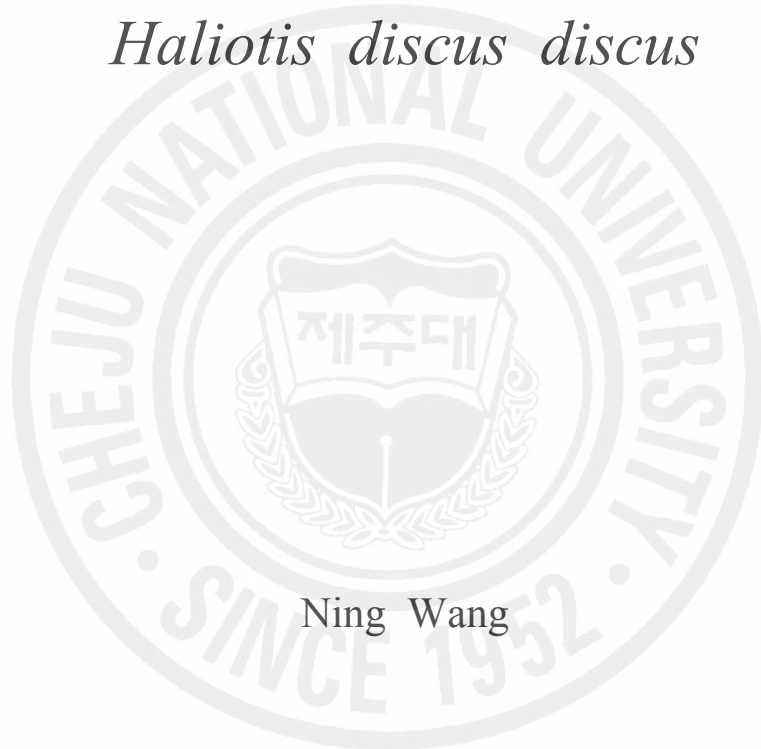


A THESIS
FOR THE DEGREE OF DOCTOR OF PHYSIOLOGY

Characterization and expression analysis
of C-type lectins from abalone,
Haliotis discus discus



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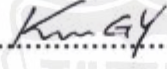
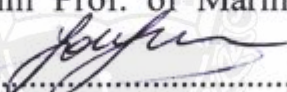
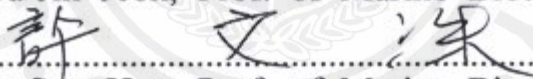
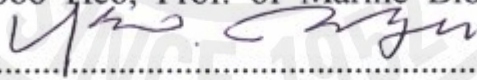
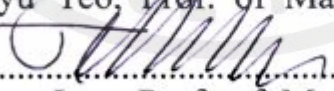
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요약문

렉틴은 세포 표면의 탄수화물 부위에 가역적으로 결합할 수 있고 특이적으로 인식할 수 있는 단백질 또는 당단백질이다. 새로운 활성들을 지니는 많은 중요한 렉틴들이 무척추동물에서 발견되었다. 무척추동물들에서 렉틴은 비자기 항원의 인지를 위해 기본적으로 사용되고, 박테리아 식균작용과 캡슐화를 할 수 있게 해준다.

이 연구에서는 비브리오 응집과 진주형성에 관계된 2개의 유전자를 까막전복의 EST cDNA library 탐색을 통해 확인하였다. 그리고 전복의 CLHd 와 perlucin complete cDNA 대해서 처음으로 연구되어졌다. 다른 종들로부터 기존에 알려진 서열의 유사성을 확인하기 위해 NCBI 의 blastX 프로그램을 이용하였다. 전복에서 생리적 작용과 그들의 기능 분석을 위해서 분자 구조의 분석, in vivo 상에서의 유전자 전사조절, 그리고 in vitro 상에서 단백질 발현, 관계된 활성 측정에 대해 조사되어졌다.

CLHd 유전자의 complete cDNA 염기서열은 151 아미노산을 암호화하는 508 bp 였다. CLHd는 연체동물과 어류로부터 밝혀진 C-type 렉틴들을 가지는 탄수화물 인식 도메인에 높은 유사성을 나타냈다. In vivo 상에서, 전복에 *Vibrio alginolyticus* 를 주입했을 때 소화관에서 CLHd의 관계된 발현 레벨이 의미있는 증가를 나타냈다. *V. alginolyticus*를 주입하고 12시간 후에 그 mRNA의 유도가 시작됐고, 24시간째에 발현률이 최고에 도달했다. 시간이 진행됨에 따라, challenge 테스트 48시간째부터 CLHd의 mRNA 전사량은 control에 비해 감소하였다. 전복에 *V. alginolyticus*를 주입했을 때 CLHd의 요구는 의미있는 증가를 나타냈다. In vitro 상에서 재조합 CLHd 단백질은 calcium에 의존하여 *V. alginolyticus* 에 특이적으로 결합하였다. CLHd 는 전복에서 미생물들이 침입했을 때 이를 억류시켰고 퍼지는 것을 저해하였다. *V. alginolyticus*에 선택적으로 결합하는 CLHd는 전복에서 내부방어와 식균작용의 증대를 위해 자기, 비자기 인식 하는데 없어서는 안될 의미를 제공할 수 있다. CLHd의 특성분석을 통해 전복 질병 조절을 위한 강한 면역 분자를 제공하고 전복의 면역 지식을 넓혀 주었다. CLHd는 *Vibrio*의 주입에 의해 높게 유도

되어졌고 이를 토대로 전복 양식장의 환경 모니터링을 위한 마커 개발과 양식장 관리에 이용 되어질 수 있다. *Vibrio* 응집에도 효과적이기 때문에 CLHd의 많은 양이 존재하면 전복의 질병 저항력을 향상 시킬 수 있다. CLHd의 강한 발현을 위한 전복의 유전자 선별 또는 전복의 형질전환 육종은 *Vibrio* 감염 으로부터 어린 전복들을 구할 수 있는 가능성을 제공한다.

전복 perlucin 유전자의 full length cDNA는 신호서열 22 아미노산과 mature protein 129아미노산을 암호화하는 1038 bp 로 구성되어졌고 greenlip 전복의 perlucin 단백질과 55 % 의 상동성을 나타냈다. 진단된 perlucin의 예측된 분자량은 15 KDa 이고 isoelectric point는 5.7로 확인됐다. 발현된 재조합 perlucin은 calcium carbonate 침전 활성화와 calcium carbonate 결정화 활성이 측정 되어졌다. 재조합 perlucin의 첨가로 인해 calcium carbonate 침전이 촉진 되어졌다. 연체동물의 진주층 성장은 aragonite crystal의 calcium carbonate 축적과 형성에 의존된다. Calcium carbonate 침전 상에서 perlucin의 positive 효과는 전복에서 진주 형성 촉진에 직접적으로 나타난다. Calcium carbonate 결정화 활성화에서 결정들은 처음에 예리한 모퉁이에 둥글게 형성됐고 4°C에서 24시간이상 처리한 결과 육면체를 형성하지 않았다. 점차적으로, calcite 크리스탈 표면은 울퉁불퉁하게 됐고 예리한 모퉁이들이 모두 사라졌다. Perlucin을 가지고 배양 72시간 후, 육면체에 가까운 크리스탈이 형성됐고 크리스탈 윗쪽에 작은 사방면체 돌기가 생성됐다. Perlucin은 예리한 모퉁이에 calcium carbonate 크리스탈이 점차적으로 둥근 형태가 되었지만 동시에 모든 모퉁이들이 그렇게 된 것은 아니다. 이 perlucin은 특이적으로 calcite 크리스탈을 인지할 수 있고 점차적으로 calcite 크리스탈의 예리한 모서리의 성질을 변화시킨다. 그 calcium carbonate의 형태적 변형은 진주와 껍질의 최종 구조에 영향을 주고 성장 속도와 적응에 역시 영향을 미친다. 본 연구에서 perlucin은 *ab*-plane 상에서 calcium carbonate 크리스탈의 성장을 촉진시켰고calcite 크리스탈이 네개의 모퉁이에 모든 단계가 완성됐다. *ab*-plane에서 크리스탈의 형성은 저해되어졌고 그 후 *c*-axis에서 성장이 시작됐다. Perlucin의 분자 구조는 한 개의 잘 보존된 탄수화물 인지 영역을

갖고 있는 전형적인 C-type lectin의 형태이다. perlucin의 RT-PCR 수행시 mantle, 아가미와 소화관에서 발현이 확인되었다. 아가미와 소화관은 외부 환경으로부터 전복내의 환경수 교환과 먹이 섭취에 상시적으로 관련되어 있으므로 이들 기관은 해양환경내 병원성 미생물의 침입에 대한 방어 영역을 형성하기 위하여 병원성 감염에 대한 민감한 감수성과 체액성 면역 반응 분자들이 필요하다.

종합적으로, perlucin 유전자는 병원성 미생물과 해로운 유기물에 대하여 면역 방어에 관여하는 물질로 추정된다. 침입한 기생충 또는 물질의 배설이 되지 않는 경우, perlucin은 비자기 항원의 비활성 수단으로 불필요한 물질들의 진주층으로의 축적을 위해 분비될 것이다. 불필요한 입자들로 인한 진주층의 성장으로 화려한 진주가 생성되는 것이다. Perlucin은 전복의 진주 형성과 내부 nacrein 성장에 관여한다. 그러나, C-type lectin에 비교하여 전복에서 perlucin 유전자의 비자기 인식과 질병 내성에 대한 더 많은 조사가 필요할 것으로 생각된다.

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1. Introduction

Lectin

The term lectin, from the Latin *legere* meaning to pick out, choose or select, was coined by Boyd (Boyd and Shapleigh, 1954). Lectin is a protein or glycoprotein substance, usually of plant origin (mostly of seeds), of non-immunoglobulin nature, capable of specific recognition of and reversible binding to carbohydrate moieties on the surface of cells, on cytoplasmic and nuclear structures, and in extracellular matrix in cells or tissues. Generally, lectins are classified into Calnexin, Galectins (S-type), L-type, I-type, P-type, R-type on structural basis (Dodd and Drickamer, 2001). Calnexin, L-type, P-type are located mostly intracellularly, in luminal compartments. They function in the trafficking, sorting, and targeting of glycoproteins in the secretory and other pathways. C-type lectins, galectins, I-type lectin and R-type lectin are found in lectins that function largely outside the cell and are either secreted or localized to the plasma membrane (Wight, 2002).

Being as the most sensitive recognition molecule to detect the carbohydrate variation in cellular environment, lectins have done great contributions to human beings in biomedical events including blood type grouping, lymphocyte activation and proliferation, drug delivery targeting and cancer or virus disease diagnosis. When cancer or virus infected cells, the abnormal change of carbohydrates on the cell membrane commonly happened. Thus the detection and quantification of glycosylation changes and carbohydrate variations provided an easy way in serological diagnosis of cancer or virus diseases (Nowell, 1960; Judd, 1980; Bird, 1989; Schumacher et al., 1994; Brooks et al., 1996; Kilpatrick, 1999; Lehr, 2000; Khan et al., 2002; Wirth et al., 2002a; Wirth et al., 2002b; Gabor et al., 2004; Kakeji et al., 2006).

Lectins exist in all animal phyla and many lectins with novel activity were isolated from invertebrate animals (Okino et al., 1995; Vazquez,1997; Denis et al., 2003; Alpuche et al., 2005). The best known and utilized animal lectin was identified from snail *Helix pomotia* (Brooks et al., 1996; Brooks, 2000; Sanchez et al., 2006). However, most of the known lectin were identified from terrestrial invertebrate animals. The marine invertebrate animals are becoming more and more popular as a source for novel lectins' isolation (Bretting et al., 1983; Kouzuma et eal., 2003; Holanda et al., 2005; Yu et al., 2006).

Lectins in marine invertebrates

In marine invertebrate animals, lectins are mostly employed to distinguish the non-self antigens, enhance or potentiate the bacterial elimination by binding to specific carbohydrate residues present on the surface of pathogens such as lipopolysachride, peptidoglycan from bacteria, and β -1, 3-glucan from fungi (Boswell, and Bayne, 1985; Chu, 1988; Fisher and Dinuzzo, 1991). Particularly, C-type lectins such as bacterial agglutinin and opsonin, have been demonstrated that they play the important roles in encapsulation and phagocytosis of potential pathogens in animals (Sminia et al., 1974; Loker et al., 1982; Renwrantz, 1983; Feng, 1988; Adema et al., 1992; Olafsen et al., 1992; Iwanaga et al., 1998; Tunkijjanukij and Olafsen, 1998). Mollusks lacks an adaptive immune system and it have to depend on its innate immunity against the invading pathogenic microbes (Loker et al., 2004). The fundamental of these innate immune responses is to recognize the conserved microbial motifs by pattern recognition proteins (PAMPs), and then to trigger the clearance of the invaders (Blalock, 1984; Medzhitov et al., 2000; Ottaviani, 2006). The two main innate immune responses are phagocytosis and encapsulation. Therefore, two genes, CLHd and perlucin, relative to these two responses are studied in this study.

CLHd

In the first part of the present study, a novel C-type lectin gene from *Haliotis discus discus* (CLHd) with *Vibrio* agglutinating activity was identified using cDNA library screening method. It contains highly conserved carbohydrate recognition domain, however CLHd does not specifically resemble any particular gene. Since it is from *Haliotis discus discus*, we designated it as CLHd. In vivo, the demand of CLHd was significantly increased when abalones were challenged by *Vibrio* species. The in vitro expressed recombinant CLHd successfully agglutinated *Vibrio alginolyticus*, the major causative pathogen for mass mortality of juvenile abalones. These suggest that CLHd behaved as an important bacterial agglutinin in abalone against the bacterial infection.

