTLP was mainly distributed in the cytoplasm of hemocytes. The mRNA expression levels of CgTLP in hemocytes were significantly up-regulated after the stimulations with mannan (13.69-fold, p < 0.05), Pichia pastoris (8.85-fold, p < 0.05) and polyinosinic-polycytidylic acid (3.62-fold, p < 0.05), but did not change significantly after stimulations with lipopolysaccharide, peptidoglycan, and Vibrio splendidus. The recombinant CgTLP protein (rCgTLP) significantly inhibited the proliferation of P. pastoris (p < 0.05), while no inhibition towards Staphylococcus aureus and V. splendidus. rCgTLP also displayed obvious β-1,3-glucanase activity, while no enzymatic activity towards chitin. These results collectively indicated that CgTLP was a homologue of TLP, which might play a vital role in defending against fungal infection in C. gigas.

Key Words: *Crassostrea gigas*; Thaumatin-like protein; Antifungal activity; Immune response

Introduction

Thaumatin was firstly isolated from the seeds of plant *Thaumatococcus danielli* for its exceptionally potent sweet taste (Noh et al., 2016). Subsequently, the proteins with similar amino acid sequences were identified in most of the plant species, which were termed as thaumatin-like proteins (TLPs) (Breiteneder et al., 2000). TLPs are low molecular-weight (20-26 kDa) proteins containing one thaumatin (THN) domain with sixteen conserved cysteine (Cys) residues. The THN domain is peculiar to the thaumatin-like protein family. The Cys form eight intra-molecular disulfide bonds to stabilize the proteins under extreme pH and temperature (Hegde et al., 2014). Most plant TLPs exhibit antifungal activity against a range of pathogenic and nonpathogenic fungi (Cao et al., 2016; Wang et al., 2017), and they are up-regulated in response to a variety of fungal infections and stress (Hegde et al., 2014). Their activities against pathogenic microorganisms are related to the activities of β-1,3-glucanase (Menu-Bouaouiche et al., 2003), glucose polymers depredating (Liu et al., 2010), chitinase (Yasmin et al., 2017), and xylanase (Fierens et al., 2007), as well as α-amylase inhibiting properties (Franco et al., 2002). Meanwhile, TLPs have also been reported in fungi, such as *Irpex lacteus* (Garcia-Casado et al., 2000) and *Cryptococcus neoformans* (Sakamoto et al., 2006) with emphases on gene structure and activities (Blouin et al., 2018). In addition, TLPs have also been reported to exhibit membrane-permeabilizing activity (Meng et al., 2017). However, the detailed mechanisms of their antifungal action are still not completely understood.

With the advances in biological technology and

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genome information, TLPSs have also been found in nematode Caenorhabditis elegans (Kitajima et al., 1999), desert locust Schistocerca gregaria (Brandazza et al., 2004), Coleoptera Diaprepes abbreviatus, Hymenoptera Lysiphlebus, and Hemiptera Toxoptera citricida (Shatters et al., 2006). For marine animals, the information about TLPSs are still very limited, and only the predicted TLPSs from C. gigas (XP_011414138.1), Crassostrea virginica (XP_02233236.1), and Mizunophopeten yessoensis (OWF35002.1) have been referred in NCBI (https://www.ncbi.nlm.nih.gov/) based on genomic sequencing.

The Pacific oyster C. gigas is one of the most important cultured mollusk species in the world, and it contributes greatly to the economic development of the global aquaculture (Yang et al., 2017). Because of its economic and ecological importance as well as biological characteristics, C. gigas is becoming a model to investigate molluscan biology, development, innate immunity, and stress adaptation (Guo et al., 2015). As a sessile marine animal living in estuarine and intertidal regions, C. gigas has to face the infections of various pathogens including bacteria, protozoans, fungi, and viruses (Paillard et al., 2004; Guo et al., 2015). Pathogenic fungi, such as Monilia, Ostracoblabe impexa, Siroplidiu zoophthorum, Democystidium marinus, and Lagendidium, can cause more than 95% mortality of the cultured Crassostrea virginica in summer (Chen et al., 2007). The characterization of antifungal molecules would benefit the knowledge of the marine animals immune defense mechanism (Franco et al., 2002; Liu et al., 2010; Yasmin et al., 2017). In the present study, a novel homologue of CgTLP was identified from oysters C. gigas (designated as CgTLP). The mRNA expression levels of CgTLP in hemocytes post immune stimulation and its antimicrobial activity were investigated. Moreover, the β-1,3-glucanase and chitinase activities of the recombinant CgTLP protein were detected in vitro, which would provide more information to understand the immune defense roles of CgTLP in C. gigas.

