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A novel C-type lectin from abalone, *Haliotis discus discus*, agglutinates *Vibrio alginolyticus*

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Bacterial agglutination;
Innate immune;
Carbohydrate recognition domain (CRD)

Summary
Owing to its specific binding to carbohydrates, lectins play important roles in pathogen recognition and clearance in invertebrate animals. In this study, a novel C-type lectin (designated CLHd) gene was isolated from abalone, *Haliotis discus discus*, cDNA library. The complete cDNA sequence of the CLHd gene is 508 base pairs in length, and encodes 151 amino acids. CLHd shares a highly conserved carbohydrate recognition domain with C-type lectins from mollusk and fish. The mRNA expressions of CLHd in healthy and bacterial-challenged abalones were examined using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). CLHd mRNA transcription was up-regulated by *Vibrio alginolyticus* challenge and reached the maximum expression at 24 h after the bacterial injection. To understand its biological activity, the recombinant CLHd gene was constructed and expressed in *Escherichia coli*. The recombinant CLHd specifically agglutinated *V. alginolyticus* at a concentration of 50 μg/ml in a calcium-dependant way. Both the gene expression analysis and recombinant protein activity assay suggest that CLHd is an important immune gene involved in the recognition and elimination of pathogens in abalones.

Introduction

Abalone is a large herbivorous marine snail valued as highly palatable seafood, widely cultured in Australia, China, Japan, Korea, Mexico, South Africa, and the United States [1]. In 2002, the world cultured abalone production was 8696 metric tons and the total value of the production was estimated at approximately US$0.8 billion [2]. However, since 2000, the mass mortality of abalone reared in grow-out

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ponds, settlement failure of larvae in the nursery ponds, and post-larvae abalone infected by *Vibrio* species, displayed a pattern of sudden collapse or "crash" in survival and forced many abalone farms to close [3–5].

Understanding of the abalone innate disease defense mechanism will promote strategies for abalone disease control and healthy abalone farming independent of antibiotics treatment. As most invertebrate animals lack an adaptive immune system, the mollusk depends on its innate immune system such as humoral factors to recognize the potential pathogens and then to trigger the clearance of the invading pathogenic microbes [6,7].

Among all the humoral factors, lectins refer to one family of important pattern recognition proteins, which mediates the non-self antigen recognition by binding to specific carbohydrate residues on the cell surface of pathogens such as lipopolysaccharides, peptidoglycans from bacteria, and β-1, 3-glucan from fungi [8,9]. On a molecular level, lectins may activate the associated enzymes or proteins to process the neutralization and elimination of pathogenic microorganisms. Functionally, lectins are powerful weapons to aggregate and opsonize pathogens in animals [10]. Many lectins, specifically agglutinating bacteria or parasites have been identified from invertebrate animals such as the horseshoe crab, horse mussel, clam, and oyster [11–17]. Recently, the characterization of lectins from commercially important species, like shrimp, crab, and oyster has attracted more attention [18–23]. However, the corresponding work on abalones has not been reported to date.

In the present study, a novel C-type lectin gene (designated CLHd) was cloned from abalone normalized cDNA library. The gene regulation of CLHd response to *Vibrio* infection and the bacterial agglutinating activity of the recombinant CLHd protein were investigated and the results showed CLHd might contribute to the recognition of pathogenic bacteria in abalones and the protection juvenile abalones from *Vibrio* diseases.

Materials and methods

Materials

Two-year-old disk abalones, *Haliotis discus discus*, with an average weight of 10–13 g were obtained from Hanlim abalone farm (Jeju, South Korea) and acclimated in laboratory culture conditions 1 week before the experiments. All the bacterial strains used in this study were from the institute of Korean Collection for Type Culture (KCTC, South Korea).

Molecular cloning and sequence analysis

The abalone cDNA library was synthesized by isolating total RNA from the whole abalone and using a cDNA library construction kit (Creator™, SMART™, Clontech, USA). To isolate the rare transcript genes, the cDNA library was normalized using a Trimmer-Direct cDNA normalization kit (Evrogen, Russia).