Perlucin

Perlucin is a functional C-type lectin and well known as pearl formation protein in abalone (Mann, 1988). When abalones can not eject the invasion parasites or particles, perlucin will be secreted to regulate nacre entomb the offending entity as a means of inactivation the non-self materials and finally produce the beautiful pearls. The interest of pearl industry, material science and medical treatment for bone repairing formation have driven great attention to pearl's constitution and biogenic mechanism (Fritz et al., 1994; Stupp and Braun, 1997; Fritz and Morse, 1998; Kaplan, 1998; Sellinger et al., 1998; Westbroek and Marin, 1998; Kamat et al., 2000; Rubner, 2003; Tang, Kotov et al. 2003; Lamghari, Berland et al. 2001; Zhang and Zhang, 2006). Known as mother of pearl, nacre is mainly composed of calcium carbonate crystals in the form of aragonite tablets arranged in a continuous parallel lamina by sheets of organic matrix. The organic matrix separates the aragonite layer in 0.5 μm thick, which is comparable to the wavelength of visible light and results in iridescent appearance of pearl. Compared with the pure aragonite crystal, the

interplay of organic and mineral phase dramatically improved the material toughness and resilience of nacre (Goldberg, 2001). Scientists believe that the functional molecules of nacre formation and pearl production should be present in nacre organic matrix, which may guide the nucleation and growth of calcium carbonate crystals at ambient conditions and associate the aragonite tablet with organic matrix in its peculiar structure. Many candidate proteins have been isolated from nacre in different mollusk species, such as mucoperlin of mussel, *Pinna Nobilis*, nacrein of oyster *Pinctada fucata*, 8 KD protein of abalone, *H. rufescens* and perlucin of abalone, *H. laevigata* (Mann et al., 2000; Marin et al., 2005; Miyamoto et al., 2005; Fu et al., 2005; Tacheuchi and Endo, 2005). Perlucin is originally isolated from nacre of abalone inner shell and improved to promote calcium carbonate precipitation at ambient conditions, nucleate the calcium carbonate crystallization and modify the morphology of calcium carbonate crystals therefore it is not only a constitutive protein of nacre but also an important functional molecule regulating shell and pearl formation (Weiss et al., 2000; Blank, et al., 2003). More important, perlucin is the only one functional C-type lectin, which contains a highly conserved carbohydrate recognition domain suggesting that it should play some important roles in specific glycocalyx recognition of abalone (Mann et al., 2000). However, its specific physiological role in abalone is still not clear. Previous studies of perlucin are confined to the protein level and as far as we know, an investigation at a molecular level is anticipated.

In this study, both the perlucin and the CLHd of abalone (*Haliotis discus discus*) were studied using molecular methods. Their full length cDNA were isolated from the expression sequence tag cDNA library of disk abalone, *Haliotis discus discus*. According to the results of their mRNA expression analysis, in vitro protein

expression and recombinant protein activity assay, the function of these two lectins were illustrated.

Abalone is a large herbivorous marine snail valued as a highly palatable seafood, widely cultured in Australia, China, Japan, Korea, Mexico, South Africa, and the United States (Gordon and Cook, 2001). 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 (Gordon and Cook, 2004). However, since 2000, the mass mortality of abalone reared in grow-out 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 (Lee et al., 2001; Cai et al., 2006; Cheng et al., 2004). The characterization of genes relative to abalone innate immunity and understanding of abalone self defense system against disease definitely will promote the management strategy of the abalone disease control and develop the healthy abalone farming system independent of the antibiotic treatments.

Part I

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

Vibrio alginolyticus

Abstract

Owing to its specific binding to carbohydrate, lectins play important roles in pathogen recognition and clearance in invertebrates. 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. However, the difference in functional amino acid residues relative to ligand binding suggested that CLHd possesses a novel specificity to carbohydrate recognition. Semi-quantitative reverse transcriptase polymerase chain reaction detected its mRNA expression in healthy and bacterial challenged abalones. CLHd mRNA transcription was up-regulated by *Vibrio alginolyticus* challenge and reached the maximum expression at 24 hr post the bacterial injection. For the understanding 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.

Key words: C-type lectin; Bacterial challenge; Protein expression; Bacterial agglutination; Innate immune.

2. Materials and methods

2.1 Materials

Two-year old female abalones, *Haliotis discus discus* (n=15), was purchased from an abalone farm. They were cultured in flat-bottomed rectangular tanks (50 L) of aerated and sand-filtered seawater at 18-20 °C with daily twice feeding. Maximum 10 abalones per tank were kept undisturbed for one week in order to acclimatize to their environment before the experimental processing.

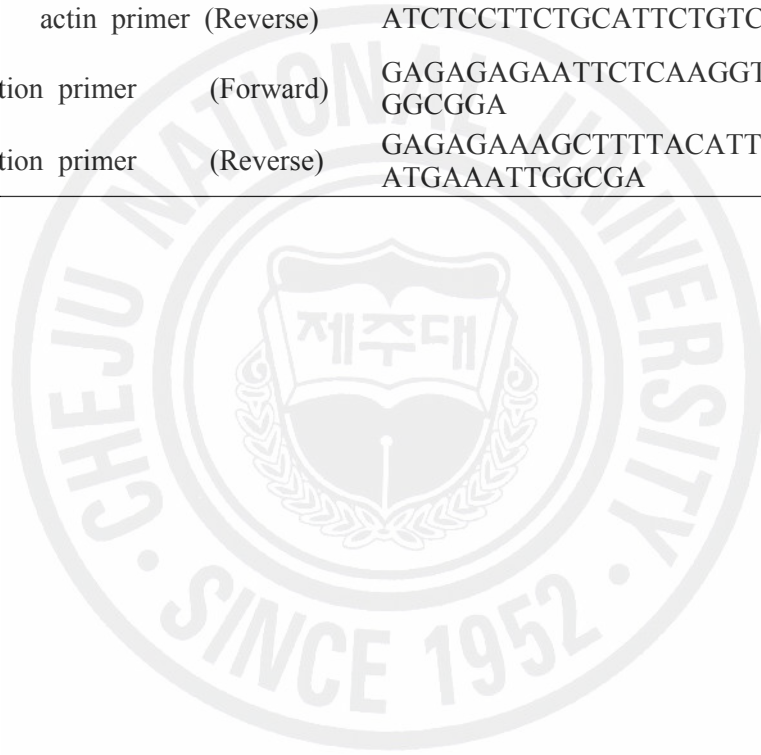
2.2 Molecular cloning and sequence analysis

The abalone (*H. discus discus*) cDNA library was synthesized by isolating total RNA from the whole abalone and using a cDNA library construction kit (Creator™, SMART™, Clontech, USA). For the purpose of isolation 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. All the primers used in the study of part I were listed in Table 1. Sequence analysis, ORF detection, signal peptide prediction, N/O glycosylation site and rare codon analysis were performed using the DNAssit 2.1, 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.

Table 1. Oligonucleotide primers used in the part I experiments.

Primer name	Sequence
RT-PCR CLHd primer (Forward)	TCTGCTGTACCCTGTTGTTGCTGT
RT-PCR CLHd primer (Reverse)	TCGTCCATATCCAGCTTGACTCCA
RT-PCR actin primer (Forward)	GACTCTGGTGATGGTGTACCCA
RT-PCR actin primer (Reverse)	ATCTCCTTCTGCATTCTGTCGGC
Recombination primer (Forward)	GAGAGAGAATTCTCAAGGTGTAGA GGCGGA
Recombination primer (Reverse)	GAGAGAAAGCTTTTACATTTTACAT ATGAAATTGGCGA



2.3. Expression of CLHd mRNA in tissue distribution and in induction by *Vibrio* challenge

Three healthy abalones as a control were sacrificed and the tissues were immediately snap-frozen in liquid nitrogen and stored at -70 °C. The total mRNA was extracted from different tissues, including gill, mantle, digestive tract, muscle, and hemocytes.

In the bacterial challenge group, 1.0 ml live *V. alginolyticus* (O.D.600 = 1.0) was collected by centrifuge at 3000 rpm for 10 min and then resuspended in 100 µl of 0.1 M phosphate buffered saline (PBS). Fifty microlitre bacterial suspension or PBS, was injected into the adductor muscle of two-year old abalones. The abalones were sacrificed at each sampling time point (12 hr, 24 hr, 48 hr post the injection).

The RNA isolation was manipulated using TRI Reagent™ (Sigma, USA) followed the manufacturer's protocol. Except hemocytes, a 100 µg of each tissue sample was sliced into the pieces small as possible in a short time. In a 50 ml conical tube, a 1.5 ml of Tri Reagent was added and then the sample was homogenized completely till no visible particles. Through 5 min of standing in room temperature and 15 min of chloroform precipitation, all the samples were centrifuged (12000 ×g) at 4 °C for 25 min. Subsequently, 0.6 ml isopropanol were mixed with the collected pellet from the centrifuge. After a 15 min incubation in room temperature, the total RNA were separated from tissue samples by a 25 min of centrifuge (12000 ×g) at 4 °C. Finally, the total RNA of samples were washed one time using a 1.5 ml of cold ethanol. The remain pellet in each tube was dried for 5~10 min. Finally, the extracted RNA were dissolved in nuclease free water for the cDNA synthesis. The total RNA concentration was determined by spectrophotometer at the wave length of 280 nm.

The mRNA expression of CLHd in different tissues of healthy and bacteria challenged abalones were measured by semi-quantitative RT-PCR. Totally, 5 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). Briefly, RNA from each sample were incubated with 1 µl of 50 µM oligo (dT)₁₂₋₁₈, 2 µl of 10 mM dNTP mix and a appropriate amount of DEPC water (adjust the total volume to 10 µl) for 5 min at 65 °C. After chilling on the ice for 2 min, 4 µl of 25 mM MgCl₂, 2 µl of the 10 × reverse transcriptase buffer, 2 µl of dithiothreitol (0.1 M, DTT), 1 µl of RNaseOUT (40 U/µl) and 1 µl of SuperScript™ III reverse transcriptase (200 U/µl) were mixed gently with the products from the first step and collected by brief centrifuge. Then the mix was incubated for 1 h at 50 °C. The PCR reaction was terminated by adjusting the temperature to 85 °C for 5 min. Finally, 1 µl of RNase H was added to each tube and was incubated for 20 min at 37°C to remove the remain template RNA, which may interfere with the further PCR reaction. Then the target cDNA amplification 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 condition was optimized by going through several pilot PCR reactions. 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 sec, 53.5°C for 30 sec, 72 °C for 30 sec 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 densities were measured using Quantity One software (BioRAD, USA). The relative mRNA levels of CLHd in abalone samples were expressed as the ratio to that of actin RNA. Statistical 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 \pm SEM (n=3), and the differences were considered significant at $P < 0.05$.

2.4. In vitro protein expression and purification

2.4-1 Cloning and construction of the recombinant CLHd

Based on the CLHd full length cDNA sequence, specific PCR primers were designed to amplify the mature protein coding region of the CLHd gene. In a total of 50 μ l of PCR reaction, 5 units of Ex Taq polymerase (Takara Korea Biomedical Inc., Korea), 5 μ l of 10X Ex Taq buffer, 4 μ l of 2.5 mM dNTP, 50 ng of template and 50 pmol of each primer were used. After initial incubation at 94 °C for 2 min, 25 cycles were carried out with 30 sec denaturation at 94 °C, 30 sec of annealing at 55 °C, and 30 sec of elongation at 72 °C, followed by a final extension at 72 °C for 5 min. The PCR product was analysed using 1 % agarose gel and ethidium bromide staining. Thereafter, it was purified by the AccuprepTM gel purification kit (Bioneer Co., Korea) and digested with *Eco* RI and *Hind* III restriction enzymes.

The expression vector, pMAL-c2X, was digested with the same restriction enzymes as the PCR product and dephosphorylated with calf intestine phosphatase (NEB, USA) according to the relative protocol. Thereafter the vector and the PCR product was purified by a 1 % agarose gel using the Qiaex-II gel purification kit (QIAGEN Inc., USA). Ligation was carried out at 16 °C, overnight with 100 ng of pMAL-c2X vector, 70 ng of PCR product, 1 μ l of 10X ligation buffer and 0.5 μ l 1X T4 DNA ligase (Takara Korea Biochemical Inc., Korea).

2.4-2 sub-cloning and transformation to the host cell for protein expression

The competent cells of *E. coli* (DH10b) were prepared using calcium chloride method. The ligated product was mixed with 10 times of competent *E. coli* cells for 30 min on ices and then the DNA / *E. coli* cell mixture was transferred into a water bath in 40 °C for 2 min heat shock. After a recovery of 2 min, 80 μ l of the

DNA/*E. coli* cells mixture was plated on LB ampicillin plates. Large amounts of colonies were grown up in the plates after overnight incubation at 37 °C. Randomly, the candidate colonies were selected to confirm the integration of target DNA insert. The cloning plasmid DNA was compared with vector in size using gel electrophoresis. The plasmid containing correct recombinant DNA inserts were sent out for sequencing to ensure the fidelity of all the cloning work. Subsequently, the plasmid were extracted again. And then they were transformed into competent cells of *E. coli* (Rosetta-gami DE3) for the protein over expression.

2.4-3 over expression of the recombinant CLHd

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 till density approached OD₆₀₀ = 0.5~0.8. Then protein expression was induced by adding 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After 6 hr of induction at 37 °C, the cells were harvested by centrifugation at 3500 rpm for 30 min at 4 °C. Subsequently, all the cells were resuspended in a 6 ml column buffer (Tri-HCl, pH 7.4 + NaCl) and stored in -70 °C overnight.

2.4-4 The purification of the recombinant CLHd

After thawing, the bacterial cells were placed in an ice-water bath and sonicated six times in short pulses of 10 sec. Having centrifuged at 9000x g for 30 min at 4 °C, the supernatant was diluted with a 1:5 column buffer. The pMAL protein fusion and purification system was followed. In brief, amylose resin was poured into a 1 x 5 cm column and washed with 8 x column volumes of column buffer. The diluted crude extract was loaded at a flow rate of 1 ml/hr. The column was then washed with 12 x column volumes of column buffer and the fusion protein was eluted with elution buffer (column buffer + 10 mM maltose). The elute was collected in 500 µl

fractions. The purified recombinant CLHd was subjected to 12 % SDS-polyacrylamide gel electrophoresis at 30 mA for 50 min. The protein separation was visualized by staining with coomassie brilliant blue R-250 (Sigma, USA). Molecular weight protein standards (Unstained Plus, Biorad, USA) were used to determine the target protein size. Through the amylose resin column, protein was purified and used for activity assay.