Materials and methods

Animals, microorganisms, and drugs

Oysters Crassostrea gigas with an average shell length of 12.0 cm were sampled from a local farm in Dalian, China, and maintained in aerated seawater at approximately 20 °C and salinity of 30‰. For the analysis of CgTLP mRNA distribution, six tissues including gonad, gill, mantle, adductor muscle, hemocytes and hepatopancreas from nine oysters were collected as three parallels. The tissues were kept in Trizol reagent at -80 °C for RNA extraction.

Four hundred and twenty oysters were randomly selected and separated into seven groups for fungus, bacteria and pathogen associated molecular patterns (PAMPs) stimulations to reveal the mRNA expression of CgTLP. Fungus P. pastoris, bacterium V. splendidus and four different PAMPs were used for immune stimulations. The oysters in the treatment groups (80 oyster in each group) individually received an injection of 100 μL live V. splendidus (1×10^8 CFU/mL in PBS), P. pastoris (1×10^8 CFU/mL in PBS), LPS, PGN, Poly (I:C) and MAN, respectively. The rest oysters in the control group received an injection of 100 μL PBS. Nine oysters were randomly sampled from each group at 0, 3, 6, 12, 24, 48 and 72 h after injection and divided into three parallels. The hemocytes collected from three oysters were pooled together as one sample. There were three parallels for each time point. The hemocytes were kept in Trizol reagent at -80 °C for RNA extraction.

RNA isolation and cDNA synthesis

The total RNA was obtained from tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen, USA) and quantified by Nanodrop 2000 (Thermo Scientific, USA). The first strand cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The cDNA mix was diluted 50-fold and stored at -80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (qRT-PCR).

The gene cloning of CgTLP

The DNA fragment of CgTLP (GenBank accession No. XM_011415836.2) (Zhang et al., 2012) was amplified using ExTaq DNA polymerase (TaKaRa, Japan). The information of the primers used in this study was listed in Table 1. The PCR products were cloned into the pMD19-T (TaKaRa, Japan) and verified by nucleotide sequencing in both directions.
**Table 1** Primers used in this study

<table>
<thead>
<tr>
<th>Primer purpose</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Clone primers</td>
<td>CgTLP-5’-F</td>
<td>TTAACATTGTGGAGATAAAGAACT</td>
</tr>
<tr>
<td></td>
<td>CgTLP-3’-R</td>
<td>TGAAAGTATTTCAACAAATTGTAAT</td>
</tr>
<tr>
<td></td>
<td>CgTLP-ORF-F</td>
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<td></td>
<td>CgTLP-ORF-R</td>
<td>TTATCCACAGAGACGACATC</td>
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<tr>
<td>qRT primers</td>
<td>CgTLP-qRT-F</td>
<td>GAAGGGCAATCGGAGAGCATA</td>
</tr>
<tr>
<td></td>
<td>CgTLP-qRT-R</td>
<td>GGGCGTATGAAAGTTGGGA</td>
</tr>
<tr>
<td></td>
<td>CgEF-qRT-F</td>
<td>CTCCACCCACATCACCCT</td>
</tr>
<tr>
<td></td>
<td>CgEF-qRT-R</td>
<td>GGATTTCCTTACGGACAG</td>
</tr>
<tr>
<td>Recombination primers</td>
<td>CgTLP-recombinant-F</td>
<td>GGAATTCCATATGCAAGAATCCATTAGAAAC</td>
</tr>
<tr>
<td></td>
<td>CgTLP-recombinant-R</td>
<td>CCGCTCGAGTCCACAGAAGACGACATC</td>
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**Bioinformatics analysis of CgTLP**

The amino acid sequence of CgTLP was analyzed using the Sequence Manipulation Suite (http://www.bio-soft.net/sms/), and domains were predicted by using the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/). The three-dimensional models of TLPs were built by homology modeling with the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/). The amino acid sequences of TLPs from other species were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) database. The Clustal x1.81 and the Sequence Manipulation Suite (http://www.bio-soft.net/sms/) were used to perform the multiple sequence alignment for TLPs. A phylogenetic tree was constructed by the neighbor joining (NJ) method using the software of MEGA 6.0.6. The reliability of the branching was tested by using bootstrap re-sampling (1000 pseudo-replicates).