After referring to single-pass random sequencing results and the sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php) analysis reports, the candidate clone was selected for further cloning. Table 1 lists all the primers used in this study. Sequence analysis, ORF detection, signal peptide prediction, N/O glycosylation site, and rare codon analysis were performed using the DNAssist2.1, webpage server (http://www.ncbi.nlm.nih.gov/BLAST/), (http://www.cbs.dtu.dk/services/SignalP/), (http://www.cbs.dtu.dk/services/NetNGlyc/), (http://www.cbs.dtu.dk/services/NetOGlyc/), and (http://www.faculty.ucr.edu/mmaduro/codonusage/usage.htm), respectively.

CLHd mRNA transcription tissue distribution and induction by *Vibrio* challenge

The mRNA expression of CLHd in different tissues of healthy and bacteria challenged abalones was measured by semi-quantitative RT-PCR. Three healthy abalones as a control were sacrificed and the total mRNA extracted from different tissues, including gill, mantle, digestive tract, muscle, and hemocytes.

For the bacterial challenge, 10 ml live *Vibrio alginolyticus* (O.D.600 = 1.0) was resuspended in 0.1 M phosphate buffered saline (PBS) at 1.0×10^9 cells ml^{-1}. A 50 μl bacterial suspension, or PBS, was injected into the adductor muscle of two-year- old abalones. After the injection at 12, 24 and 48 h, three abalones were sacrificed at each sampling time point.

Totally, 1 μg of RNA from each tissue sample was used for cDNA synthesis following the manual of the superscript III cDNA PCR synthesis kit (Invitrogen, USA). Then RT-PCR was performed using two specific primers from the CLHd coding sequence. A 492 bp fragment of the actin sequence was amplified as an internal control. The PCR conditions were optimized by going through several pilot experiments. Finally, the PCR condition followed this protocol: one cycle of 94 °C for 2 min, 30 cycles (or 24 cycles for actin) of 94 °C for 30 s, 53.5 °C for 30 s, 72 °C for 30 s, and one cycle of 72 °C for 5 min. The RT-PCR products were separated on agarose gels, stained by ethidium bromide, and then detected under ultraviolet light. The amplified PCR fragments were measured using Quantity One software (Bio-Rad, USA). The relative mRNA expression levels of CLHd in abalone samples were expressed as the ratio to that of actin RNA. Statistical

Table 1 Oligonucleotide primers used in the present experiments.

Primer name	Sequence
RT-PCR CLHd primer (forward)	5′–TCTGCTGTACCCTGTTGTTGCTGT–3′
RT-PCR CLHd primer (reverse)	5′–TCGTCCATATCCAGCTTGACTCCA–3′
RT-PCR β-actin primer (forward)	5′–GACTCTGGTGATGGTGTCACCCA–3′
RT-PCR β-actin primer (reverse)	5′–ATCTCCTTCTGCATTCTGTCGGC–3′
Recombination primer (forward)	5′–GAGAGAGAATTCTCAAGGTGTAGA-GGCGGA–3′
Recombination primer (reverse)	5′–GAGAGAAAGCTTTTACATTTTACAT-ATGAAATTGGCGA–3′

analysis was determined using one-way analysis of variance (ANOVA) followed by Tukey's test in sigma plot 10.0 (Sigma, USA). Data were represented as the mean ± SEM ($n = 3$), and the differences were considered significant at $P<0.05$.

Protein expression and purification

Based on the CLHd full-length cDNA sequence, specific PCR primers were designed to amplify the mature protein coding region of the CLHd gene. PCR products were digested by restriction endonucleases and ligated to expression vector pMAL-c2X according to the instructions of the kit manual (NEB, USA). After the recombinant plasmids were propagated in *E. coli* (DH10b), they were transformed into *E. coli* (Rosetta-gami DE3) for protein expression. A 5 ml starter culture was inoculated into 100 ml of Luria broth with 100 μL ampicillin (100 mg/ml) and 10 mM glucose (2% final concentration) and kept at 37 C with continuous rotary shaking at 180 rpm until density approached $OD_{600} = 0.5$–0.8. Then protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of induction at 37 C, the cells were harvested by centrifugation at 3500 rpm for 30 m at 4 C. Subsequently, all the cells were resuspended in a 6 ml column buffer and stored in −70 C overnight. After thawing, the bacterial cells were placed in an ice-water bath and sonicated. Having been centrifuged at 9000*g* for 30 m at 4 C, the supernatant was loaded into a maltose-binding resin column. Through the amylose resin column, the protein was purified and used for activity assay. The purified recombinant CLHd was subjected to 12% SDS-polyacrylamide gel electrophoresis at 30 mA for 50 m. The protein separation was visualized by staining with Coomassie brilliant blue R-250 (Sigma, USA). Molecular weight protein standards (Bio-Rad, USA) were used to determine the target protein size. Protein concentration was measured by the Bradford method [24].

