Cloning, Expression, Purification, And Assessment Of Vibrio Parahaemolyticus-Agglutinating C-Type Lectin From Litopenaeus Vannamei Fused To GST Tag

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Abstract: C-type lectins (CTLs) are a group of Pattern Recognition Receptors (PRR) that contains at least one carbohydrate recognition domain. CTLs play important roles in innate immunity of invertebrates by identifying and eliminating invading microorganisms. Recent studies have shown that a new type of CTL, LvLdrlCTL (Litopenaeus vannamei Low-Density Lipoprotein Receptor C-type Lectin) helps shrimp to resist to Acute Hepatopancreatic Necrosis Disease (AHPND) caused by Vibrio parahaemolyticus, which is currently no effective preventing and treatment available. LvLdlrCTL protein was fused with GST by cloning the LvLdlrCTL gene from Litopenaeus vannamei into pGEX-5X-1 vector and being expressed by host strain E. coli BL21 (DE3). The expression of GST-tagged LvLdlrCTL protein confirmed by SDS-PAGE and Western blot probed with GST antibody. The purified protein then was used for in vitro binding and agglutinating assay with V. paraheamolyticus. The result showed that GST-tagged LvLdlrCTL protein expressed in soluble form. Protein purification step could reach the purity above 85%. Lastly, purified protein could bind to and agglutinate AHPND-causing V. parahaemolyticus. This initial trial created the groundwork for expression and purification in large quantities of LvLdlrCTL. The recombinant protein could be used for further developing on anti-AHPND in white leg shrimps.

Keywords: AHPND, C-type lectin, Litopenaeus vannamei, LvLdlrCTL, recombinant protein, Vibrio parahaemolyticus

1. INTRODUCTION

With nearly 700,000 ha of cultivated land, favorable domestic and global markets, along with the transformation of crop structure, shrimp farming industry has made Vietnam the fourth largest shrimp-export country in the world, valued at billions of dollars annually [1]. Furthermore, the development of shrimp industry has created a job market for millions of Vietnamese workers. In addition to the huge profits brought by its potential, the shrimp farming industry has faced many challenges and difficulties in recent years. One of the leading challenges is shrimp diseases such as white spot disease, yellow head disease, and a newly emerging disease named acute hepatopancreatic necrosis disease (AHPND) [1]. According to the Directorate of Fisheries' report in 2012; 100,776 ha in Vietnam were damaged by different diseases. In particular, AHPND is the most affected agent, causing damage to millions of dollars [2], and has been a major threat to shrimp farming in Southeast Asia in the recent years. Since the disease first reported in China in 2009, the epidemic quickly spread to other areas. In Vietnam, AHPND was first documented in 2010 [3]. The disease occurs in L. vannamei about 10 days after stocking [4]. Shrimp die quickly, about 3-7 days after infection [5]. AHPND spreads very quickly and the mortality rate can be up to 100% in the whole crop. Similar to other invertebrates, shrimp relies on innate immunity to protect itself from infectious agents [6]. The innate immune response against invading agents is triggered by immune recognition [7]. A large number of molecules encoded by germ cells is called Pattern Recognition Receptors (PRR), capable of identifying molecular patterns related to pathogens. Pathogen Associated Molecular Patterns (PAMP) are conserved during evolution and play an important role in the invasion of pathogens [8]. These PRR molecules can recognize various types of PAMP such as lipopolysaccharide (LPS) of Gram-negative bacteria, peptidoglycan and lipoteichoic acid (LTA) from Gram-positive bacteria, double-stranded RNAs or glycoproteins from viruses, and mannans from fungi, then trigger a series of reaction to kill the invading pathogens [9]. According to recent studies, a group of C-type lectin (CTL) recognition receptors that plays an important role in innate immune responses in invertebrates has been discoverd. CTL is capable of recognizing and eliminating infectious microorganisms. Research shows that a new type of CTL, LvLdrlCTL (Litopenaeus vannamei Low Density Lipoprotein Receptor C-type Lectin), is capable of supporting shrimp's innate immune system in combating the effects of V. parahaemolyticus. After immune stimulation, LvLdrlCTL is not only enhanced the expression in hemocytes but also increases the phagocytosis of hemocyte to remove pathogen such as agglutinating AHPND-causing V. parahaemolyticus. In addition, the regulated expression of immune factors-coding genes and signaling pathway components in shrimp knocked-down LvLdrlCTL-coding gene indicates LvLdlrCTL relation to immune regulation. LvLdlrCTL associates with enhancement of the immune system's responsed capacity by decreasing the mortality of V. parahaemolyticus-infected shrimps. Because LvLdrlCTL is a eukaryotic protein, its expression in E. coli in a soluble form is a huge challenge. In recombinant protein expression, correct folding of protein is crucial to the success of expression process. One of the strategies for increasing protein folding efficiency is to use fusion tag; in particular, glutathione S-transferase (GST) tag which has both solubility and affinity support functions [10]. For those reasons, in this