2.4-5 Quantification of the protein concentration

Protein concentration was measured by the Bradford method (Bradford, 1976). A 50 mg coomassie blue G-250 (Sigma, USA) was dissolved in 20 ml of 95 % ethanol. With the addition of 50 ml of 85 % phosphoric acid (ortho-phosphoric-acid)-cation nasty stuff and distilled water, the final volume of coomassie blue solution were made up to 500 ml. The completely dissolved solution was filtered through a whatman No. 1 filter paper to remove the redundant particles. Then it is ready for the measurement of protein concentration. Although it can be stored in a amber bottle for four weeks principally, all the coomassie blue G-250 solution used in this study were prepared within one week. The standard absorbance curve was attained through the measurement of a 1 mg/ml of bovine serum albumin (BSA) solution in series dilution. The formular between the absorbance and protein concentration was finally constructed as : $Y = (23.63x - 0.048) / 5$. Every protein sample was measured 2~60 min after the addition of the coomassie blue G-250 solution.

2.5. Bacterial agglutination assay

The bacterial recognition of CLHd was assessed by a bacterial agglutination test following a similar method described in (Maruyama et al., 1975; Tunkijjanukij and Olafsen, 1998; Yang et al., 2007). The gram negative non-pathogenic *E. coli* (DH5 α), 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 $\mu\text{g}/\text{ml}$) 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 a light microscopy.

2.6 Hemocyte agglutination assay

The hemocyte agglutination assay was manipulated in the similar method described in (Tunkijjanukij et al., 1998; Yang et al., 2006) with a little modification. Fresh blood sample were taken out from human ('o' blood type) or horse by syringe preloaded sodium citrate (1/9 volume). Erythrocytes were separated from plasma by centrifuge at 2500 rpm within 10 minutes. Then we washed the erythrocytes three times in phosphate buffered saline (PBS) (pH 7.4, 0.19 M NaCl; 1.01 mM NaH_2PO_4 ; 2.35 mM Na_2HPO_4) and suspended at 2 % in the same buffer (v/v). Four microliter of 25 % glutaraldehyde was carefully added to per milliliter erythrocyte suspensions, incubated for 15 min and washed three times with PBS before use or storage at 4 °C. On 96 well plate we add 20 μl 2 % blood sample and mixed with 60 μl PBS as control or 30 μl CLHd (0.6mg/ml) supplemented by 30 μl TBS calcium solution (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM CaCl_2). Hemocyte agglutination was observed at room temperature under a microscope.

3. Results

3.1 EST cDNA library screening

Sequencing of the abalone normalized cDNA library yielded more than 8000 EST sequences. With the assistance of sequence assembly software, all the sequence were

arranged as contig or singleton of genes. Nine candidate clones homologous to C-type lectin genes were detected according to their expect value in BlastX analysis (Table 2). Since their identity with known gene sequence are not high (40~55 %), the sequence analysis is manipulated to ensure the selected clone with high integrity in functional cis-elements.



Table 2. C-type lectins in Normalized cDNA library of abalone (*Haliotis discus discus*).

Homologous Gene	NCBI Access No.	Clone number	Full size	ORF size	E-Value (Blast X) (in 2007.11)
Perlucin (<i>Haliotis discus discus</i>)	P82596	K431	523bp	381bp	2e-45
		53-C11* (Perlucin)	1129bp	453bp	3e-40
		50-E11* (CLHd)	526bp	453bp	6e-31
		K413	808bp	495bp	2e-18
		15-E07	656bp	495bp	3e-17
		42-C04	1025bp	486bp	1e-11
Galactose-specific C-type lectin (<i>Aedes aegypti</i>)	XP_001657848	65-G10	615bp	465bp	7e-19
C-type lectin A (<i>Chlamys farreri</i>)	ABB71672	69-G04	644bp	495bp	2e-17
		17-F10	1444bp	489bp	7e-17

* labelled the clones used in the following study

3.2 Sequence analysis of the full length CLHd

BlastX 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*. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 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 152 amino acid residues, a 3' UTR of 29 bp and a polyA tail. In N terminal, a putative cleavage site was predicted after the position 22 (GAEG-SRC) by webpage server (<http://www.cbs.dtu.dk/services/SignalP/>). The deduced mature CLHd protein had a theoretical mass of 15 KDa and an isoelectric point of 9.1. In order to optimize the in vitro protein expression of recombinant CLHd, the glycosylation site and translation codon usage of CLHd was analyzed using the relative computer server. The coding sequence of CLHd contained six arginine condons, which were in low frequency (< 0.1 %) of *E. coli* usage. In the CLHd mature protein sequence, no significant glycosylation site was predicted through the computer software analysis.

<i>TCTTCCCCAAG</i>	ATG	AAA	ACG	TTC	TGC	TGT	ACC	CTG	TTG	TTG	CTG	TTG	47		
	<u>M</u>	<u>K</u>	<u>T</u>	<u>F</u>	<u>C</u>	<u>C</u>	<u>T</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>L</u>	12		
GGT	TGT	GCC	CTC	CAC	CGT	GGT	GCT	GAA	GGT	TCA	AGG	TGT	AGA	GGC	92
<u>G</u>	<u>C</u>	<u>A</u>	<u>L</u>	<u>H</u>	<u>R</u>	<u>G</u>	<u>A</u>	<u>E</u>	<u>G</u>	<u>S</u>	<u>R</u>	<u>C</u>	<u>R</u>	<u>G</u>	27
GGA	TTT	CAC	AAG	CAC	GGA	GGT	TCC	TGC	TAT	TGG	TTC	TCC	AAC	ATA	137
<u>G</u>	<u>F</u>	<u>H</u>	<u>K</u>	<u>H</u>	<u>G</u>	<u>G</u>	<u>S</u>	<u>C</u>	<u>Y</u>	<u>W</u>	<u>F</u>	<u>S</u>	<u>N</u>	<u>I</u>	42
CGG	GGA	ACA	TTT	GCC	GAG	GCA	AGA	TCA	ATA	TGC	CGC	TTC	CTT	GGG	182
<u>R</u>	<u>G</u>	<u>T</u>	<u>F</u>	<u>A</u>	<u>E</u>	<u>A</u>	<u>R</u>	<u>S</u>	<u>I</u>	<u>C</u>	<u>R</u>	<u>F</u>	<u>L</u>	<u>G</u>	57
AGT	GAC	CTT	GCA	TCC	ATC	ACC	AGT	GCA	GCT	GAA	GAT	GTC	TTT	ATC	227
<u>S</u>	<u>D</u>	<u>L</u>	<u>A</u>	<u>S</u>	<u>I</u>	<u>T</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>V</u>	<u>F</u>	<u>I</u>	72
AGA	GGG	TAT	GCG	ACC	CAG	AGA	GGC	AAA	GCT	AAG	GTT	TAC	TAC	CTG	272
<u>R</u>	<u>G</u>	<u>Y</u>	<u>A</u>	<u>T</u>	<u>Q</u>	<u>R</u>	<u>G</u>	<u>K</u>	<u>A</u>	<u>K</u>	<u>V</u>	<u>Y</u>	<u>Y</u>	<u>L</u>	87
GGA	GGC	GCC	GAC	CTC	GGT	CTG	GAG	TCA	AGC	TGG	ATA	TGG	ACG	AGG	317
<u>G</u>	<u>G</u>	<u>A</u>	<u>D</u>	<u>L</u>	<u>G</u>	<u>L</u>	<u>E</u>	<u>S</u>	<u>S</u>	<u>W</u>	<u>I</u>	<u>W</u>	<u>T</u>	<u>R</u>	102
AAC	AAA	CCC	TTT	ACT	TTC	ACC	AAC	TGG	GGA	TCT	GGG	CAA	CCT	GGA	362
<u>N</u>	<u>K</u>	<u>P</u>	<u>F</u>	<u>T</u>	<u>F</u>	<u>T</u>	<u>N</u>	<u>W</u>	<u>G</u>	<u>S</u>	<u>G</u>	<u>Q</u>	<u>P</u>	<u>G</u>	117
AAC	TCA	AAA	AAT	AAT	GAA	CAC	TGT	CTT	GCA	CTT	CAA	AGT	AGT	GAT	407
<u>N</u>	<u>S</u>	<u>K</u>	<u>N</u>	<u>N</u>	<u>E</u>	<u>H</u>	<u>C</u>	<u>L</u>	<u>A</u>	<u>L</u>	<u>Q</u>	<u>S</u>	<u>S</u>	<u>D</u>	132
GGA	TAT	CGT	TGG	CAT	GAT	TAC	AAC	TGT	GAT	TTT	ATC	GCC	AAT	TTC	452
<u>G</u>	<u>Y</u>	<u>R</u>	<u>W</u>	<u>H</u>	<u>D</u>	<u>Y</u>	<u>N</u>	<u>C</u>	<u>D</u>	<u>F</u>	<u>I</u>	<u>A</u>	<u>N</u>	<u>F</u>	147
ATA	TGT	AAA	ATG	TAA	<i>CTCGATCAATTAAACTATATCTGCAGGACAAAAAAAAAAAAA</i>										508
<u>I</u>	<u>C</u>	<u>K</u>	<u>M</u>												151

Figure 1. The nucleotide and deduced amino acid sequence of CLHd from abalone, *Haliotis discus discus*. The nucleotides of untranslated region are in italics. Amino acid residues of predicted signal peptide are underlined. The nucleotides and amino acids are numbered along the right margin.

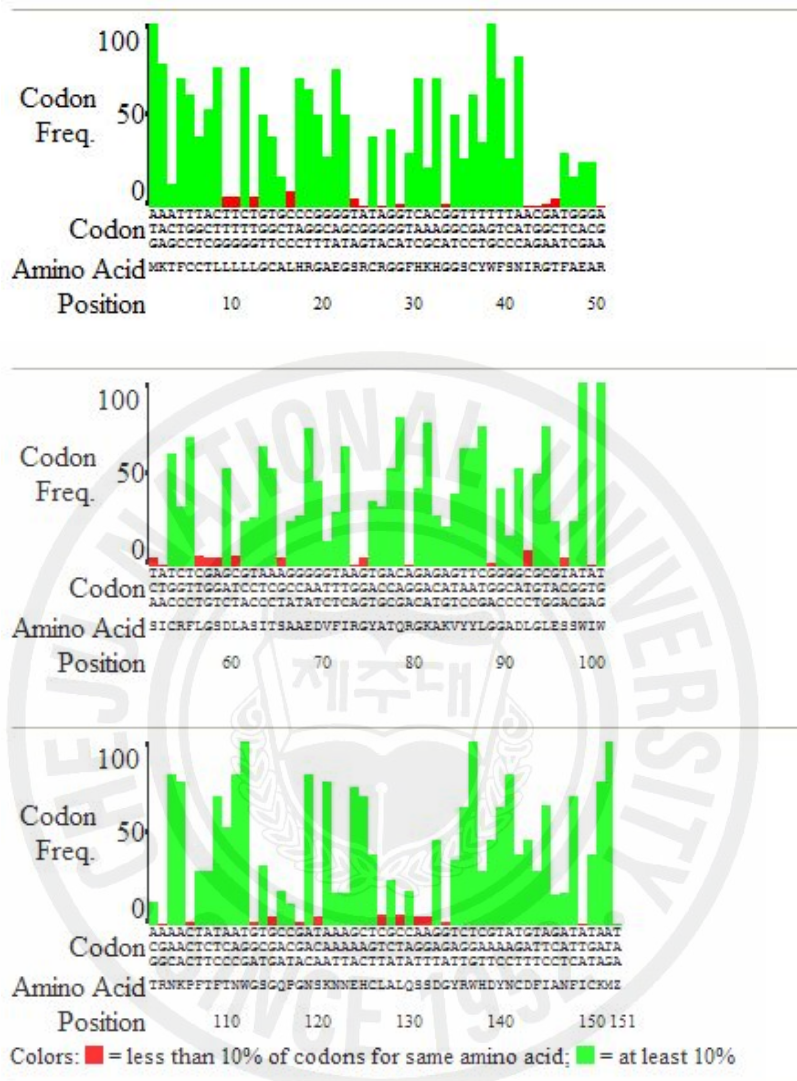


Figure 2. The *E. coli* codon usage analysis of the CLHd coding sequence. Note the arginine codon in the deduced amino acid residue list, the codon usage frequency of the position 24 (AGG), 26 (AGA), 50 (AGA), 73 (AGA), 79 (AGA) and 102 (AGG) are lower than 0.1 %.

3.2. Homology comparison and characterization of CLHd

When the CLHd sequence was blasted against the database of national center of biotechnology information (NCBI), the comparatively low expect value of its alignment with some C-type lectins indicated its homology to this group protein in the view of molecular structure. The multiple alignments with the homologous proteins showed that CLHd was highly conserved in six cysteine residues and the carbohydrate recognition domain (CRD) (Fig. 3).

In most typical C-type lectins, there were several signature sequences: 4 disulfide-bonded cysteine residues, which defined the CRD, and 2 additional cysteine residues at the amino terminus (Drickamer, 1993). In CRD regions, there are in total 23 conserved residues, including 6 cysteine residues, 4 tryptophan residues and 4 glycine residues (Kolatkar and Weis, 1996; Sujatha, and Balaji, 2004). The "QPD" and the invariant "WND" motif near the C-terminal region (boxed by broken lines in Fig. 3) are extremely important in polysaccharide recognition and the calcium binding of lectins. Mostly, the "QPD" motif means that galactose binding, if it is substituted with "EPN," has specificity for mannose (Iobsts and Drickamer, 1994). CLHd exhibited highest amino acid sequence similarity (48 %) with perlucin in the CRD region. However, the full amino acid sequence of CLHd shared not more than 40% similarity with that of any homologous C-type lectin.