**Quantitative real time PCR analysis (qRT-PCR) of the mRNA expression**

The relative mRNA expression levels of CgTLP were measured by SYBR Green fluorescent qRT-PCR, which was performed with an ABI 7500 Real-time Thermal Cycler (Applied Biosystems, USA) according to the manufacturer’s instruction with SYBR Premix Ex Taq (Tli RNaseH Plus) (TaKaRa, RR420A). A pair of specific primers, CgTLP-qRT-F and CgTLP-qRT-R (Table 1), was used to amplify a fragment of 202 bp. The C. gigas elongation factor (CgEF, NM_001305313.2) fragment amplified with primers CgEF-qRT-F and CgEF-qRT-R (Table 1) was employed as the internal control (Zhang et al., 2012). The mantle and the time of 0 h were used as the reference group, respectively. The mRNA expression levels of CgTLP were determined by 2ΔΔCt method (Livak et al., 2001). All the data were given in terms of relative mRNA expression as mean ± S.D. (N=3).

**The expression and purification of recombinant CgTLP**

The ORF fragment of CgTLP without signal peptide sequence was cloned with primers CgTLP-recombinant-F and CgTLP-recombinant-R (Table 1) with an Nde I and Xho I site at their 5' end, respectively. After cloned into pMD19-T simple vector (TaKaRa, Japan) and transformed into E. coli Trans50, the target fragment was obtained by completely digestion with restrictive enzymes Nde I and Xho I, and subsequently cloned into the Nde I/Xho I sites of expression vector pET-28a (Novagen, USA). The recombinant plasmids pET-28a-CgTLP were transformed into E. coli BL21 (DE3) (TransGen, China) for prokaryotic expression of CgTLP. The recombinant proteins of CgTLP (rCgTLP) were purified by Ni2+ chelating sepharose column (Sangon Biotech, Canada) and dialysed with the dialysate. The purified rCgTLP was examined by 15% SDS-PAGE, and visualized with Coomassie Bright Blue R250. The purified protein was quantified by Bradford Protein Assay Kit (Beyotime, China) and stored at -80 °C.

**Preparation of polyclonal antibodies, western blotting, and subcellular localization of CgTLP in hemocytes**

Three six-week-old female mice were immunized with rCgTLP protein to generate polyclonal antibody as the method described in the previous report (Sun et al., 2014). In short, 300 μL of recombinant protein (about 200 μg) was mixed with an equal volume of Freund’s complete adjuvant, and injected into the abdomen of mice. Two weeks later, three immunizations were conducted weekly with an equal volume of Freund’s incomplete adjuvant. One week after the fourth immunization, the serum was collected.

rCgTLP was electrophoretically transferred onto a piece of nitrocellulose transfer membrane after a 15% SDS-PAGE. After blocked in TBS-T (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.05% Tween
The hemolymph was collected from healthy oysters with the equal volume of anti-aggregation solution (Zhang et al., 2014). After washed with sterile seawater for three times, the hemocytes were seeded into cell culture dishes (NEST, USA), and incubated in a humidifying box at RT for one hour. The hemocytes were fixed by 4% paraformaldehyde for 20 minutes, and treated with 0.5% Triton X-100 (prepared with PBS) for 20 minutes to permeabilize the cell membrane. After washed with PBS for three times, the samples were blocked with 20 μL of 3% BSA at RT for 30 minutes, and then incubated with polyclonal antibody of rCgTLP diluted with 3% BSA in the ratio of 1:500 at 37 °C for one hour. The hemocytes were incubated with Alexa Fluor® 488 (Life technologies, USA) labeled rabbit anti-rat secondary antibody (1:1000) at 37 °C for 30 minutes. Then 4',6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, China) was added at the ratio of 1:1000 and incubated for five minutes to dye the nucleus. After washed three times with TBS-T, hemocytes were observed with an LSM 710 laser confocal scanning microscope (Carl Zeiss Jena, Germany).