present study LvLdlrCTL protein was fused with GST by cloning the coding gene into pGEX-5X-1 vector to produce recombinant LvLdrlCTL protein. The recombinant protein was then used to test the ability to bind and agglutinate V. parahaemolyticus in vitro.

2. MATERIALS AND METHODS

This study, from March 2018 to January 2020, was conducted in the Faculty of Biology and Biotechnology, University of Science, Vietnam National University, Ho Chi Minh city, Vietnam. E. coli strains DH5α {F′[proAB+lacIq lacZΔM15 Tn10(TetR)TM Δ(ccdAB)] mcrA Δ(mrr-hsdRMS-mcrBC) F80lacZΔM15 Δ(lacZYA-argF) U169 endA1recA1 supE44 thi1 gyrA96 relA1 tonA panD} and BL21 (DE3) [F-dcm ompT hsdSB (rB-mB-) gal met] were used as the host cell to clone recombinant plasmids and express the GST-LvLdlrCTL target protein, respectively. Local L. vannamei shrimps from Tien Giang Province were used as a source for LvLdlrCTL mRNA isolation [7].

2.1 Extraction of total RNA and synthesis of cDNA

Local healthy L. vannamei prepared one day before experiment and kept frozen for total RNA extract [9] by using NucleoSpin RNA Plus kit (Macherey-Nagel, Germany). 2 µg RNA were used as a template in reverse transcription polymerase chain reactions (RT-PCR) for synthesis of cDNA using MyTaq™ One-Step RT-PCR kit (Bioline, USA). The reaction were conducted according to the instructions of manufacture as following programs: 45 min at 45°C, 1 min at 95°C; and 30 cycles of 95°C for 15s, 60°C for 15s, 72°C for 10s; and then final extension for 10 min at 72°C to amplify LvLdlrCTL gene using forward (CTL-F BamH) and reverse (CTL-R Xho) primers (Table 1).

Table 1: *Nucleotide sequences of primers used for amplification.*

Primers	Sequence (5'-3')
CTL-F BamH	GGATCCCCGAGTGTACCAACAGGGACCAG
CTL-R Xho	CTCGAGTTACGCCCTCTCACTGGGTTCC
pGEX 5'	GGGCTGGCAAGCCACGTTTGGTG
pGEX 3'	CCGGGAGCTGCATGTGTCAGAGG

*Underlined characters showed restriction enzyme

2.2 Construction of pGEX-5X-1-LvLdlrCTL containing E. coli strain DH5α

Purified LvLdlrCTL fragment and pGEX-5X-1 plasmid were joined together to produce a recombinant plasmid by T4 ligase after being digested with NdeI and XhoI (Thermo Scientific, USA). Then, the recombinant pGEX-5X-1-LvLdlrCTL plasmid was transformed into E. coli host strains DH5α. The positive transformants obtained on LB medium containing Ampicillin (Amp) at the final concentration of 100 µg/ml were further screened by colony PCR with primer on pGEX-5X-1 plasmid (pGEX 5' and pGEX 3'). The result of cloning was confirmed by sequencing.