CLHd	<i>H. discus discus</i>	CRGGFHKHGGSCYWFVSNIRGTFAEARSIORFLGSDLASITSAAD 45
Perlucin	<i>H. laevigata</i>	CPLGFHQNRRCYWFVSTIKSSFEEAAGYCRYLESHELAII SNKDED 45
Serum lectin isoform 2	<i>S. salar</i>	CPTGWFQFGSRCFMFVETARSWFLAERHOVSLGANLASVHSSADD 45
C-type MBL-2 protein	<i>O. mykiss</i>	CPDSDWHPYGRSFRFVSIPQSWSDSBQNCLALGGNLA SVNNLLEY 45
C-type MBL	<i>O. mykiss</i>	CPDSDWYTYGSHCFKFVSIQSFVDSBQNCLALGGNLA SVHSLLEY 45
C-type lectin A	<i>C. farreri</i>	CPEGVWSNKNACYHISRETEEWVAEAMOKIYGALVHLETTAED 45
C-type lectin 2	<i>A. japonica</i>	CPEGWKDFNGSCYKHFDDLKNWREAESHOMTQGGHLASVHSNVEY 45
C-type lectin 1	<i>A. japonica</i>	CPEGWKGFNGCCYKHFDDLKNWREAIFYOMIRGGHLASVHSNVEY 45
CLHd	<i>H. discus discus</i>	VFIRGYATQR--GKAKVYYLGSADLGLLESSWITWT-RNKPFTFTN 86
Perlucin	<i>H. laevigata</i>	SFIRGYATRL--GEAFNYWLGASDLNIEGRWLWE-GQRRMNYTN 86
Serum lectin isoform 2	<i>S. salar</i>	QFLQAIAGCKT--GAFSTTWIGGFDWQDRLWFWS-DGSEFDYQN 87
C-type MBL-2 protein	<i>O. mykiss</i>	QFMQALTKNTN--GHLPTWIGGFDWVKEGLWVWS-DGSRFDYTN 87
C-type MBL	<i>O. mykiss</i>	QFMQALTKDAN--GHLHSTWLGGFDAIKEGTWVWS-DGSRFDYTN 87
C-type lectin A	<i>C. farreri</i>	NFLSEYLRNNSIVYNDHQYWIQLSDWEFEGFTIWWPEGVTPGYTN 90
C-type lectin 2	<i>A. japonica</i>	EFLRELKASD--PWDSTIWIQLTDIQKEGTWVWS-DGSAVDFTT 87
C-type lectin 1	<i>A. japonica</i>	QFLRELNKASD--PQDSMFWIGLTDIRKEGTWVWS-DGSAVDFTT 87
CLHd	<i>H. discus discus</i>	WGSGQDFGNSKNEHCLALQSSDG-YRWHDTNDFIANFTCKM 127
Perlucin	<i>H. laevigata</i>	WSPGQPDNAGGIEHCLELRRLDGNLYWVNDYQQKPSHFICEK 128
Serum lectin isoform 2	<i>S. salar</i>	WAKGEPNNSGGREPCIVINWGDYRWHNDIKGNSFPPSVGSK 128
C-type MBL-2 protein	<i>O. mykiss</i>	WNTGEPNNAEGEGEDCLQMNAASE-KLWFDVPEWKFVSLCSR 128
C-type MBL	<i>O. mykiss</i>	WDTDEPNNAEGEGEDCLHMNAASA-KLWFDVPEWKFVSLCSR 128
C-type lectin A	<i>C. farreri</i>	WPGGEPDNNHQNHQECTIIHTQEH-YGWFDRMONEQYSYICEL 131
C-type lectin 2	<i>A. japonica</i>	WDSKQPDNNAEGEGEDCVHANVPEQ-KLWVNDMSQSESYRFTICAL 128
C-type lectin 1	<i>A. japonica</i>	WNPQEPDNNNAEGEGEDCVHANVPEQ-KLWVNDMSQSTPYRFTICAL 128

Figure 3. Multi amino acid sequence alignment of the CRD region from CLHd and its homologous C-type lectins. Six highly conserved cysteine residues were labeled by asterisks; two motifs for the ligand binding were boxed using broken lines. All the conserved amino acid residues were shaded for the homologous comparison. All the referred sequences are from NCBI: C-type lectin 2 from *Anguilla japonica*, BAC54021; C-type lectin 1 from *Anguilla japonica*, BAC54022; C-type MBL-2 protein from *Oncorhynchus mykiss*, CAJ14130; C-type mannose-binding lectin from *Oncorhynchus mykiss*, AAM21196; Perlucin from *Haliotis laevigata*, P82596; C-type lectin A from *Chlamys farreri*, ABB71672.

3.3. 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 (Fig. 4). In *V. alginolyticus* challenged abalones, the relative expression level of CLHd was significantly up-regulated in the digestive tract (Fig. 5). At 12 hr post *V. alginolyticus* injection, the induction of mRNA was initiated and reached maximum expression at 24 hr. As the time progressed, mRNA transcription of CLHd dropped to the control value at 48 hr post the *Vibrio* challenge. All the gel electrophoresis results were quantitatively measured using Quantity One software (BioRAD, USA). At 24 hr post *Vibrio* challenge, it showed 2.5 times more CLHd mRNA expression than the control group and significant up-regulation in one-way analysis of variance ($P < 0.05$) (Fig. 6).

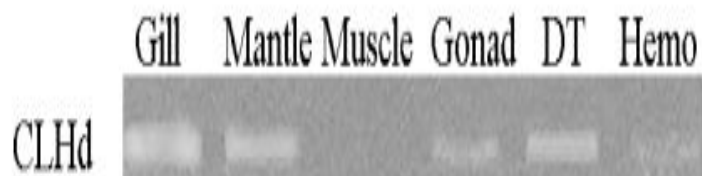
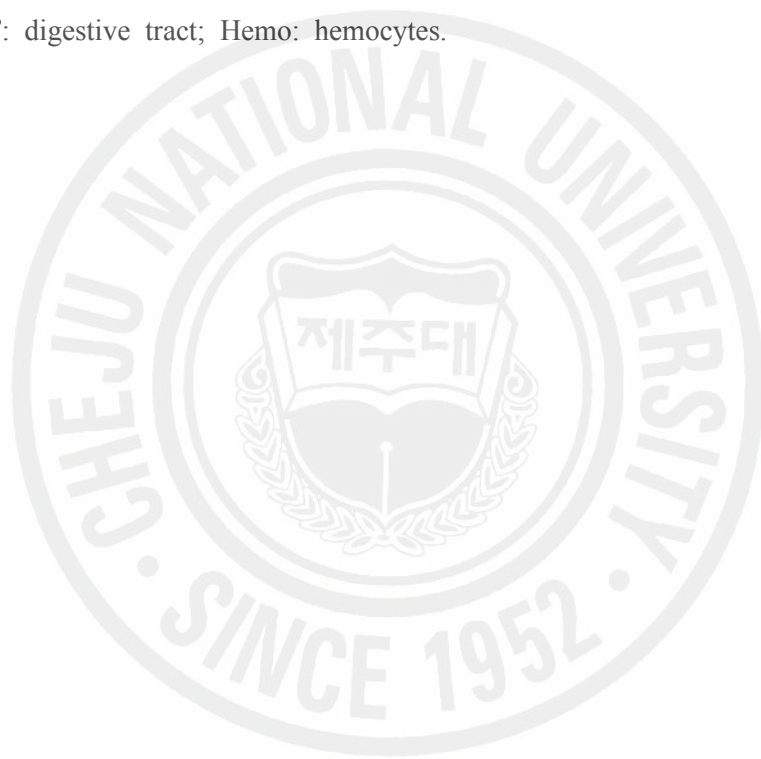


Figure 4. Tissue distribution of CLHd mRNA transcript in abalone. All the PCR products were run 1.2% agarose gel electrophoresis. Actin was selected as internal control; DT: digestive tract; Hemo: hemocytes.



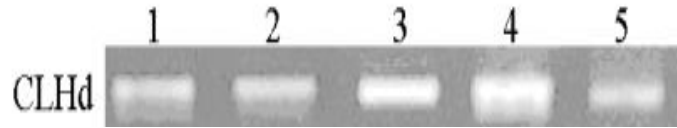


Figure 5. The mRNA temporal transcript profile of CLHd induced by *V. alginolyticus* challenge. 1: control; 2: PBS injection; 3: 12 hr post *Vibrio* injection; 4: 24 hr post *Vibrio* injection; 5: 48 hr post *Vibrio* injection.



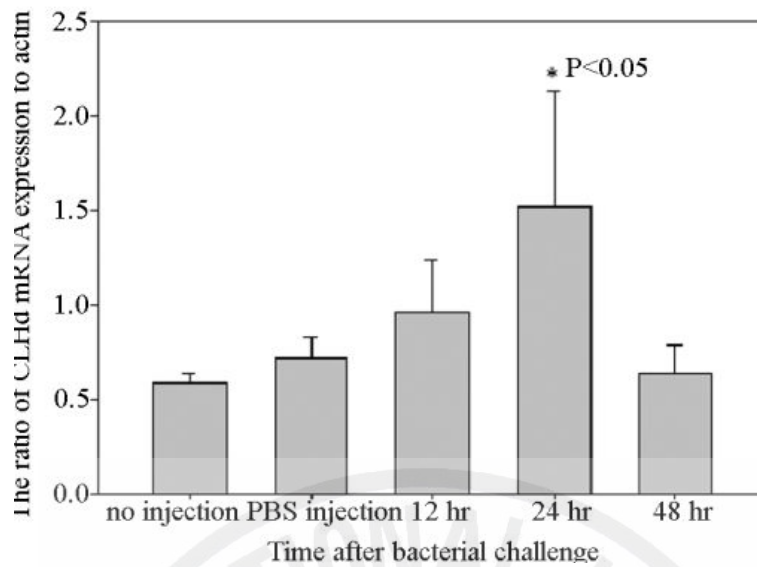
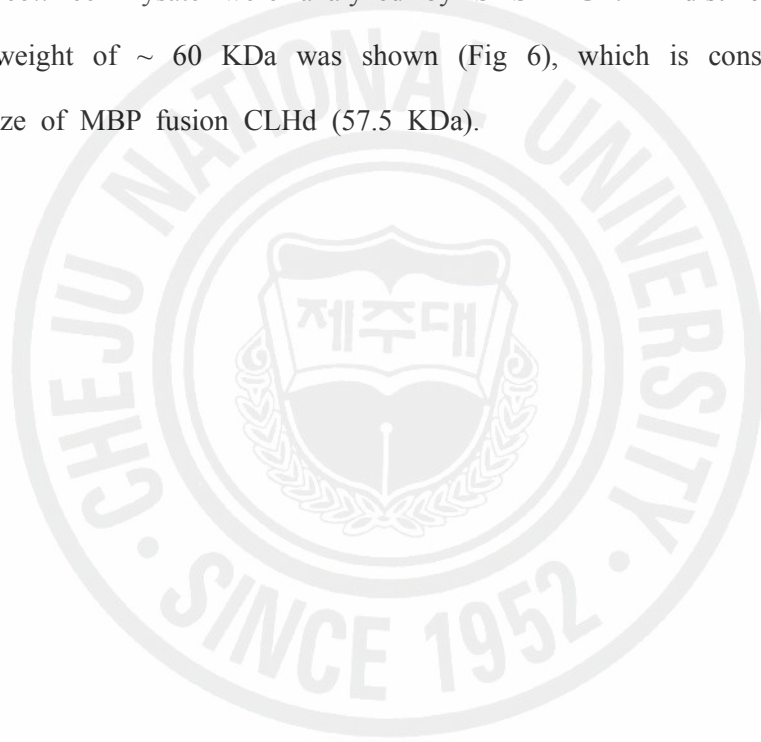


Figure 6. The relative CLHd mRNA expression in the digestive tract of abalone after *V. alginolyticus* injection. Semi-quantitative PCR was repeated three times. The CLHd levels were expressed as the ratio between CLHd mRNA and the mRNA of actin (internal control) in the same sample. Data were presented as means \pm SEM. Asterisk indicates statistically significant differences ($p < 0.05$ by Tukey test, $n=3$).

3.4. Recombinant expression of CLHd in *E.coli*

With the aim of characterization the function of CLHd, the coding sequence of CLHd was ligated into a pMal-c2X protein expression system (NEB, USA). The recombinant protein was expressed in *E. coli* (Rosetta-gammi DE3). In the pMal-c2X expression system, it provided the fusion MBP in the N terminal, which might enhance the solubility of recombinant CLHd and make the protein purification in a easy way. After IPTG induction of 3 hr, 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 shown (Fig 6), which is consistent with the predicted size of MBP fusion CLHd (57.5 KDa).



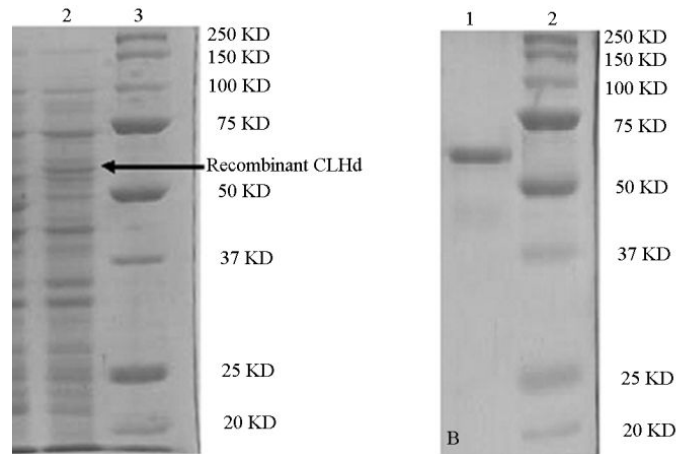
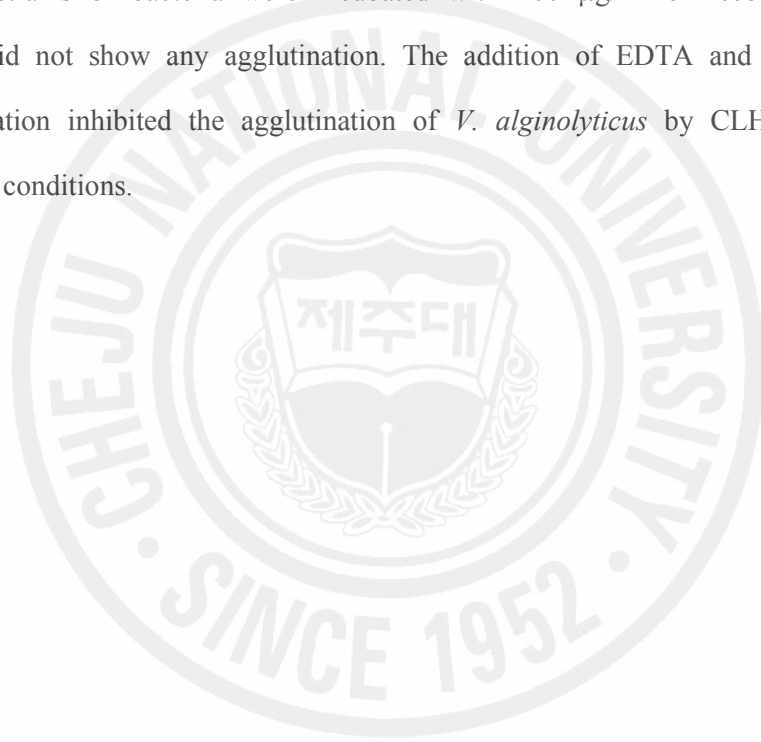


Figure 7. SDS-PAGE gel analyses of recombinant CLHd. (A) The SDS-PAGE analysis of recombinant CLHd expressed in *E. coli* (Rossetta gammi). Lane 1: proteins of *E. coli* (Rossetta gammi) without induction; lane 2: the recombinant CLHd expression induced by 0.5 mM IPTG in *E. coli* (Rossetta gammi); lane 3: Precision plus protein unstained standards (Bio-Rad, USA). The specific band of recombinant CLHd is indicated by the arrow. (B) The 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.