Bacterial agglutination assay

The bacterial recognition of CLHd was assessed by a bacterial agglutination test following a similar method described in [25,26]. The Gram-negative non-pathogenic *E. coli* (*DH5α*), Gram-negative pathogenic *V. alginolyticus*, and Gram-positive *Bacillus subtilis* were suspended in a Tris-buffered saline (TBS) (50 mM Tris–HCl, 100 mM NaCl, pH 7.5) at 5.0×10^9 cells ml^{-1}. A 10 μl of bacteria suspension was added to 40 μl of recombinant CLHd in series dilution (5, 25, 50, and 100 μg/ml) by TBS containing 10 mM $CaCl_2$, or only TBS calcium chloride solution as a control. The mixtures were incubated overnight at 4 C. To determine whether the agglutination was calcium dependent, the *V. alginolyticus* was incubated with CLHd in TBS–EDTA solution (50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5) under the same condition described above. Cells were observed in light microscopy.

Bacterial agglutination inhibition assay

To test carbohydrate-binding specificity for CLHd, 20 μL of serial dilutions (1, 10, 100, and 200 mM) of various carbohydrates (Sigma, USA) were premixed with 20 μL of 50 μg/ml CLHd at room temperature for 30 min. Then 10 μL of *V. alginolyticus* (5.0×10^9 cells ml^{-1}) was added into each of the carbohydrate–CLHd mixture for overnight incubation at 4 C.

Results

Sequence analysis of the full length CLHd

Sequencing of the abalone normalized cDNA library yielded more than 6500 EST sequences. Blast analysis detected a 508 bp clone, which was homologous to C-type lectins and thereby it was designated as CLHd, since it was from *H. discus discus*. The nucleotide sequence and deduced amino acid sequence are shown in Figure 1. The complete cDNA sequence of CLHd consisted of a 5'-untranslated region (UTR) of 11 bp, an open reading frame of 456 bp encoding 151 amino acid residues, a 3' UTR of 29 bp, and a polyA tail. In N terminal, a putative cleavage site was predicted after position 22 (GAEG$^{22\text{-}23}$SRC) and the amino acid residues of the signal peptide were underlined in Figure 1. The deduced mature CLHd protein had a theoretical mass of 15 kDa and an isoelectric point of 9.1. To optimize the in vitro protein expression of recombinant CLHd, the glycosylation site and translation codon usage were analyzed. The coding sequence of CLHd contained six arginine codons, which were in low usage frequency (<0.1%) of *Escherichia coli*. In the CLHd mature protein sequence, no significant glycosylation site was predicted through computer software analysis.

Characterization and homology comparison of CLHd with CRD region of C-type lectins

When the CLHd sequence was blasted against the database of NCBI, the comparatively low expect values of its