2.3 GST-LvLdlrCTL protein expression

The recombinant pGEX-5X-1-LvLdlrCTL plasmid with correct sequencing was transformed into E. coli BL21 (DE3) and spread on LB medium containing Amp at the final concentration of 100 µg/ml. E. coli BL21 (DE3) clones carrying the recombinant pGEX-LvLdlrCTL plasmid will be screened through colony PCR with primers pGEX 5' and pGEX 3'. E. coli strains BL21 (DE3) containing pGEX-5X-1- LvLdlrCTL plasmid were cultured at 37°C in LB medium containing ampicillin (100 µg/ml). After 16 hours, bacteria were sub-cultured with 1:20 (v/v) and continued to be shaken at 37°C. Until OD_{600} of bacterial solution reached 0.4 to 0.6; isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to the final concentration of 0.1 mM and continued to shake at 25°C. After 16 hours of induction, cell biomass was collected and the cell membrane was disrupted by ultrasonic waves to obtain proteins in the total, soluble and insoluble fractions. The expression of the GST-LvLdlrCTL protein was confirmed by SDS-PAGE and Western blot probed with GST-tag antibody. E. coli strain BL21 (DE3) containing pGEX-5X-1 plasmid with IPTG induction was used as negative control.

2.4 Purification of GST-LvLdlrCTL protein

Protein solution obtained in previous step was used to purify recombinant GST-LvLdlrCTL protein using GSTrap FF (GE Healthcare) column. All of the steps were carried out according to user manual. The recombinant protein purification was analyzed using SDS-PAGE. Gel-Pro Analyzer and Braford assay were used to determine the protein purity and concentration.

2.5 Assessment the capability of binding to AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL

In this study, a modified Dot blot technique was applied to assess the ability of binding to AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL. First, the GST-LvLdlrCTL protein were incubated with V. parahaemolyticus bacteria for 1 hour at 25°C prior to transferring onto a nitrocellulose membrane. Then, the complex was incubated with anti-GST antibody to capture GST-LvLdlrCTL protein, and incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibody. The blue precipitate of 3,3′,5,5′-Tetramethylbenzidine (TMB) appeared on the cellulose membrane via HRP enzyme conversion of TMB.

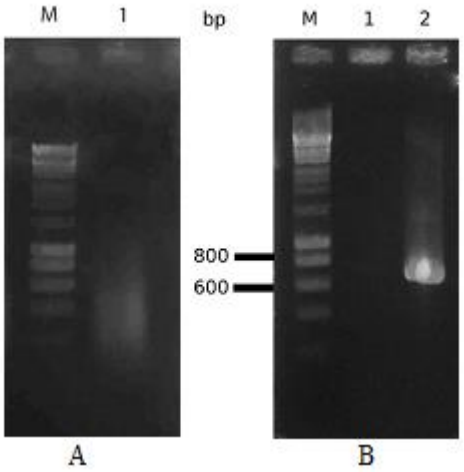
2.6 Evaluating the capacity of agglutination with AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL

Firstly, AHPND-causing V. parahaemolyticus bacteria were labelled with Calcein AM 1 hour at room temperature at 10^6 CFU/ml in TBS-Ca buffer (50 mM Tris-HCl; 100 mM NaCl; 10 mM $CaCl_2$; pH 7.5). Secondly, 20 µl of purified GST-LvLdlrCTL protein or control protein GST (0.1 mg/ml) was mixed with 10 µl of labelled V. parahaemolyticus. Then the mixtures were incubated at 25°C for 1 hour. Finally, agglutination was observed at 490 nm excitation under fluorescence microscope (Nikon, Japan) [9]. In addition, to determine calcium-dependent agglutination, the Calcein-labelled bacteria were incubated with the GST-LvLdlrCTL protein in calcium-depleting buffer, TBS-EDTA (50 mM Tris-HCl; 100 mM NaCl; 4 mM EDTA; pH 7.5).

3. RESULTS

3.1 Extraction of total RNA and synthesis of cDNA

We collected LvLdlrCTL gene from the genome of L. vannamei by extracting total mRNA from shrimp tissue (Fig. 1A, lane 1) and synthesized cDNA by RT-PCR with specific primers CTL-F BamH and CTL-R Xho. The result showed that it had only one band about 729 bp (Fig. 1B, lane 2), which matched the size of LvLdlrCTL gene. In addition, the PCR to amplify LvLdlrCTL target gene was not contaminated because of the absence of any DNA band on agarose gel in negative control (Fig. 1B, lane 1).