The assay of microbe growth suppression activity of rCgTLP

The antimicrobial activities of rCgTLP against gram-positive bacterium (S. aureus), gram-negative bacterium (V. splendidus) and fungus (P. pastoris) were determined according to the method described in previous report (Li et al., 2015). S. aureus, V. splendidus and P. pastoris were grown in LB medium 37 °C for 20 hours, 2216E medium 28 °C for 20 hours, YPD medium 28 °C for 24 hours to mid-logarithmic phase, respectively. The cultures were centrifuged at 800 g, 4 °C for 10 minutes, and the microorganism pellets were re-suspended with accordingly medium at the concentration 10⁵ CFU/mL. Fifty microliter of gradient diluent rCgTLP (protein concentrations at 0.500 mg/mL, 0.250 mg/mL, 0.125 mg/mL, and 0.000 mg/mL in PBS, respectively), 50 μL microorganism resuspension, and 1000 μL of accordingly medium were added into a 96-well microtiter plate, respectively, and the plates were placed an ELISA reader at 28 °C with a shake uninterrupted. OD₅₆₀ was measured every one hour to detect the growth of bacteria and fungi. Accordingly mediums were used as blank controls. There were three replicates for each group.

The phylogeny of Cg TLP

Multiple sequence alignment was performed on the basis of the deduced amino acid sequences of CgTLP and some other TLPs downloaded from NCBI. The deduced amino acid sequence of CgTLP shared higher similarity with TLPs from other organisms, such as 94% similarity with thaumatin-like proteins from Solarrbio, China. After washed with sterile seawater for three times, the samples were blocked with 20 μL of 3% BSA at RT for 30 minutes, and then incubated with polyclonal antibody of rCgTLP. Briefly, 100 μL of reagent 1 was added to 100 μL rCgTLP (protein concentrations were 0.500 mg/mL) in a centrifuge tube. The same volume of distilled water was employed as negative control. After incubated at 37 °C for one hour, 600 μL of reagent 2 was appended to the tube and incubated at 100 °C for five minutes. Two hundred microliter of reaction mixture was added to a 96-well microtiter plate, which was measured absorbance at 540 nm in an ELISA reader. Each experiment was repeated in triplicate.

The chitinolytic activity of rCgTLP was measured with a Chitinase Assay Kit (BC0820, Solarbio, China) according to the instruction. In brief, 200 μL extracting solution and 400 μL reagent 1 was added to 400 μL rCgTLP (protein concentrations were 0.500 mg/mL), and 600 μL extracting solution was added to 400 μL rCgTLP (protein concentrations were 0.500 mg/mL) in a centrifuge tube as negative control. The tube was incubated at 37 °C for one hour followed by centrifugation at 5000 rpm, 4 °C for ten minutes. Two hundred microliter of reagent 2 was added to the reaction mixture followed by incubation at 100 °C for seven minutes. Two hundred microliter of reagent 3 and 400 μL of reagent 4 were added to the tube followed by incubated at 37 °C for 15 minutes. Two hundred microlitre reaction mixture was added to a 96-well microtiter plate, and the absorbance at 585 nm was measured in an ELISA reader. Each experiment was repeated in triplicate.

Statistical analysis

The data were analyzed by using SPSS 18.0 and expressed as means ± standard deviation (N=3). The statistically significant differences among groups were designated at p < 0.05 by one-way analysis of variance (ANOVA) (labeled with *).

Results

The molecular feature, domain and spatial structure of Cg TLP

The coding sequence of CgTLP was retrieved from NCBI (XM_011415836.2). The complete cDNA of CgTLP was of 913 with a 98 bp 5’ untranslated regions (UTR), an 80 bp 3’ UTR and an open reading frame (ORF) of 735 bp (Fig. 1A). The ORF encoded a putative polypeptide of 244 amino acid residues with a predicted molecular weight of 25.97 kDa and theoretical isoelectric point of 7.84. There was a signal peptide (from M₁ to G²⁵) in the deduced amino acid sequence of CgTLP, and a classic SMART THIN domain of TLP protein family from G²³ to G²⁴⁴ (Fig. 1A and Fig. 1B).
Fig. 1 The molecular feature, domain and spatial structure of CgTLP. A: The cDNA and amino acid sequences of gene encoding CgTLP from C. gigas. The nucleotides and amino acids were numbered along the left margin. The putative signal peptide was underlined. The initiation codon and termination codon were boxed in red. B: The structural domain of CgTLP predicted by SMART. The red area represented the signal peptide. C: The spatial structure of CgTLP predicted by SWISS-MODEL program.