3.5. Bacterial agglutination activity of CLHd

The gram negative no-pathogenic *E. coli* (DH5 α), pathogenic gram negative *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 $\mu\text{g/ml}$, was obvious agglutination observed in *V. alginolyticus* solution (Fig. 8B) but not in *E. coli* and *B. subtilis*. Even when the latter two strains of bacteria were incubated with 100 $\mu\text{g/ml}$ 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.



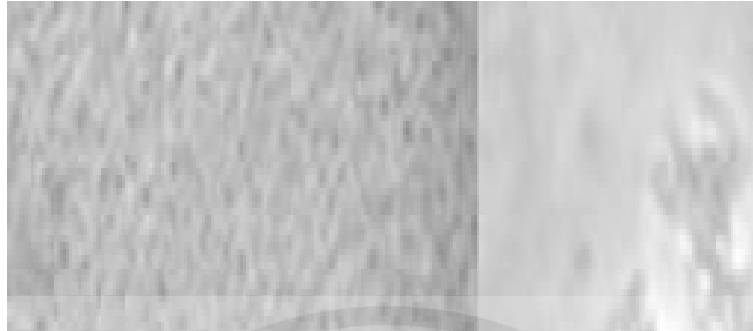


Figure 8. The agglutination of *V. alginolyticus* induced by CLHd. (A) the control *V. alginolyticus* with pure TBS buffer; (B) the *V. alginolyticus* incubated with 50 µg/ml recombinant CLHd in TBS-Ca buffer.

3.6 Hemocyte agglutination activity of CLHd

No visible agglutination was detected in the "O" type human blood cells or horse hemocytes, even when the addition of CLHd reached a concentration of 100 $\mu\text{g}/\mu\text{l}$ and the incubation time was extended for several hours.

4. Discussion

One of the most intriguing characteristics of the immune system is its competence to recognize and respond to pathogens with considerable specificity. It can be postulated that the need for the organisms to recognize potential pathogens and distinguish between "self" and "non-self" arose at the time of the appearance of the first multicellular organisms and has played an essential role in the maintenance of their homeostasis. In vertebrates, the recognition of pathogens is carried out by two distinct types of immune receptors. The adaptive immune system is characterized by the generation of antibodies and T-cell receptors which have the capacity to recognize an innumerable repertoire of allogenic molecules. On the other hand, innate immunity relies on a restricted number of genotypically encoded molecules such as toll-like receptors (TLRs), integrins, nucleotide oligomerisation domain molecules (NODs) and scavenger receptors (known as pattern recognition receptors, PRRs) to recognize and respond to pathogens or their pathogen-associated molecular patterns (PAMPs) (Medzhitov, 2001; Zipfel and Felix, 2005). Pattern recognition receptors can be secreted proteins present in the body fluids. They can also be expressed on the cell surface or expressed in the cytoplasm. Functionally, pattern recognition receptors can be involved in: i) opsonization of bacteria and viruses for phagocytosis or activation of the lectin pathways of complement; ii) uptake of pathogens by phagocytes and dendritic cells; or iii) triggering of the signaling pathways that result in the induction of transcription of a variety of immune response genes, such as the

antimicrobial peptides and inflammatory cytokines (Fraser et al., 1998; Stahl and Ezekowitz, 1998; Buffer et al., 2003).

Invertebrates do not have antibodies and therefore they have to rely on their innate immune response to efficiently protect themselves from a variety of pathogens (Vazquez, 1997; Loker et al., 2004). Recognition of nonself materials in innate immune is mediated by PRPs, which could recognize and bind to different molecules on the surface of invading microorganisms and then trigger a series of immune responses.

There are six sub-groups of PRP in invertebrate animals, peptidoglycan recognition protein (PGRP), thioester-containing protein (TEP), gram-negative binding protein (GNBP), mulidomain scavenger receptor (SCR), C-type lectin (CTL), and galectin (GALE) (Christophides et al., 2002). C-type animal lectins represent an important recognition mechanism for extensive oligosaccharides and proteins in the serum and extracellular matrix, and for proteins and lipids on the surface of cells (Drickamer, 1988). C-type lectins in invertebrates are proved to be involved in various biological responses, for instance activation of the prophenoloxidase system, antibacterial activity , promotion of phagocytosis, and nodule formation (Suzuki et al., 1990; Tateno et al., 1998; Koizumi et al., 1999; Yu et al., 1999; Yu and Kanost, 2000; Yu et al., 2005; Luo et al., 2005). The most important one is its contribution in invertebrate immunity that C-type lectin may recognize and bind to terminal sugars on glycoproteins and glycolipids, and distinguish nonself antigens and initiate the clearance of invaders (Iwanaga and Kawabata, 1999; Yu and Kanost, 2003). In mollusks, the main mechanisms of neutralization and elimination of potential pathogens are phagocytosis and encapsulation (Sminia et al., 1974; Loker et al., 1982; Feng, 1988; Adema et al., 1992). These cellular defense reactions are closely to the activity of humoral factors and are intermediated by lectins such as

agglutinins, opsonins, and lysins (Boswell and Bayne, 1985; Chu, 1988; Fisher and Dinuzzo, 1991; Ottaviani et al., 2004).

In the present study, one C-type lectin with novel *Vibrio* agglutinating activity was identified from abalone, *H. discus discus*. Unlike the traditional ways of lectin identification, the screening of 8000 EST cDNA library clones may detect all the C-type lectin genes in one time including some novel lectins, which can not be isolated using protein purification and sequencing method owing to its low translation level or unknown polysaccharide binding specificity. For example, CLHd is a novel lectin, which does not agglutinate human being blood cells or horse hemocytes as the other lectins from mollusk (Yakovleva, et al., 2001).

In the view of its molecular structure, the highly conserved CRD and six disulfide bond cysteines classified CLHd as a C-type lectin structurally. The 508 bp nucleotide cDNA of CLHd may encode 151 amino acid premature proteins. 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 as a C-type lectin structurally and provide Ca²⁺ dependent sugar-recognition activity to initiate a broad range of biological processes, such as adhesion and pathogen neutralization. In the functional residues for ligand binding, CLHd shows QPG at the residues of 97-99, which is different with the typical "QPD" or "EPN", and the amino acid residues of 136-138 is not the invariant "WND" but "WHD". All these structural differences of CLHd with its known homologues suggest that CLHd is a novel C-type lectin.

In healthy abalones, CLHd mRNA transcript was mainly distributed 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 were more susceptible to pathogen infection. The tissue distribution of CLHd suggested it might offer the initial response to an invasion of marine pathogenic micro-organisms. 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 hr. Therefore, CLHd is inducible and sensitive to *Vibrio* infection. The abrupt increase of CLHd mRNA transcription can be a dangerous signal indicating the pathogenic infection in abalones.

To understand the specific biological activity of CLHd, it was expressed heterologously in *E. coli* and the bacterial agglutination assay tested. The recombinant CLHd specifically agglutinated *V. alginolyticus* in a calcium dependent way. The dependence of calcium indicated CLHd was functionally a typical C-type lectin.

Basically, the bacterial agglutination immobilized the invading *Vibrio* and inhibited its further tissue invasion. Through its CRD, CLHd may selectively bind to the conserved carbohydrate residues on the surface of invading cells and thereby discriminated the pathogens in an efficient way. This provided the self and non-self-recognition necessary for the activation of abalone complement system, since abalone lacks immunoglobulins (Coombe. et al., 1984). Although the specific role of CLHd in innate immunity still need to be further investigated, the lectin pathway has been well established in its homologous C-type lectins such as mannose binding lectin and ficolin (Ofek and Sharon, 1988). After lectins bind to the non-self antigens, the lectin complement pathway is activated and then it will trigger the opsonization of pathogens, chemotaxis and activation of cytokines, and direct killing of pathogens (Teiz, 2002; Sodhi and Kesherwani, 2007). Taken all together, CLHd was an important agglutinin in abalones for the recognition and clearance of *Vibrio* pathogens.

The characterization of CLHd enriched the knowledge of abalone immunity and provided a powerful immune molecule for abalone disease control. Since it is highly inducible by *Vibrio* infection, in future applications, CLHd can be a useful marker for the monitoring of abalone farming environment and developing the husbandry management. Being as an effective *Vibrio* agglutinin, the presence of large amount of CLHd may definitely enhance the disease resistance of abalone. Gene selection or transgenic breeding of abalones with intensive expression of CLHd will provide a promising way to save juvenile abalones from the infection of *Vibrio* disease.



Part II

Recombinant perlucin nucleates the growth of calcium carbonate crystals: molecular cloning and characterization of pearl formation gene from disk abalone, *Haliotis discus discus*

1. Abstract

Perlucin was well known as the pearl formation protein in abalone and a good research model of bio-mineralization and pearl formation. In this study, we isolated perlucin gene by screening disk abalone (*H. discus discus*) cDNA library. The full length cDNA of abalone (*H. discus discus*) perlucin gene consisted of 1038 bp nucleotide encoding putative signal peptide of 22 amino acids and a mature protein of 129 amino acids, which shared 55 % identity with greenlip abalone perlucin gene. The coding sequence was inserted into pMal-c2X expression vector and expressed the recombinant protein in *Escherichia coli* (Rosetta-gammi DE3). In protein activity assay, the in vitro expressed perlucin successfully promoted calcium carbonate precipitation and directed crystal morphological modification in super saturated calcium carbonate solution. Reverse transcription polymerase chain reaction (RT-PCR) results showed that perlucin gene was expressed in gill, mantle, and digestive tract, where directly confront outside environment. It implies perlucin may play some protective roles against invasive pathogenic micro-organisms like other similar C-type lectins. Further characterization of perlucin in non-self recognition is promising and anticipated.

Key words: Perlucin; Abalone (*Haliotis discus discus*); Protein expression; *Escherichia coli*.

2. Materials and Methodology

2.1 Materials

Healthy adult abalones, *H. discus discus* ranging from 8 to 9 cm in body size (wet body weight at about 90 g) were purchased from abalone farms. To ensure the abalone was in natural growth condition, all the abalone were dissected on site and stored in liquid nitrogen immediately.

2.2 Molecular cloning and sequencing

The abalone (*H. discus discus*) cDNA library was synthesized by isolating total RNA from a whole abalone and using a cDNA library construction kit (Creator™, SMART™, Clontech, USA). Referred to single-pass random sequencing result and sequence assembly program (<http://pbil.univ-lyon1.fr/cap3.php>) analysis report, we chose the candidate clone of perlucin from our abalone cDNA library. Then we designed specific internal primers and completed its full length cDNA sequencing (Table 3). ORF detection, signal peptide prediction, N/O-glycosylation site and rare codon analysis were performed by DNAssit 2.1, webpage server (<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.

Table 3. Oligonucleotide primers used in the part II experiments.

Primer name		Sequence
Internal sequencing primer	(Forward)	ATATGAGGTGTGGTGTGC
RT-PCR perlucin primer	(Forward)	GCTGCTCACATTTGCATCATTGC
RT-PCR perlucin primer	(Reverse)	GTTGGTGTACGTCATGGCCTTGTT
RT-PCR actin primer	(Forward)	GACTCTGGTGATGGTGTACCCCA
RT-PCR actin primer	(Reverse)	ATCTCCTTCTGCATTCTGTGCGC
Recombination primer	(Forward)	GAGAGAGAATTCTGTCCTCTGGGAT TCTTTCG
Recombination primer	(Reverse)	GAGAGAAAGCTTTTAAAGGTTCTTCTCA CAGATAAAGTTTAAG



2.3 Protein expression and purification

Based on the perlucin full length cDNA sequence, specific PCR primers were designed to amplify the mature protein coding region of the perlucin gene. PCR products were digested by restriction endonucleases and ligated to expression vector pMAL-c2X under the instruction of kit manual (NEB, USA). Finally, the recombinant plasmids were transformed into *E. coli* (Rosetta-gammi DE3) for protein expression. A volume containing 5 ml of starter culture was inoculated into 100 ml 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 till density approached $OD_{600} = 0.5\sim 0.8$. Then protein expression was induced by adding 0.5 mM IPTG. After 6 hr induction at 37 °C, the cells were harvested by centrifugation at 3500 rpm for 30 min at 4 °C. Subsequently, all the cells were resuspended in 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 centrifuged at 9000 x g for 30 min at 4 °C, the supernatant was loaded into a maltose binding resin column. Through amylose resin columns, proteins were purified and used for activity assays. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed under reducing condition (with 2-ME) to analysis the expression and purification of the recombinant perlucin. The gel (8 × 10 cm and 1.5 mm thick) was subjected to electrophoresis at 30 mA for 50 min. Molecular weight protein standards (Unstained Plus, Biorad, USA) were used. The gel was stained with coommasie brilliant blue R-250 (Sigma, USA). Protein concentration was calculated by Bradford method (Bradford, 1976).

2.4 Protein activity assay

To make sure the success of cloning and expression, here we tested our recombinant perlucin in two ways as mainly used in previous researches (Samata et

al., 1999 Weiss et al., 2000; Zhang et al., 2003; Suzuki et al., 2004). In pMal-c2X expression system, it provided the fusion MBP in N terminal, which might enhance the solubility of recombinant perlucin and simplify the protein purification. Since the recombinant perlucin has more attractive interests in further application, for example, color modification of pearls by recombinant perlucin gene transfer, the N terminal MBP fusion perlucin was directly tested by calcium carbonate precipitation and crystallization.