A B

Figure 1*: total RNA isolation and cDNA synthesis.*
(A): Lane M, 1kb DNA ladder; lane 1, L. vannamei total RNA.
(B): Lane M, 1kb DNA marker; lane 1, negative control; lane 2, LvLdlrCTL gene synthesised by RT-PCR

3.2 Construction of pGEX-5X-1-LvLdlrCTL plasmid-containing E. coli strain DH5α

LvLdlrCTL gene and pGEX-5X-1 plasmid were joined together by T4 ligase after being double digested by BamHI and XhoI enzyme and transformed into E. coli DH5α. Because the ampicillin antibiotic resistance gene was on the plasmid pGEX-5X-1, so the transformants carrying the recombinant pGEX-5X-1-LvLdlrCTL plasmids were screened by spreading on an LB culture medium containing ampicillin antibiotics. Only colony containing the plasmid could be resistant to ampicillin and grow on the plates. These selected colonies were further screened to confirm the presence of the LvLdlrCTL gene by PCR colonies with pGEX 5'/ pGEX 3' primers on plasmids. The recombinant-vector harboring colonies gave the exact size of 1029-bp product (Fig. 2, lane 3 and 5). The PCR colonies with non-inserted plasmid gave a 300-bp product (Fig. 2, lane 2 and 4). PCR negative control had no band (Fig. 2, lane 1). Thus, we had successfully created the E. coli strain DH5α carrying recombinant pGEX-5X-1-LvLdlrCTL plasmids.

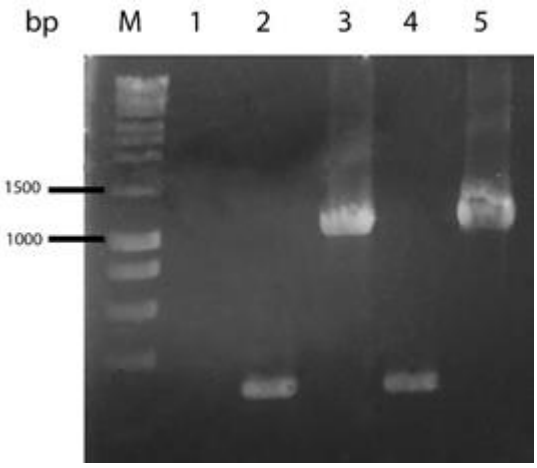


Figure 2*: Confirmation of recombinant clones of E. coli DH5α by colony PCR analysis with pGEX primers. Lane M, 1 kb DNA ladder; lane 1, PCR negative control without DNA; lane 2 and 4, negative control (E. coli DH5α containing pGEX-5X-1); lane 3 and 5, recombinant clones*

The result of cloning confirmed by sequencing showed that the cloned gene had 100% homology to the published LvLdlrCTL gene sequence of L. vannamei (Fig. 3)

3.3 Expression of LvLTLC1

The recombinant GST-LvLdlrCTL protein was induced to express from the E. coli strain BL21 (DE3)/pGEX-5X-1-LvLdlrCTL as described in the method section. Samples of total, soluble, and insoluble proteins were collected and analyzed for expression of GST-LvLdlrCTL protein by SDS-PAGE and Western blot. The analyzed results in Figure 5 showed that there was an overexpressed protein band about 53 kDa in lane 4 which was equal to the predicted size of the GST-LvLdlrCTL protein. There was no appearance of this band in the negative control, E. coli strains BL21 (DE3)/pGEX-5X-1 with IPTG induction (Fig. 5, lane 2), E. coli BL21 (DE3) (Fig. 5, lane 1), and E. coli BL21 (DE3)/pGEX-5X-1-LvLdrlCTL non-induction (Fig. 5, lane 3). In addition, because the recombinant LvLdlrCTL protein was expressed as fusion with the GST tag available on pGEX-5X-1 vector, so the presence of the GST-LvLdlrCTL protein could be indirectly comfirmed via the GST tag.