protein from C. virginica (XP_022333236.1), 51% similarity with pathogenesis-related protein 5 like protein from M. yessoensis (XP_021343380.1), 49% similarity with Arabidopsis thaliana pathogenesis-related protein 5 (NP_177641.1) and 37% similarity with Rhizoctonia solani 123E pathogenesis-related protein PR5K (KEP50242.1), respectively (Fig. 2). In the phylogenetic tree, 28 TLP proteins from C. elegans, S. gregaria, Aleuroglyphus ovatus, M. yessoensis, A. thaliana and R. solani 123E et al. were gathered separately as three major distinct branches including fungi, Plantae, and animalia. There were three distinct clades in the branch of animalia, nemathelminthes, molluscs and arthropods. CgTLP was firstly clustered with TLP from C. virginica and PR-5 from M. yessoensis to form molluscan branch, which was a sister branch to nemathelminthes and arthropods (Fig. 3).

The distribution of CgTLP in different tissues

The mRNA transcripts of CgTLP were detected in all the examined tissues, including mantle, gonad, hepatopancreas, hemocytes, adductor muscle, and gill (Fig. 4). The highest mRNA expression level of CgTLP was detected in adductor muscle, which was 64.88-fold (p < 0.05) of that in mantle. The mRNA expression levels of CgTLP in hepatopancreas (45.64-fold, p < 0.05) and hemocytes were also significantly higher (34.58-fold, p < 0.05) than that in mantle, respectively. Nevertheless, the mRNA expression levels of CgTLP were relatively lower in gonad and gill, which were 2.46-fold (p < 0.05) and 1.13-fold (p > 0.05) of that in mantle, respectively (Fig. 4).

The mRNA expression pattern of CgTLP in hemocytes after different immune stimulations

The temporal mRNA expression of CgTLP in oyster hemocytes was examined at 0, 3, 6, 12, 24, 48 and 72 h after LPS, PGN, Poly (I:C), or MAN stimulation, respectively. Compared with PBS control group, the mRNA expression level of CgTLP in hemocytes was significantly increased at 12 h (6.56-fold, p < 0.05), maintained higher levels from 24 h to 48 h, and then peaked at 72 h (13.69-fold, p < 0.05) after MAN stimulation. The mRNA expression level of CgTLP in hemocytes was significantly up-regulated to the peak level at 6 h (3.62-fold of the control group, p < 0.05) and then recovered to the original level at 12-72 h (p > 0.05) after Poly (I:C) stimulation (Fig. 5A). However, there was no significant change of CgTLP mRNA expression in hemocytes after LPS or PGN stimulation (p > 0.05) (Fig. 5A).

The temporal change of CgTLP mRNA expression levels in hemocytes were also monitored after live V. splendidus or P. pastoris stimulation. The mRNA expression of CgTLP in hemocytes was significantly up-regulated to 5.45-fold (p < 0.05) at 12 h post P. pastoris challenge, and then continuously
Fig. 2 Multiple alignment of CgTLP with other TLP family proteins deposited in GenBank. The same amino acids were shaded in dark and similar amino acids were shaded in grey. Species and gene accession numbers are as follows: *Crassostrea gigas* (XP_011414138.1), *Crassostrea virginica* (XP_022333236.1), *Mizuhopecten yessoensis* (OWF35002.1), *Schistocerca gregaria* (AA97603.1), *Acyrthosiphon pisum* (NP_001313585.1), *Caenorhabditis briggsae* (CAP30301.1), *Arabidopsis thaliana* (NP_001313585.1), *Theobroma cacao* (EOY24665.1), *Rhodotorula* (KWU43254.1), and *Folsomia candida* (XP_021955579.1).

increased to the peak level at 72 h (8.85-fold, \( p < 0.05 \)) (Fig. 5B). There was no significant change of CgTLP expression (\( p > 0.05 \)) after the injection of *V. splendidus* during the whole experimental process (Fig. 5B).