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TCTTCCCCAAG ATG AAA ACG TTC TGC TGT ACC CTG TTG TTG CTG TTG    47
            M   K   T   F   C   C   T   L   L   L   L   L     12
GGT TGT GCC CTC CAC CGT GGT GCT GAA GGT TCA AGG TGT AGA GGC    92
 G   C   A   L   H   R   G   A   E   G   S   R   C   R   G     27
GGA TTT CAC AAG CAC GGA GGT TCC TGC TAT TGG TTC TCC AAC ATA   137
 G   F   H   K   H   G   G   S   C   Y   W   F   S   N   I     42
CGG GGA ACA TTT GCC GAG GCA AGA TCA ATA TGC CGC TTC CTT GGG   182
 R   G   T   F   A   E   A   R   S   I   C   R   F   L   G     57
AGT GAC CTT GCA TCC ATC ACC AGT GCA GCT GAA GAT GTC TTT ATC   227
 S   D   L   A   S   I   T   S   A   A   E   D   V   F   I     72
AGA GGG TAT GCG ACC CAG AGA GGC AAA GCT AAG GTT TAC TAC CTG   272
 R   G   Y   A   T   Q   R   G   K   A   K   V   Y   Y   L     87
GGA GGC GCC GAC CTC GGT CTG GAG TCA AGC TGG ATA TGG ACG AGG   317
 G   G   A   D   L   G   L   E   S   S   W   I   W   T   R    102
AAC AAA CCC TTT ACT TTC ACC AAC TGG GGA TCT GGG CAA CCT GGA   362
 N   K   P   F   T   F   T   N   W   G   S   G   Q   P   G    117
AAC TCA AAA AAT AAT GAA CAC TGT CTT GCA CTT CAA AGT AGT GAT   407
 N   S   K   N   N   E   H   C   L   A   L   Q   S   S   D    132
GGA TAT CGT TGG CAT GAT TAC AAC TGT GAT TTT ATC GCC AAT TTC   452
 G   Y   R   W   H   D   Y   N   C   D   F   I   A   N   F    147
ATA TGT AAA ATG TAA CTCGATCAATTAAACTATATCTGCAGGACAAAAAAAAAAAA 508
 I   C   K   M                                               151
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Figure 1 Nucleotide and deduced amino acid sequence of CLHd from abalone, *Haliotis discus discus*. The nucleotides of un-translated region are in italics. Amino acid residues of predicted signal peptide are underlined. The nucleotides and amino acids are numbered along the right margin.

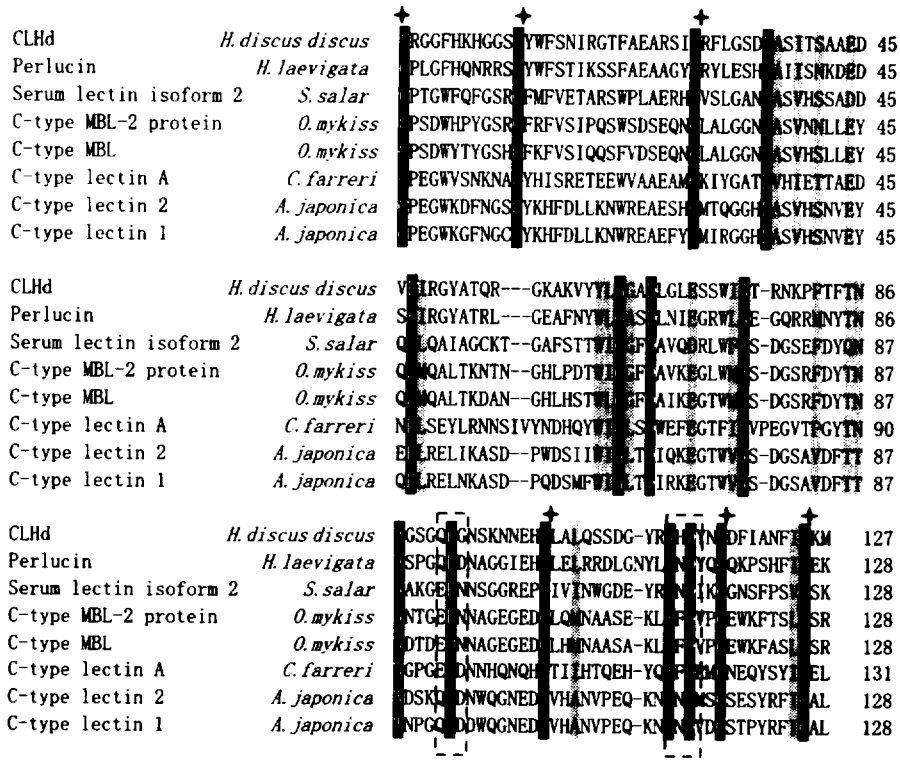

Figure 2 Multiple amino acid sequence alignment of CLHd and CDR region of its homologous C-type lectins. Six highly conserved cystine residues are labeled by asterisks; two motifs for the ligand binding are boxed using broken lines. All the conserved amino acid residues are shaded for homologous comparison. All the referred sequences are from NCBI: C-type lectin 2 of *Anguilla japonica*, BAC54021; C-type lectin 1 of *Anguilla japonica*, BAC54022; C-type MBL-2 protein of *Oncorhynchus mykiss*, CAJ14130; C-type mannose-binding lectin of *Oncorhynchus mykiss*, AAM21196; Perlucin of *Haliotis laevigata*, P82596; C-type lectin A of *Chlamys farreri*, ABB71672.

alignment with some C-type lectins indicated its homology to this group protein in the view of molecular structure. Multiple amino acid sequence alignment of CLHd and carbohydrate recognition domain (CRD) regions in its homologous C-type lectins showed that six cysteine residues and two ligand-binding motifs were conserved (Figure 2). CLHd exhibited highest amino acid sequence similarity (48%) with perlucin in the CRD region. However, the full amino acid sequence of CLHd shared no more than 40% similarity with that of any homologous C-type lectins.