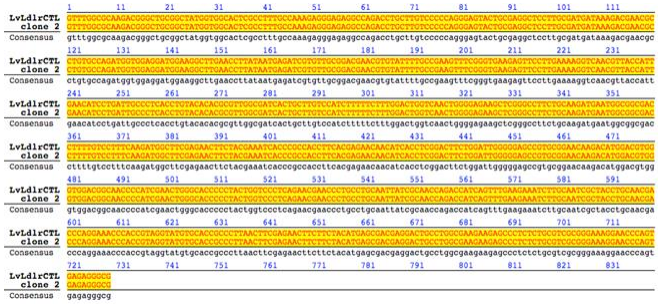


Figure 3: *The aligning of sequence between one positive clone with published LvLdlrCTL gene*

Figure 5B showed that the overexpressed protein band in the SDS-PAGE electrophoresis was GST-LvLdlrCTL. This protein was mostly expressed in the soluble fractions (Fig. 5, lane 5). Thus, the recombinant LvLdlrCTL protein fused to GST was successfully expressed on E. coli strain BL21 (DE3).

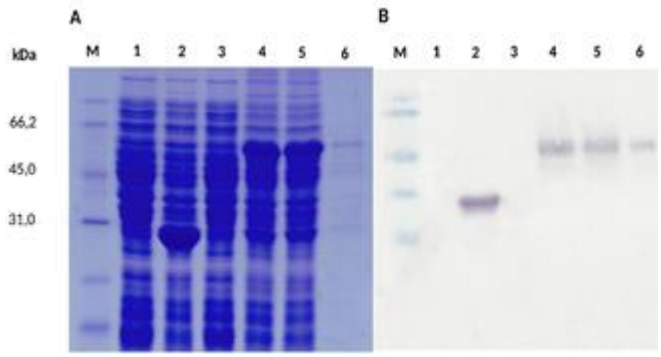


***Figure 5**: Confirming the expression of GST-LvLdlrCTL; (A): SDS-PAGE (A), (B): Western blot*

Lane M, protein ladder; lane 1, E. coli BL21 (DE3) (IPTG +); lane 2, E. coli BL21 (DE3)/pGEX-5X-1 (IPTG +); lane 3, E. coli BL21 (DE3)/pGEX-5X-1-LvLdrlCTL (IPTG-); lane 4-6, E.coli BL21 (DE3)/pGEX-5X-1-LvLdrlCTL (IPTG +) total protein samples (4), soluble samples (5), insoluble samples (6)

3.4 Purification of GST-LvLdlrCTL protein

When the total protein sample was passed through purification column, the GST-carrying proteins would be retained through GST's affinity in the presence of GSH in the column. Then, the target protein was eluted from the column and analyzed for purity by SDS-PAGE. The result showed that the elution sample (Fig. 6, lane 5) gave a band about 53 kDa, similar to the predicted size of GST-LvLdlrCTL protein band. Thus, we had initially purified and successfully obtained the GST-LvLdlrCTL protein with purity more than 85%. However, in order to obtain purified GST-LvLdlrCTL protein in large quantities, further investigation during purification was needed.

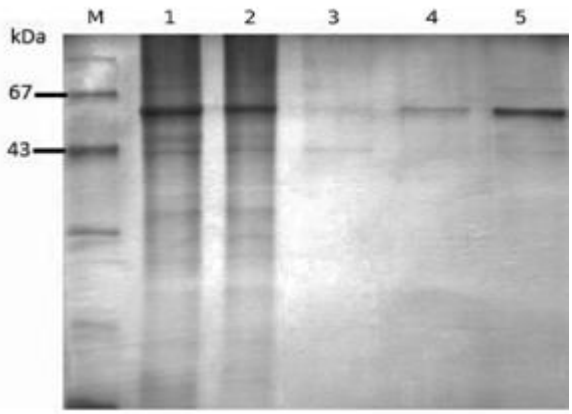


***Figure 6:** Analysis of GST-LvLdlrCTL protein purification.*

Lane M, protein ladder; lane 1, total protein samples from E. coli BL21 (DE3)/pGEX-LvLdlrCTL (+) IPTG; lane 2, flow-through sample; lane 3, washing sample; lane 4-5, elution samples