2.4-1 Calcium carbonate precipitation test

A calcium carbonate precipitation experiment was performed according to previous work with a few modifications (Morales et al., 1996). Briefly, 20 mM NaHCO₃ was adjusted to pH 8.70 by adding 1 N HCl and 1 N NaOH and supplemented with 0.5 M KCl to keep the total ionic strength close to that of seawater. Then 6 ml 20 mM CaCl₂ was immediately mixed with the same volume NaHCO₃ solution in 50 ml conical tubes. Fifty microlitre 1 mg/ml maltose binding protein fusion perlucin (MBP-perlucin) was added to the calcium carbonate solution. As the control, distilled water or protein buffer solution (1.0 M Tris-HCl, pH 7.4 and 200 mM NaCl) was added into the calcium carbonate super saturated solution. The pH variation was determined by istek (720P model) pH meter every ten seconds.

2.4-2 Calcium carbonate crystallization test

About 30 ml of 100 mM NaHCO₃ was added dropwise under permanent stirring to 120 ml of a 40 mM CaCl₂ solution (Blank, et al. 2003). As soon as the solution became turbid, the addition of CaCl₂ was stopped and the pH was adjusted to 8.2 with 1 N NaOH. If the pH value of the crystallization solution is too low (less than 7.7) or too high, it will cause pH drift during incubation (Walters et al. 1997). Thus we chose pH 8.2 for the crystallization test after several pilot experiments. The saturated calcium carbonate solution was then filtered using a 0.22 µm filter. 50 µl

perlucin of 1 mg/ml was added into 300 μ l to the above prepared calcium carbonate solution in 24 well plates to promote calcium carbonate crystallization. The addition of the same volume of protein buffer (1.0 M Tris-HCl, pH 7.4; 200 mM NaCl) or 1 mg/ml Bovine serum albumin (BSA) was used as the control group treatment. Samples were incubated at 4 °C and the typical crystal morphology was recorded by digital camera under a stereo invert microscope.

2.5 RT-PCR

Total mRNA was isolated individually from mantle, gill, the digestive tract, muscle, and hemocytes by Tri reagent (Sigma, USA). In total, 2.5 μ g RNA was used for cDNA synthesis by following the manual of superscript III cDNA PCR synthesis kit (Invitrogen, USA). RT-PCR was then performed using two specific primers from the perlucin coding sequence. A 492 bp fragment of the actin sequence was amplified as internal PCR control. In order to avoid saturation of the PCR product the PCR condition was optimized according to several pilot experiments. Finally, the PCR condition followed the protocol: one cycle of 94 °C for 2 min, 30 cycles (or 23 cycles for actin) of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and one cycle of 72 °C for 5 min.

3. Results

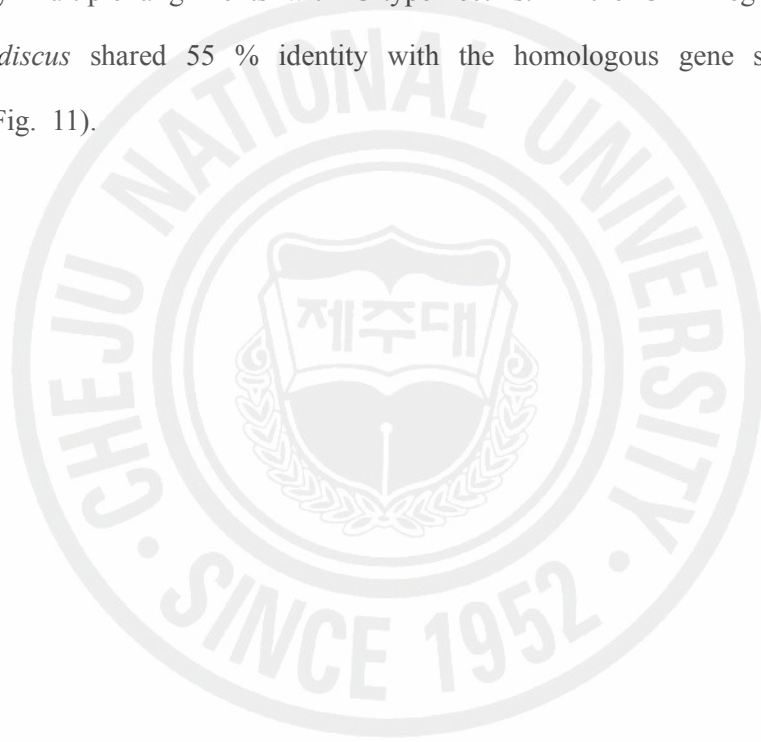
3.1 Perlucin cDNA Sequence and analysis

The full length cDNA of perlucin from abalone, *H. discus discus* consisted of 29 bp of 5' untranslated region (UTR), 456 bp of open reading frame, and 506 bp of 3' untranslated region (Fig. 9). The perlucin cDNA encoded a polypeptide of 151 amino acids with a putative signal peptide of 22 amino acid residues and a mature protein of 129 amino acids.

<i>ATTCAAGTCTGACGCTGCCAACCAAGC</i>	ATG AGA CTC GAA GTC CTA TCA TTC CTG CTG CTC	62
	<u>M R L E V L S F L L L</u>	11
ACA TTT GCA TCA TTG GCT CAA GCA CGC GGC CGG TGT CCT CTG GGA TTC TTT CGG CAC		119
<u>T F A S L A Q A R G R C P L G F F R H</u>		30
GGG AAA TCC TGC TAC TGG ATC TCA ACC ACT GTG GCG TCG TTC GCT GAA GCG AAG ACA		176
G K S C Y W I S T T V A S F A E A K T		49
TAC TGT CAG TAT ATG CAG AGT CAC CTG GCG AGG ATA ACC AGC AGA TAT GAG GAT GCC		233
Y C Q Y M Q S H L A R I T S R Y E D A		68
TTT CTG AGG GGC CAA GTC AGA CGT CGT GGC AAG GGT ACT GAT TAC TGG CTT GGT GCT		290
F L R G Q V R R R G K G T D Y W L G A		87
ACT GAC TTG AAC GTT GAA GGC GTG TGG CTG TGG GAG GGG AAC AAG GCC ATG ACG TAC		347
T D L N V E G V W L W E G N K A M T Y		106
ACC AAC TGG GGT GGT GAC AAC CCT AAT AAT TAC AAA GGA GTT GAA AAC TGT CTG GGA		404
T N W G G D N P N N Y K G V E N C L G		125
CTG AGA AAG GAG AAT AAC TAC CAG TGG AAT GAT TTT CAG TGT CAT CAC CGC TTA AAC		462
L R K E N N Y Q W N D F Q C H H R L N		144
TTT ATC TGT GAG AAG AAC CTT TAAAATCAGAATCAGTCCTTAAAAATATCAGTAAATGTTGGGCAAC		530
F I C E K N L		151
<i>ACCTTGCTTTTCTTAAAATAAAACATAACCACAATCTTTGAATAAGGTATCATAATGGTATAATGTCTAACAAAT</i>		605
<i>TCCTTGACACTTGGATAGGGAAAACACTGTGCAAATAACAGCAACAAAATCATTCCATAATCATAAGAAATA</i>		680
<i>TCTGCTAAAAGCAGAGTCCTTGTACTATTTGACTATATCATGAGCATGTGTTATGTGTTCCCTGCGCCTTGC</i>		755
<i>GTGGAGGTGTGGCATGATGTGCTGTTGCAGCATATAGGTGAGGTGTGGTGTGCTGTTGCAGCATATATGTGAGGT</i>		830
<i>GTGGTGTGCTCATGTATCTGGAATCTGGAAACATTTATGAACTGTACACATACATTTCTTTTCTTCAAGA</i>		905
<i>ATGTAAGGTGTTGCTGAACATCTTCTGAGTAAGGTAAACTGCATATGTAATTGCCTTCTTAATAAGAAATAGAC</i>		980
<i>TGCATTTACAAGAA</i>		1038

Figure 9. The nucleotide sequence of the cDNA clones and deduced amino acid sequence of perlucin from abalone, *Haliotis discus discus*. Amino acid residues of predicted signal peptide are underlined; the nucleotide of untranslated region are in italic.

The predicted perlucin has a molecular weight of 15 KD, and an isoelectric point of 5.7. Although perlucin from abalone *H. discus discus* is an extracellular protein, there was no glycosylation site detected in the perlucin cDNA sequence of abalone *H. discus discus*. In total, 20 rare transcription codon of *E. coli* was distributed in a deduced amino acid sequence of perlucin. Six arginine codon are almost not available in a normal *E. coli* cell (Fig. 10). Six highly conserved cysteine residues and one single carbohydrate recognition domain (CRD) were detected in perlucin amino acid sequence by multiple alignments with C-type lectins. In the CRD region, perlucin of *H. discus discus* shared 55 % identity with the homologous gene sequence of *H. laevigata* (Fig. 11).



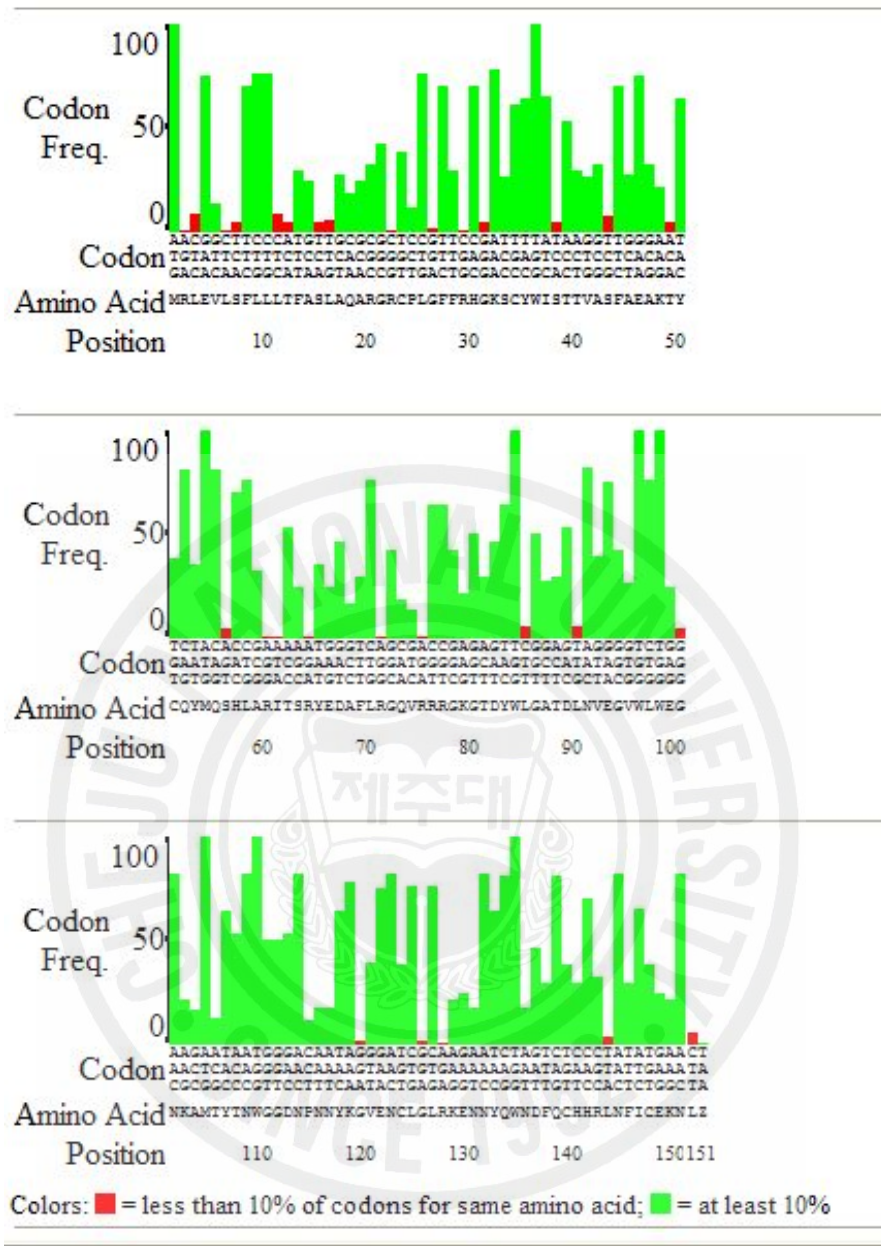


Figure 10. The *E. coli* codon usage analysis of the perLucin coding sequence. Note the arginine codon in the deduced amino acid residue list the condon frequency of the position 2 (AGA), 60 (AGG), 64 (AGA), 71 (AGG), 75 (AGA) and 127 (AGA) are lower than 0.1 %.

```

                                     +       +       +
Perlucin (H. Laevigata)                GCPLGFHQNRRCYWFSTIKSSFAEAAGYC 30
Perlucin (H. discus discus) MRLEVLSFLLLTFASLAQARG RCPLGFFRHGKSCYWISITVASFAEAKTYC 51

RYLESHLAIISNKDEDSFIRGYATRLGEAFNYWLGASDLNIEGRWLEWQRRMNYTNWSPGQPDNAGGIEHCL 103
QYMQSHLARITSRYEDAFLRGQVRRRGKGTDYWLGAIDLNVEGVWLVEGNKAMTYTNWGGDNPNNYKGVENCL 124

ELRRDLGNYLVNDYQCQKPSHFICEKERIPYTNSLHANLQQRDSLHANLQQR 155
GLRKEN-NYQVNDFQCHRLNFICEK-----NL 151

```

Figure 11. Alignment of the amino acid sequence of perlucin from *Haliotis discus discus* and the homologous sequence from *Haliotis laevigata*. Perlucin of *Haliotis laevigata* (GenBank accession No. P82596). All the conserved amino acid residues were shaded. Asterisks label the highly conserved cystine residue; dots point out six highly conserved residues involved in calcium dependent carbohydrate binding in C-type lectin group proteins.