CLHd mRNA expression induced by *V. alginolyticus* challenge

The CLHd mRNA tissue distribution in healthy abalone and its induction pattern in abalone challenged by *V. alginolyticus* were determined using semi-quantitative PCR. In the healthy tissue, CLHd expression was mainly detected in the gill, mantle, and digestive tract, subordinately in the gonad and hemocytes, but with no signal in muscle (Figure 3). In *V. alginolyticus* challenged abalones, the relative expression level of CLHd was significantly up-regulated in the digestive tract (Figure 4A). The induction of mRNA was initiated at 12 h after *V. alginolyticus* injection and it reached maximum expression within 24 h. As time progressed, mRNA transcription

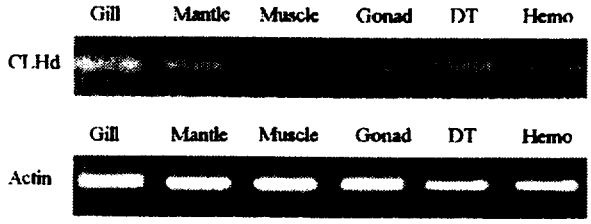

Figure 3 Tissue distribution of CLHd transcript revealed by RT-PCR. Actin was amplified as an internal control. All the PCR products were run on 1.2% agarose gel electrophoresis. DT: digestive tract; Hemo: Hemocytes.

of CLHd decreased to the control value at 48 h post *Vibrio* challenge. All the gel electrophoresis results were quantitatively measured using Quantity One software (Bio-Rad, USA). At 24 h post *Vibrio* challenge, it showed 2.5 times more CLHd mRNA expression than the control group and significant up-regulation in ANOVA ($P<0.05$) (Figure 4B).

Recombinant expression of CLHd in *E. coli*

With the aim of characterizing the function of CLHd, the coding sequence of CLHd was ligated into a pMal-c2X protein

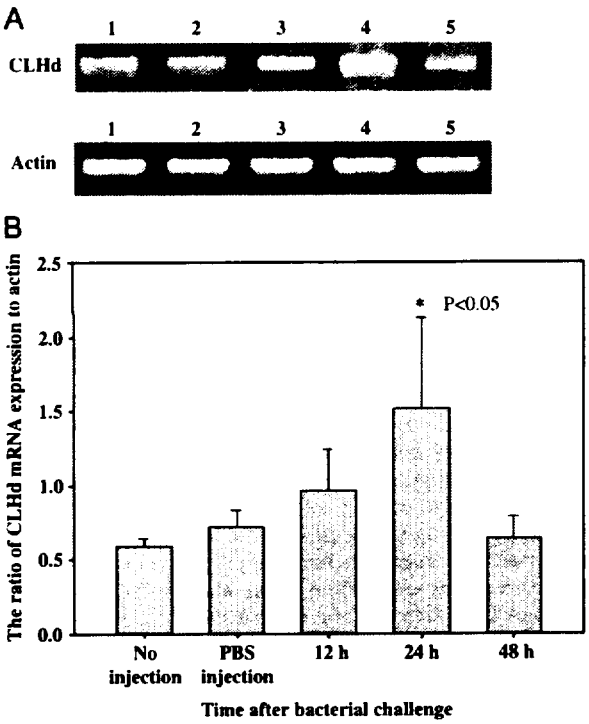

Figure 4 (A) mRNA temporal transcript profile of CLHd induced by *V. alginolyticus* challenge. (1) control, (2) PBS injection, (3) 12 h post *Vibrio* injection, (4) 24 h post *Vibrio* injection, (5) 48 h post *Vibrio* injection. (B) Relative CLHd mRNA expression in the digestive tract of abalone after *V. alginolyticus* injection. Semi-quantitative PCR was repeated three times. The CLHd transcript levels were expressed as the ratio between CLHd mRNA and the mRNA of actin (internal control) in the same sample. Data were presented as means ±SEM. Asterisk indicates statistically significant differences ($p<0.05$ by Turkey test, $n = 3$).