3.5 Assessment the capability of binding to AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL

After purification, GST-LvLdrlCTL protein was tested the interaction with V. parahaemolyticus. Dot blot technique was applied to assess the ability of binding with AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL protein on nitrocellulose membrane. The negative control was a mixture of GST and V. parahaemolyticus. Only target proteins bound to V. parahaemolyticus were retained, interacted with anti-GST primary antibody, and detected by HRP-conjugated secondary antibody. Although GST also had the ability to bind to primary antibody, it did not have the ability to bind to bacteria and would be washed away. Thus, it could be seen that the GST-LvLdrlCTL protein bound to the bacteria and be retained (Fig. 7, dot 1). In the negative control, GST protein was not able to bind with the bacteria and was easily washed out (Fig. 7, dot 2). Two samples with no primary antibody added had no signal (Fig. 7, dots 3 and 4) showing that secondary antibody did not bound to bacteria or target protein. Therefore, it could be concluded that the LvLdrlCTL protein had successfully bound to membrane of V. parahaemolyticus. In other words, the LvLdrlCTL protein was capable of binding to AHPND-causing V. parahaemolyticus.

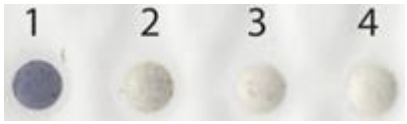


***Figure 7:** Dot blot-based binding assay.*
1, V. parahaemolyticus + GST-LvLdrlCTL + primary antibody + secondary antibody;
2, V. parahaemolyticus + GST + primary antibody + secondary antibody;
3, V. parahaemolyticus + LvLdrlCTL with GST + secondary antibody;

3.6 Evaluating the capacity of agglutination with AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL

Under fluorescent microscope, V. parahaemolyticus bacteria could be agglutinated by GST-LvLdrlCTL protein only in TBS-Ca buffer (Fig. 8D). On the other hand, the agglutination was not observed in both calcium-depleted TBS-EDTA buffer (Fig. 8C) and GST control protein (Fig. 8B). These results supported the hypothesis that only if calcium was supplemented LvLdlrCTL was able to agglutinate AHPND-causing V. parahaemolyticus as prediction.

4, V. parahaemolyticus + GST + secondary antibody

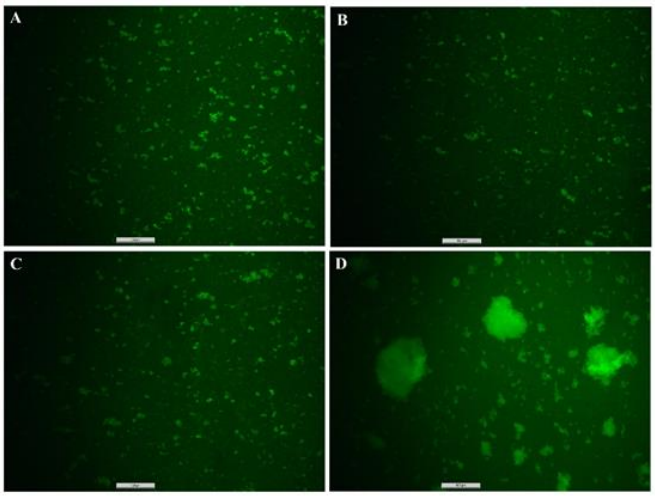


***Figure 8**: GST-LvLdlrCTL protein could agglutinate V. parahaemolyticus.*
A, V. parahaemolyticus + TBS;

B, V. parahaemolyticus + GST;
C, V. parahaemolyticus + GST-LvLdlrCTL + TBS-EDTA;
D, V. parahaemolyticus + GST-LvLdlrCTL + TBS-Ca