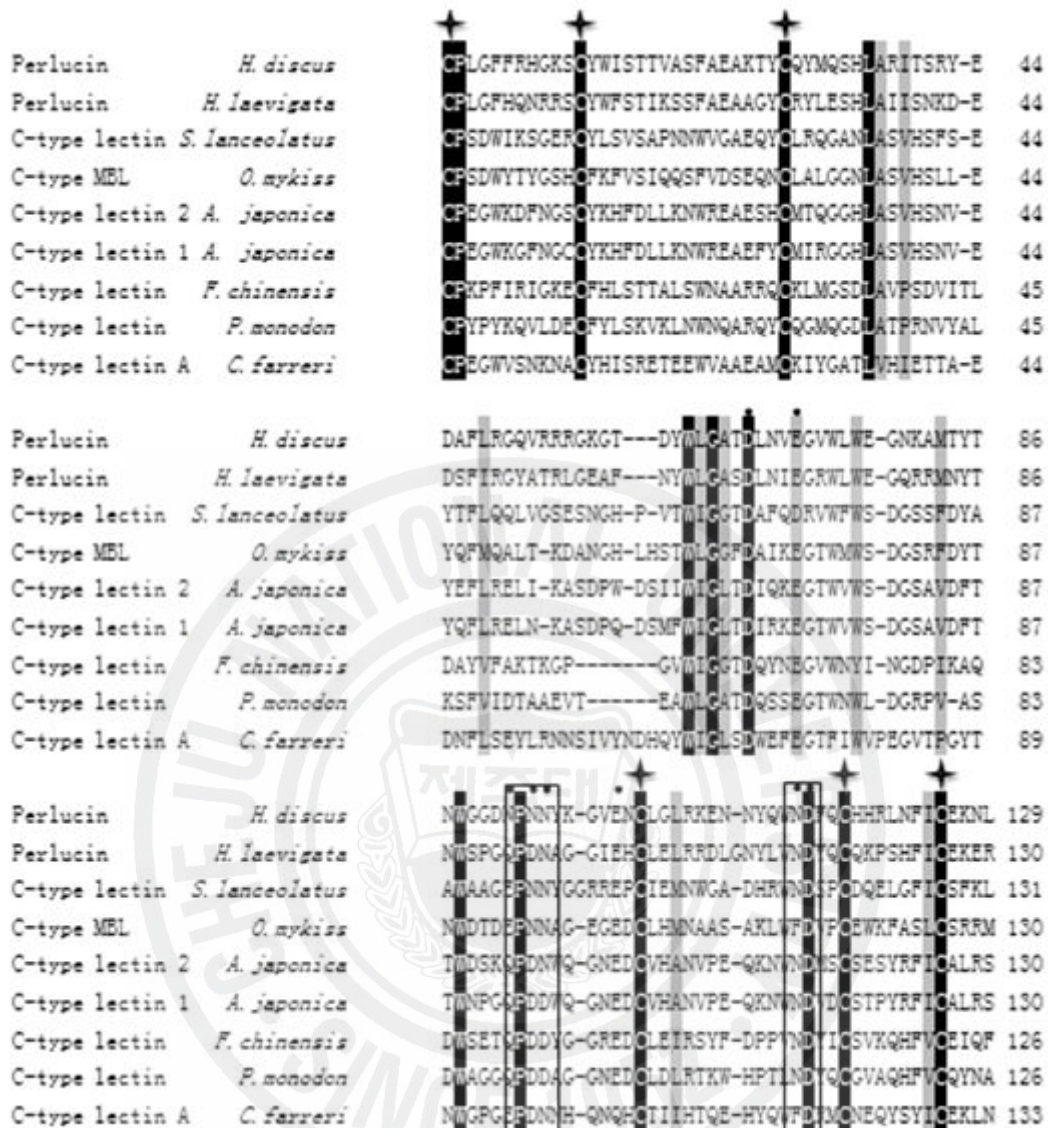


Figure 12. Comparison of carbohydrate recognition domains from C-type lectins homologous to perlucin. Asterisks label highly conserved cystine residue dots point out the important conserved residues in carbohydrate recognition domain of C-type lectins rectangular box the motif "NPN" and "WND", which are proposed to determine carbohydrate binding specificity of lectins. All the conserved amino acid residues were shaded for the homologous comparison. All the referred sequences are from NCBI: *Haliotis laevigata* P82596, *Spirinchus lanceolatus* BAE45334,

Oncorhynchus mykiss AAM21196, *Anguilla japonica* BAC54021, *Anguilla japonica* BAC54022, *Fenneropenaeus chinensis* AAX63905, *Penaeus monodon* ABI97373, *Chlamys farreri* ABB71672.



The carbohydrate recognition domain (CRD) of C-type lectins contains 4 disulfide-bonded cysteine residues that define the CRD and 2 additional cysteine residues at the amino terminus (Drickamer 1993). There are in total 23 conserved residues among these proteins, including 6 cysteine residues, 4 tryptophan residues and 4 glycine residues (Kolatkhar and Weis 1996; Sujatha and Balaji 2004). The "QPD" and the invariant "WND" motif near the C-terminal region (labeled by rectangular symbol in Fig. 12) are extremely important in polysaccharide recognition and the calcium binding of lectins. Mostly, the "QPD" motif means that galactose binding, if it is substituted by "EPN," has specificity for mannose (Iobsts and Drickamer 1994). However, in the corresponding sites, "NPN" and "WND" suggest the specific carbohydrate binding of perlucin from abalone (*H. discus discus*) cannot be predicted by this way.

3.2 Protein expression and purification

The molecular weight of the maltose binding protein fusion with perlucin was predicted to be 57.5 KD. In the SDS PAGE gel, a clear band was detected between 50 KD and 75 KD that matched with our prediction (Fig. 13). The pMAL-c2X maltose binding expression system successfully expressed soluble MBP-perlucin and provided a convenient purification method. Generally, 1 mg of purified soluble proteins could be isolated from 50 ml of bacterial cells.

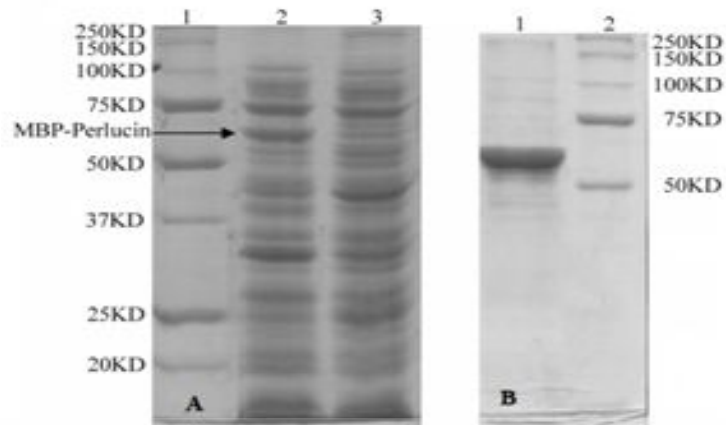


Figure 13. SDS-PAGE gel analyses of MBP fusion perlucin. (A) The perlucin expression induced by 0.5 mM IPTG. Lane 1: protein marker lane; 2: proteins of *E. coli* (Rossetta gammi); lane 3: MBP fusion perlucin induced by IPTG for 3 h. The molecular mass in KDa is shown in the left side of gel and the band of MBP fusion perlucin was indicated by arrow. (B) MBP fusion perlucin purified by resin column. Lane 1: protein marker; lane 2: purified MBP fusion perlucin. The molecular mass in KDa is shown in the right side of gel.

3.3 Recombinant perlucin activity

Mollusk nacre growth depended on calcium carbonate accumulation and formation of aragonite crystal. The effect of perlucin on calcium carbonate precipitation directly reflected its contribution in pearl formation. When the appropriate amount of CaCl_2 was added to NaHCO_3 , the pH value dropped suddenly from 8.7 ± 0.03 to 8.45 ± 0.05 in the first 10 seconds and then slowly decreased to 8.07 ± 0.18 . As shown in Fig. 14, pure MBP without perlucin, did not induce any changes in the tendency of pH variation, while in the presence of MBP-perlucin, pH value dramatically decreased to 8.30 ± 0.03 in the first 10 seconds and reached 8.07 within 150 ± 30 seconds.

In the assay of calcium carbonate crystallization, the morphology of calcium carbonate crystals was recorded daily. After 24 hr incubation at 4°C , large amount of calcite crystals were present. Since the polymers of calcite crystals always showed variant figures and sizes, here we just recorded and compared the morphological modification of a single crystal.

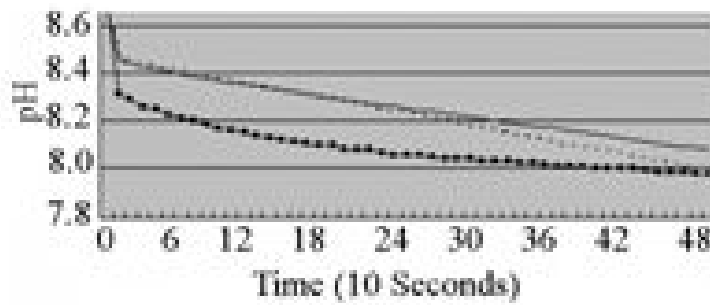


Figure 14. Recording of Calcium carbonate precipitation with or without perlucin in super saturated solution. Note the addition of recombinant perlucin in the saturate solution resulted in the rapid decrease of pH, which indicated the accelerated precipitation of calcium carbonate.

—Saturated calcium carbonate solution — MBP Prtotein ; ··· MBP-Perlucin.

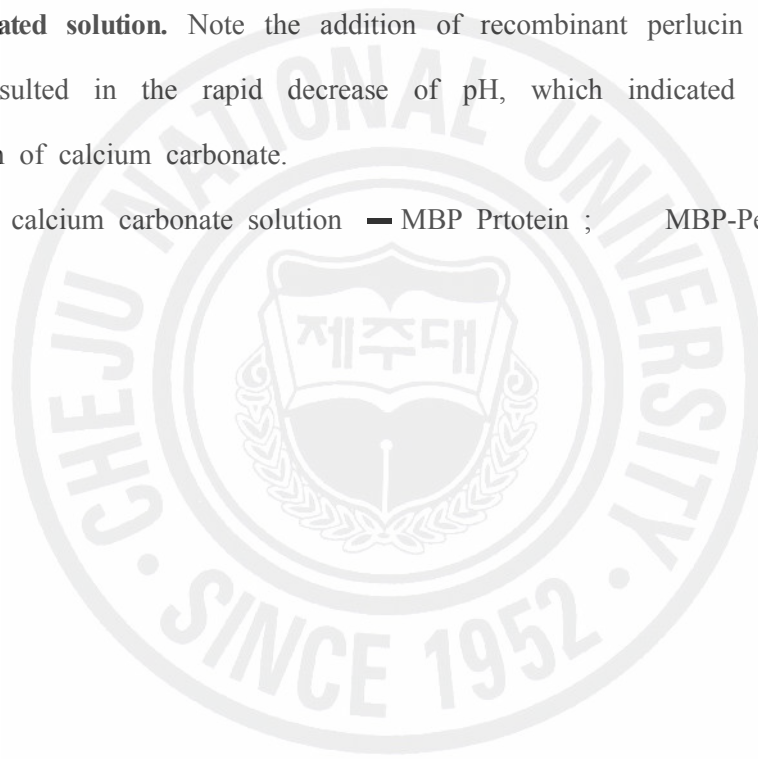




Figure 15. Light microscopic images of crystals grown in presence or absence of recombinant perlucin. (A) The control calcite crystal after incubation at 4 °C for 24 h; (B) the calcium carbonate crystal after perlucin treatment for 24 h (200 ×); (C) the calcium carbonate crystal after perlucin treatment for 24 h (400 ×); (D) the calcium carbonate crystal after perlucin treatment for 72 h (200 ×); (E) the calcium carbonate crystal after perlucin treatment for 72 h. Note the presence of recombinant perlucin rounded the edges of calcite crystals gradually and all the sharp corners disappeared after incubation for 72 h and accompanied a rhomohedral protuberance occurrence in the top of crystals, which was indicated by arrow. Scale bar: 20 μm.

As shown in Fig. 15A, the calcite was in standard rhombohedral shape, which has six parallelogram-shaped faces; each face was conjoined to two neighboring faces at an acute angle (78°) and two other faces at an obtuse angle (102°). The addition of BSA in protein elution buffer or MBP without perlucin did not induce distinguished morphological modifications. At the acute corner, the crystal became round firstly and was not rhombohedral any more after 24 hr perlucin treatment at 4°C (Fig. 15B). Gradually, the calcite crystal surfaces were roughed and lost all the sharp corners. After 72 hr incubation with perlucin, an approximate hexagonal crystal was promoted (Fig. 15D and 15E). Interestingly, a small rhombohedral protuberance occurred on the top of the crystal with the crystal growth (Fig. 15C and 15E).

3.4 Tissue distribution of perlucin mRNA transcription

To get the better understanding of the physiological role of perlucin gene in abalone, its tissue distribution was investigated by RT-PCR. In healthy adult abalone, the perlucin gene is mainly expressed in mantle, gill and subordinately expressed in the digestive tract; on the contrary no expressions were detected in muscle, gonad and hemocytes (Fig. 16).

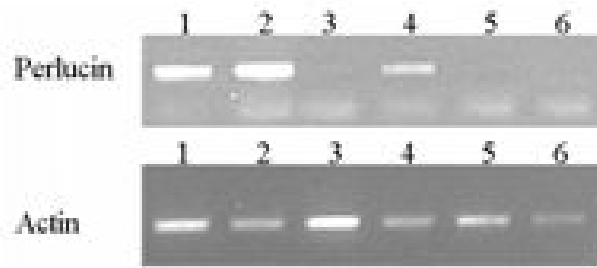


Figure 16. Tissue distribution of perlucin transcript revealed by RT-PCR in abalone *H. discus discus*. 1: Gill; 2: Mantle; 3: Adductor muscle; 4: Digestive Tract; 5: Gonad; 6: Hemocytes.



4. Discussion

The full length perlucin cDNA of abalone *H. discus discus* is 1038 bp and encoded a mature protein of 129 amino acids. Although it is shorter than the perlucin sequence from greenlip abalone *H. laevigata*, they share a high identity (55 %) in amino acid sequence and are conserved in almost all important functional residues. The most abundant amino acid residue of perlucin was glycine. In total, 12 glycine and 5 aspartic acid residues occupy 12 % of all the 129 amino acids, which are proposed to alter the surface equilibrium thermodynamics of calcium carbonate crystal growth by their terminal carboxyl group (Teng et al., 1998; Orme et al., 2001). Perlucin of abalone *H. discus discus* contains one single carbohydrate recognition domain and six highly conserved cysteines. Therefore, it is a typical C-type lectin, however, the specificity of polysaccharide binding still needs to be determined by further experiment, such as thru hemocyte agglutination assay.