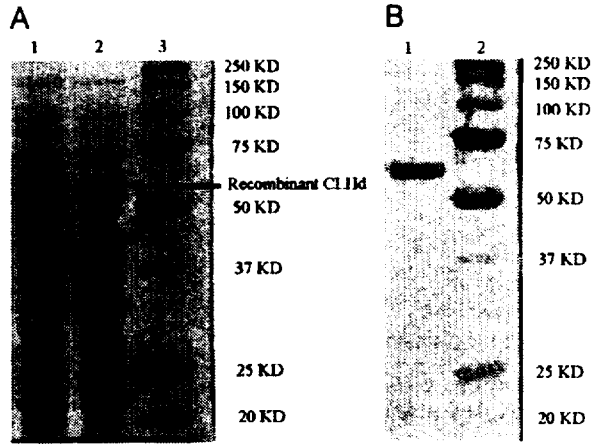

Figure 5 (A) SDS-PAGE analysis of recombinant CLHd expressed in *E. coli* (Rossetta gami) Lane 1: Lysate of *E. coli* (Rossetta gami) without induction; lane 2: Recombinant CLHd expression induced by 0.5 mM IPTG in *E. coli* (Rossetta gami); lane 3: Precision plus protein unstained standards (Bio-Rad, USA). The specific band of recombinant CLHd is indicated by an arrow. (B) SDS-PAGE analysis of purified recombinant CLHd Lane 1: Purified recombinant CLHd; lane 2: Precision plus protein unstained standards (Bio-Rad, USA). The molecular mass of each protein marker band is labeled in the right side of the acrylamide gel.

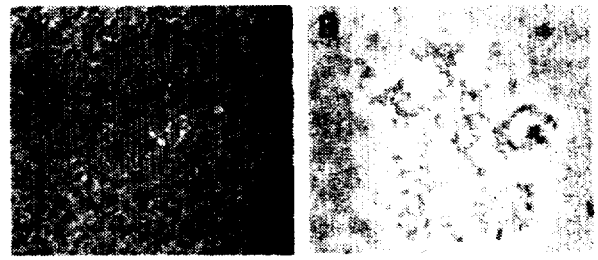

Figure 6 Agglutination of Gram-negative pathogenic *V. alginolyticus* induced by CLHd A: control, *V. alginolyticus* with TBS-Ca buffer B: *V. alginolyticus* incubated with 50 μg/ml recombinant CLHd in TBS-Ca buffer.

expression system (NEB, USA). The recombinant protein was expressed in *E. coli* (Rosetta-gami DE3). In the pMal-c2X expression system, it provided the fusion maltose-binding protein (MBP) in the N terminal, which might enhance the solubility of recombinant CLHd and make the protein purification in an easy way. After 3 h IPTG induction, the whole cell lysate, purified CLHd, and control *E. coli* cell lysate were analyzed by SDS-PAGE. A distinct band with a molecular weight of ~60 kDa was detected (Figure 5), which is consistent with the predicted size of CLHd (15 kDa) and MBP (42.5 kDa) fusion protein.

Bacterial agglutination activity of CLHd

The Gram-negative non-pathogenic *E. coli* (*DH5α*), Gram-negative pathogenic *V. alginolyticus*, and Gram-positive *B. subtilis* were selected to test CLHd bacterial agglutination activity. The incubation with a Tris buffer or MBP solution did not induce any visible agglutination. Only when the addition of recombinant CLHd reached a concentration higher than 50 μg/ml, was obvious agglutination observed in the *V. alginolyticus* solution (Figure 6B), but not in *E. coli* and *B. subtilis*. Even when the latter two strains of bacteria were incubated with 100 μg/ml of recombinant CLHd, they still did not show any agglutination. The addition of EDTA and no-calcium ion supplementation inhibited the agglutination of *V. alginolyticus* by CLHd in the same experiment conditions.