4. DICUSSION

LvLdlrCTL gene is encoding for a 243-amino acid protein which is largely detected in brain, hepatopancreas, stomach, heart, gill, and hemocytes with the highest expression in hepatopancreas [11]. The location of LvLdlrCTL protein distribution may be related to its functions. Furthermore hepatopancreas, the main organ in the shrimp digestive system, stomach and intestine are tissues directly combat against pathogenic infections [7]. Therefore, the presence of LvLdrlCTL in the shrimp gastrointestinal tract predicts that it can prevent the invasion of V. parahaemolyticus bacteria by agglutinating them and blocking the bacteria-attached sites on shrimp epithelial cells. Necessarily, this research was conducted to evaluate the possibility in vitro agglutination with AHPND-causing V. parahaemolyticus of LvLdlrCTL before similar in vivo experiments in shrimps conducted. The name CTL is come from the fact that it can bind specifically to carbohydrates on the microbial cell surface through the CRD (Carbohydrate Recognition Domain) in the presence of calcium [12]. CRD has a folded coil region and is stabilized by two disulfide bonds besides four Ca^{2+} binding regions involved in maintaining the linkage between carbohydrates and PAMP [13]. The second Ca^{2+} binding region existing in most CTL has a conserved structure called EPN or QPD, which plays an important role in binding to mannose or galactose sugars [14]. LvLdlrCTL has been identified belonging to the CTL group [11], presumably it possesses the full biological activity of this group. Expectedly, LvLldrCTL contains at least one CRD motif recognizing and binding carbohydrate [14]. The results of binding and agglutination to AHPND-causing V. parahaemolyticus bacteria of the recombinant protein showed that it was capacity to bind mannose or galactose sugars presenting on the cell surface of bacteria. The ability to agglutinate Gram negative bacteria V. parahaemolyticus was Ca^{2+}-dependence, in consistency with the fact that agglutination disappeared when TBS-Ca was replaced by TBS-EDTA buffer (Fig. 8). Similarly, to the previously reports, the novel identified LvLdlrCTL has the potential antibacterial activities in vitro on V. parahaemolyticus by binding and agglutinating the bacteria. However, their antibacterial roles in immunity remain elusive. Moreover, the advantage of using dot blot assay to evaluate the capability of binding to AHPND-causing V. parahaemolyticus of LvLdlrCTL is faster and cheaper than western blot, a similar immunoblotting assay. Because the binding after incubation between V. parahaemolyticus and GST-LvLdlrCTL were analyzed using SDS-PAGE and detected using anti-GST and HRP-conjugated secondary antibody, hence both methods are the same except that western blot requires SDS-PAGE while dot blot does not. Moreover, it does not require expensive equipment for documentation such as a chemiluminescence capture but it is still possible to visualize specific binding between the recombinant LvLdlrCTL protein and V. parahaemolyticus prior to conducting agglutination test. Additionally, in comparison to bacteria agglutination assay, dot blot-conducted bacteria binding assay is simpler yet faster in predicting agglutination potential if the required material such as fluorescence microscope or cell-permeant dye is not available. On the other hand, taking advantage of the ability to bind carbohydrate, the purified LvLdlrCTL protein from this study can be used as a source not only for evaluation the ability of binding and agglutinating with other pathogenic bacteria, but also for in vivo researches to remove harmful bacteria for developing bio-products to prevent and treat AHPND in shrimp.

5. CONCLUSIONS

This study succeeded in obtaining the gene encoding for LvLdrlCTL protein from L. vannamei; constructing the E. coli strains DH5α and BL21 (DE3) carrying the target gene in the recombinant pGEX-5X-1-LvLdlrCTL plasmid; expressing successfully GST-LvLdrlCTL in soluble form with above 85% purity; confirming the capacity of binding and agglutination with AHPND-causing V. parahaemolyticus of GST-LvLdlrCTL only if calcium was present. This was the initial result to express, isolate purified protein to support the treatment of AHPND in shrimp when applying LvLdlrCTL protein from L. vannamei, thereby showing a great potential of CTL in general and LvLdlrCTL in particular in supporting the treatment of V. parahaemolyticus-causing AHPND on shrimp cultured in Vietnam.

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