The polyclonal antibody of CgTLP and its subcellular localization in hemocytes

The recombinant plasmid pET-28a-CgTLP was transformed and expressed in *E. coli* BL21 (DE3). After Isopropyl β-D-Thiogalactoside (IPTG) induction, the whole cell lysate was analyzed by 15% SDS-PAGE, and an obvious band about 25.0 kDa was observed (Fig. 6A). The band was highly consistent with the predicted molecular mass (25.97 kDa) of CgTLP. The purified rCgTLP was injected into the mice to obtain the immune sera. A clear primary reaction band was revealed by western blotting assay, indicating the high recognition specificity of the polyclonal antibody against CgTLP (Fig. 6B).

The localization of CgTLP protein in the hemocytes was analyzed by immunofluorescence assay with the polyclonal antibody acquired from
**Fig. 3** The phylogenetic tree of CgTLP and TLP homologues of other species. The phylogenetic tree analysis of the amino acid sequences of TLPs was constructed by the neighbor-joining method and was bootstrapped 1000 times using the MEGA 6.06 software. The CgTLP was marked by a red triangle.

**Fig. 4** The mRNA expression levels of CgTLP in different tissues of adult oyster. The CgEF (elongation factor) gene expression was used as an internal control and mantle sample was used as the reference sample. Each value was shown as mean ± S.D. (N = 3), and bars with different characters were significantly different (p < 0.05).
Fig. 5 The mRNA expression patterns of CgTLP in hemocytes. A: Temporal mRNA expression of CgTLP in hemocytes after LPS, PGN, Poly (I:C) and MAN stimulation at 0, 3, 6, 12, 24, 48, and 72 h, respectively. B: Temporal mRNA expression of CgTLP in hemocytes after V. splendidus and P. pastoris stimulations at 0, 3, 6, 12, 24, 48, and 72 h, respectively. The relative expression values were shown as mean ± SD (N=3). Asterisk indicated significant difference from control (p < 0.05).

The activity of rCgTLP to inhibit the growth of microbes

The microbe growth inhibition activity of rCgTLP was assessed by detecting the microbe growth curve. Within the detection time (12 hours) and concentration range (0.000-0.500 mg/mL), there was no obvious growth suppression activity against S. aureus and V. splendidus (p > 0.05) (Fig. 8A and 8B). However, the growth of P. pastoris in 0.250 mg/mL and 0.500 mg/mL rCgTLP treatment groups was significantly suppressed (p < 0.05), compared with that in the blank group from 7 h to 16 h post treatment. Moreover, the growth level of P. pastoris in the 0.500 mg/mL rCgTLP treatment group was significantly lower (p < 0.05) than that in the 0.250 mg/mL rCgTLP treatment group during 7-16 h (Fig. 8C). The antifungal effect of rCgTLP to P. pastoris was strengthened with the increase of protein concentration.

Fig. 6 SDS-PAGE and western blotting analysis of rCgTLP protein. A: SDS-PAGE of rCgTLP protein. M: Protein molecular standard (kDa); Lane 1: Negative control for rCgTLP; Lane 2: IPTG induced rCgTLP; Lane 3: Purified rCgTLP. B: Western blotting of rCgTLP protein. Lane 4: Western blotting of rCgTLP. M: Pre-dyed protein molecular standard (kDa)
**Fig. 7** Fluorescent microscopy analysis of CgTLP (green) distribution in hemocytes of *C. gigas*. A: The hemocytes were observed in light field; B: The CgTLP was labeled green by polyclonal antibody of CgTLP and Alexa Flour 488 labeled second antibody in single section; C: The nuclei of hemocytes were stained blue by DAPI in single section; D: The merged chart. The scale bar was 20 μm.

**The β-1,3-glucanase and chitinase activities of rCgTLP**

In the β-1,3-glucanase activity assay, the OD_{450} of experimental group with rCgTLP (0.250 mg/mL) was significantly higher (4.10-fold, \( p < 0.05 \)) than that in control group without rCgTLP (Fig. 9A). However, the OD_{450} of experimental group with rCgTLP showed no significant change compared with control group in the chitinase activity assay (\( p > 0.05 \)) (Fig. 9B).