Carbohydrate-binding specificity of CLHd

The carbohydrate-binding specificity of CLHd was examined by competitive inhibition of various sugars on bacterial agglutination. Table 2 shows the minimum inhibition concentration of each sugar. The presence of more than 10 mM D-galactose inhibited the bacterial agglutination activity of

Table 2 Inhibition of bacterial agglutinating activity of CLHd by carbohydrates.

Saccharide	Minimum inhibition concentration
D-Fucose	NI
D-Galactose	10 mM
D-Glucose	NI
D-Mannose	NI
N-Acetyl-D-galactosamine	NI
N-Acetyl-D-glucosamine	NI

NI: No inhibition effects were observed till the concentration of sugar reached 200 mM.

CLHd. In contrast, fucose, glucose, mannose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine did not show any inhibitory activity.

Discussion

In the present study, one C-type lectin with novel *Vibrio* agglutinating activity was identified from abalone, *H. discus discus*. The 508 bp nucleotide cDNA of CLHd was reported in the first time. The signal peptide in its N terminal indicates that the mature CLHd will be transferred out of cell and function in intercellular communication. CLHd does not share high amino acid identity with any particular gene. However, in the CRD region, CLHd shows high conservation. The presence of one homologous CRD and six highly conserved cysteines suggest CLHd is a C-type lectin structurally and possesses a Ca^{2+}-dependent sugar-recognition activity to initiate a broad range of biological processes, such as adhesion and pathogen neutralization [27,28]. In the functional residues for ligand binding, CLHd shows QPG at the residues of 97–99, which differs from the typical "QPD" or "EPN", and the amino acid resides of 136–138 are not the invariant "WND" but "WHD". In the CRD of most known C-type lectins, the "QPD" or "EPN" and "WND" together will bind to the carbohydrates with 3- and 4-hydroxyl groups in the pyranose ring in the presence of calcium [29]. They are critical to the carbohydrate-binding specificity. The presence of "QPD" prefers galactose binding. If it is substituted with "EPN," the lectin has specificity for mannose [30–32]. All these structural differences of CLHd with its known homologs suggest that CLHd is a novel C-type lectin.

In healthy abalones, CLHd mRNA transcript is distributed mainly in the digestive tract, gill, and mantle. These three tissues are involved in a continuous water exchange and food uptake from the outside environment to abalones and are more susceptible to pathogen infection. The tissue distribution of CLHd suggests that it may offer the initial response to an invasion of marine pathogenic microorganisms. The direct injection of *V. alginolyticus* significantly increased the CLHd mRNA expression and attained the maximum transcript level in the digestive tract of abalones within 24 h. Therefore, CLHd is inducible and sensitive to *Vibrio* infection. The abrupt increase of CLHd mRNA transcription can be a warning to abalones of pathogenic infection.

To understand the specific biological activity of CLHd, the recombinant CLHd was expressed in *E. coli* and the bacterial agglutination assay was tested. The recombinant CLHd specifically agglutinated *V. alginolyticus* in a calcium-dependent way. The dependence of calcium indicated that CLHd was a typical C-type lectin [33]. The bacterial agglutination supported that CLHd may efficiently immobilize the invading bacteria and inhibit its further tissue invasion. However, CLHd did not agglutinate all the Gram-negative bacteria. It suggested that the bacterial agglutination activity of CLHd should not depend on recognizing the lipopolysaccharides of bacterial cell walls. The bacterial agglutination inhibition test showed that D-galactose was the preferential-binding substrate for CLHd. Through its CRD, CLHd may selectively bind to the conserved galactose residues on the surface of invading cells and thereby efficiently distinguish the pathogens. This provides the self- and non-self-recognition necessary for the activation of abalone complement system, since abalone lacks immunoglobulins [34]. Although the specific role of CLHd in innate immunity still requires further investigation, the lectin pathway has been well established in its homologous C-type lectins such as mannose-binding lectin and ficolin [35]. After lectins bind to the non-self-antigens, the lectin complement pathway is activated and then it will trigger the opsonization of pathogens, chemotaxis, cytokine activation, and then direct killing of pathogens [10,36].

Taken all together, CLHd was an important agglutinin in abalones for the recognition and clearance of *Vibrio* pathogens. The characterization of CLHd gene enriched the knowledge of abalone immunity and provided a powerful immune gene for *Vibrio* disease control.

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