**Discussion**

Thaumatin-like proteins (TLPs) have been reported to be involved in host responses against a wide range of stresses, such as pathogen/pest invasion, wounding, drought, and cold hardness (Leone et al., 2006). In this study, a homologue of TLP with a theoretical molecular weight of 25.97 kDa was identified in oyster *C. gigas*. It was consistent with the previous reports that TLPS are about 200 amino acids (Zhang et al., 2012) with the molecular weight of 21-26 kDa (Petre et al., 2011). There was a THN domain in CgTLP contained, which was an exclusive domain of TLP families (Leone et al., 2006). The deduced amino acid sequence of CgTLP shared high similarity with other known TLPS, such as the thaumatin-like protein from *C. virginica* (XP-022333236.1) and the pathogenesis-related protein 5 from *M. yessoensis* (OWF35002.1). The putative mature polypeptide of CgTLP was of cysteine-rich, which was consistent with the previous report that most TLPS contained 16 conserved cysteine residues (Fierens et al., 2009). These cysteine residues could be paired to form disulfide bonds, which were expected to be a relatively stable
The mRNA expression of TLPs could be induced by the presence of fungal molds (such as MAN, chitin, and β-1,3-glucan) and fungi Puccinia triticina (Zhang et al., 2017). TLPs could also be induced by some kind of virus or viral simulacrum (Kinkema et al., 2000). In this study, the mRNA expression of CgTLP in hemocytes was up-regulated after Poly (I:C) stimulation from 3 h (1.51-fold change, \( p > 0.05 \)) and peaked at 6 h (3.62-fold change, \( p < 0.05 \)). These results demonstrated that the CgTLP might also play an important role in the immune response to virus infection in C. gigas. No significant changes were observed in the mRNA expression levels of CgTLP among control group, V. splendidus group, LPS group, and PGN group during the whole experimental process, indicating CgTLP could not be induced by the presence of bacteria or the bacterial simulacrum. It was consistent with the findings that bacteria could not induce the mRNA expression of TLP in Pinus thunbergii (Solano et al., 2013). Similar to plant TLPs, CgTLP protein was mainly distributed in the cytoplasm of the oyster hemocytes, indicating that the mature CgTLP might perform as cytoplasmic protein to play important roles in the innate immune response of C. gigas against fungal and viral infection, and resistance to stresses (Gómez-Casado et al., 2014).

TLP family members have been reported to inhibit several kinds of fungi including Taphrinomycotina, Saccharomycotina, and Basidiomycota (Anžlovar et al., 2003; Jung et al., 2005; Hayashi et al., 2014). In the present study, the antifungal and antibacterial activities of rCgTLP were investigated in vitro. rCgTLP could inhibit the growth of P. pastoris, which was in line with previous reports that most of plant TLPs exhibited antifungal activities (Liu et al., 2010). Interestingly, rCgTLP could not inhibit the growth of gram-positive bacteria, S. aureus and gram-negative bacteria, V. splendidus. Similarly,
some plants TLPs, such as *Oryza sativa* TLP, *A. thaliana* TLP, and *Pinus monticola* TLP, also did not display inhibitory effect on bacteria (Futamura et al., 2005; Zhang et al., 2007; Singh et al., 2017). These results suggested that *Cg* TLP might exert selective inhibitory activity to fungi.

It has been reported that the inhibition activities of TLPs to microorganisms owe to their β-1,3-glucanase or chitinase activities (Menu et al., 2003). For instance, TLP from *Musa acuminata* could hydrolyze β-1,3-glucan (Menu-Bouaouiche et al., 2003), and TLP from *Picea glauca* possessed chitinase activity (Beleneva et al., 2011). Some TLPs even did not possess enzymatic activity (Hernández-Blanco et al., 2007; Borad et al., 2008; Miura et al., 2013), which exert their antifungal activities by permeabilizing cell membranes (Brandazza et al., 2004; Meng et al., 2017). In order to explore the possible antifungal mechanism of *Cg*TLP, the β-1,3-glucanase and chitinase activities of r*Cg*TLP were evaluated in vitro. r*Cg*TLP displayed obvious β-1,3-glucanase activity, but no chitinase activity, suggesting that the antifungal activity of r*Cg*TLP might be ascribed to its β-1,3-glucanase activity rather than chitinase activity. This study expanded the knowledge on the functions of TLP in the antifungal immune defense system in oyster, and threw light on the evolutionary of TLPs.

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