The existence of a signal peptide indicates that perlucin is an extracellular protein. However, it is poorly glycosylated. Only one candidate N glycosylation site is present in the perlucin sequence of greenlip abalone *H. laevigata* and not even one single glycosylation site is detected in the perlucin cDNA sequence of abalone *H. discus discus* (Mann et al., 2000). No intensive glycosylation makes it possible to express perlucin in prokaryotic protein expression, which is cost-efficient but incapable to perform glycosylation.

In order to obtain optimal protein expression, the codon usage bias of *E. coli* has to be considered. The presence of codons used rarely in *E. coli* may inhibit the protein expression properly (Zhang et al., 1991; Wada et al., 1992; Kane, 1995; Gao et al., 1997). In our pilot experiment, we failed to induce perlucin expression in common *E.coli* (K12TB1). The recombinant proteins were well induced in host cell of Rosetta gammi (DE3), which could supply the most rare transcription codons

necessary for eukaryotic protein expression in prokaryotic cells.

In mollusk, its calcifying epithelium of mantle may extrude abundant calcium and bicarbonate ions (about 5 mM) into the extra-pallial space, a micro-volume extra-cellular cavity, and where it is possible for calcium and bicarbonate ions to reach a super saturated condition (Giles et al., 1995; Weiner and Addadi, 1997; Callil and Mansur, 2005). By stimulations of the invaded foreign particles or abalone growth hormone, large mounts of extra-pallial fluids secreted from mantle were released into extra-pallial space to precipitate calcium carbonate in the form of calcite or aragonite crystal (Young, et al., 1977; Krampitz, 1982; Eyster and Morse, 1984; Weiner, 1984; Wheeler and Sikes, 1989; Wilbur and Bernhardt, 1984; Lowenstam and Weiner, 1989; Hattan et al., 2000; Westermann et al., 2005; Moueza et al., 2006). Under the organization of relative proteins, these calcite or aragonite tablets either were deposited on a shell surface for shell growth or encapsulate the offending entity by pearl formation. The accumulation and precipitation of calcium carbonate supply the materials for a shell bio-mineralization and a pearl formation (Nair and Robinson, 1998; Comps et al., 2000; Checa, 2000). Therefore, the recombinant perlucin was firstly tested by calcium carbonate precipitation assay.

At all the measuring time points in the first three minutes, the recombinant perlucin accelerated the pH decrease of calcium saturated CaCO_3 solution that was consistent with previous research results, although our control pH variation curve was in absence of a pH upward drift of saturated calcium carbonate solution mentioned in previous studies (Weiss et al., 2000; Zhang et al., 2003). In fact, the similar pH drift happened in our primary test. However, it was alleviated after we adjusted the ionic strength with potassium chloride and avoided fierce stirring, since loss of CO_2 from solution to atmosphere and low ionic strength might cause pH measurement unstable and thereby accompanied the upward pH drift (Covington et al., 1985

Walters et al., 1997; Bates, 1973). In CaCO_3 solution, the pH value depended on the concentration of calcium carbonate. The promotion of pH decrease suggested perlucin possessed some positive effects on the precipitation of CaCO_3 and made it possible to accumulate sufficient calcium carbonate for pearl formation in abalones at short time.

However, as mentioned in my introduction part, the extraordinary property of pearl mostly is due to the unique structure of aragonite crystals assembled from organic matrixes. Therefore, calcium carbonate crystallization assay was performed to test the interaction between perlucin and calcium carbonate crystals. In ambient temperature, calcite is the dominant stable polymorph of calcium carbonate whilst aragonite presence need higher temperature (over than $50\text{ }^\circ\text{C}$) or mental iron addition (Aizenberg et al., 1999; Raz et al., 2000; Gower and Odom, 2000). Calcite possesses standard rhombohedra shape and aragonite crystal is in needle like style. (shown in Fig. 17 refereed from Treccani, 2006).

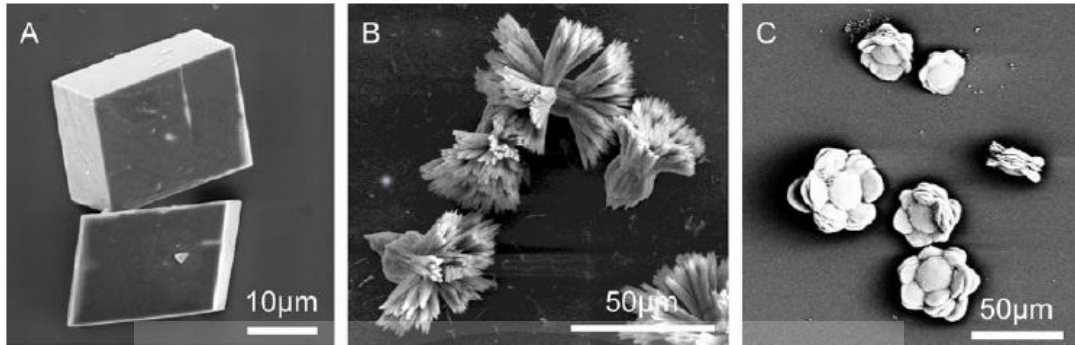


Figure 17. SEM images of allotropic calcium carbonate crystals precipitated from saturated calcium carbonate solutions (Refereed from Treccani, 2006). (A) Calcite crystals are characterized by a rhombohedral shape. Calcite is the most abundant and thermodynamically stable polymorph at ambient conditions. (B) Aragonite typically forms needle-like crystals. (C) Vaterite florets. Vaterite is the most thermodynamically unstable polymorph.

In this study, calcium carbonate crystallization with perlucin was tested at 4 °C to ensure the recombinant protein stable. In Figure 15, perlucin showed its activity in morphological modification of calcium carbonate crystal. Perlucin gradually rounded calcium carbonate crystal at acute corners but not at all the corners at once. This indicated that perlucin may recognize a specific calcite crystal surface and modify the sharp edges of a calcite crystal, gradually. The calcium carbonate of morphological modification was not only contributed to the final structure of a pearl or a shell but also the growth velocity and orientation. According to thermodynamic analysis, theoretically the rounded surface edges may lower the energy barrier of crystallization. In step growth of the Ca-CO₃, the ion pair while moving will encounter a lower energy barrier on the obtuse step than it in an acute step, if we count in all the ion pairs moving direction (Mann et al., 1993; Duckworth et al., 2004).

Another noteworthy point in our results was that we detected one growing rhombohedral protuberance on the c-axis of the calcite crystal. On the calcite surface, every flat region is called a terrace and the raised partial layers are called steps. In these step corners there are kink sites where molecules may attach kinks that will make more bonds to neighboring molecules than the ones that attach to the terraces or to other places of the step (Fig. 18).

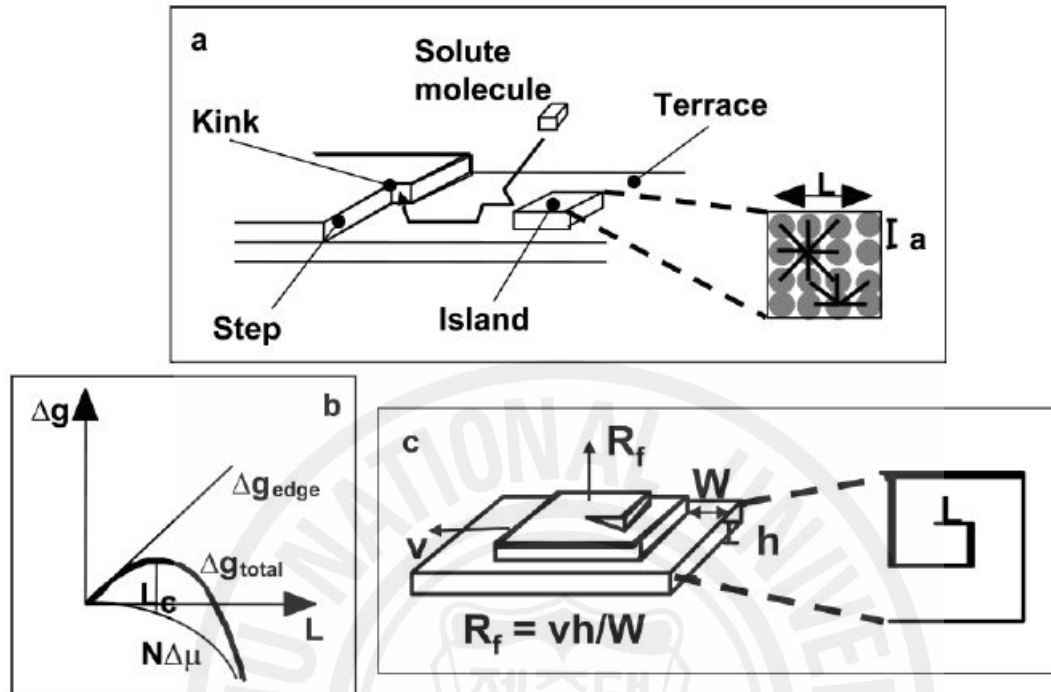


Figure 18. The illustration of kink sites (Referred from Yoreo and Vekilov 2003). (a) Illustration of the atomic processes during crystal growth. Solute molecules enter kinks either directly from solution or after adsorbing and diffusing across terraces. Islands can nucleate on the terraces provided they overcome the free energy barrier to 2D nucleation illustrated in (b). (c) shows the geometry of a dislocation hillock. The existence of a critical size leads to the formation of the spiral structure, since the new segment of the step cannot move until it reaches that size.

As the result, the rate at which molecules can be added to a crystal, for a given solute concentration, scales with the kink density (Yoreo and Vekilov 2003). Rough surface and irregular shape obviously will provide more kink sites for molecular binding. To be simple, the rough surface and irregular shape will do better in calcium crystal nucleation. Therefore, perlucin initially rounded the corner of calcite and roughed the adjoining surfaces which resulted in a promotive effect on the crystal growth in the *ab*-plane. When all the corners became rounded, the growth becomes inhibited due to kink site limits on the *ab*-plane, while the new rhombohedral step provides plenty of new kink sites to continue the calcite growth on the *c*-axis. We proposed that perlucin promoted the growth of calcium carbonate crystals on *ab*-plane first and when calcite crystals completed every step in four corners, the crystal growth in *ab*-plane was inhibited and then triggered the growth in *c*-axis.

Although in our experiments, the calcium carbonate was in the form of calcite not aragonite, it still exhibited the contribution of perlucin to nucleate calcium carbonate crystallization, template the crystals growth and modify the calcium carbonate crystal structure. The recombinant perlucin showed similar activity with perlucin isolated from greenlip abalone shell in promotion of calcium carbonate precipitation and modification of crystal morphology at ambient conditions. These results confirmed that our cloning and heterologous expression of perlucin in *E.coli* was successful. The highly purified soluble perlucin provided the material precondition for further characterization of its biochemical activity. N terminal fusion with maltose binding proteins did not block its activity of the regulation of calcium carbonate crystals in two assays. It suggested that recombinant perlucin gene transfer probably was practical to improve pearl's value, such as color modification.

In the view of protein structure, perlucin is a typical C-type lectin, which contains a highly conserved carbohydrate recognition domain (Mann et al., 2000). Many C-type lectin of invertebrate animals serve as recognition molecules involved in defensive

biological events such as anti-bacteria, promotion of phagocytosis, bacteria agglutinating and nodule formation (Suzuki et al., 1990; Luo et al., 2006; Wang et al., 2007; Koizumi et al., 1999). Therefore, perlucin probably plays some important roles in specific glycocalyx recognition of abalone. RT-PCR results showed strong expression in mantle indicating perlucin was synthesized by mantle and mixed with other proteins and polysaccharide as extrapellial fluids for abalone nacre growth. However, mantle was not the only place for perlucin mRNA intensive expression. The expression of the perlucin gene in the gill and digestive tract suggested perlucin may also play a similar protective role in non-self antigen recognition by specific glycocalyx binding as other similar C-type lectins from scallop, shrimp and fish (Mistry et al., 2001; Galliano et al., 2003; Hosono et al., 2005; Nikolakopoulou and Zarkadis 2006; Luo et al., 2006; Tsutsui et al., 2006; Liu et al., 2007). Since gill and digestive tract always involved in continuously water exchange and food uptake from outside environment to abalone, these tissues were more susceptible to pathogen infection and need humoral immune molecules to create barriers against the invasion pathogenic micro-organisms in ocean.

In a conclusion, it should be a reasonable speculation that perlucin is a kind of defensive molecule against pathogenic microorganisms and damaging detritus. When abalones can not eject the invaded parasites or particles, perlucin will be secreted to regulate nacre entomb the offending entity as a means of inactivation the non-self materials. With the growth of nacre layer on the offending particle, a beautiful pearl is produced. Perlucin played central roles in abalone pearl formation and inner nacrein growth. However, in the view of C-type lectin, more study about the function of perlucin in abalone non-self recognition and disease resistance is still remained to investigate.

SUMMARY

Lectin is a protein or glycoprotein substance capable of specific recognition of and reversible binding to carbohydrate moieties on the surface of cells. Many important lectins with novel activities were from invertebrate animals. In invertebrate animals, lectins were basically employed to recognize the non-self antigens, enhance or potentiate the bacterial phagocytosis and encapsulation.

In this study, two genes relative to the *Vibrio* agglutination and the pearl formation were identified using EST cDNA library screening method. The complete cDNA of CLHd and perlucin of abalone, *Halotis discus discus*, were reported in the first time. The homologous comparison with known genes from other species was performed using blastX in the National Center for Biotechnology Information (NCBI) web server. The molecular structure analysis, the in vivo gene transcription and regulation, and the in vitro protein expression and the relative activity assay were investigated in order to characterize their function and physiological roles in abalones.

The complete cDNA sequence of the CLHd gene is 508 base pair in length, and encodes 151 amino acids. CLHd shares a highly conserved carbohydrate recognition domain with C-type lectins from mollusk and fish. The highly conserved CRD and six disulfide bond cysteines classified CLHd as a C-type lectin structurally. However, CLHd does not specifically resemble any particular gene.

In vivo, the relative expression level of CLHd in the digestive tract was significantly up-regulated when abalones were injected *Vibrio alginolyticus* in phosphate-buffered saline (PBS). At 12 hr post *V. alginolyticus* injection, the induction of mRNA was initiated and reached maximum expression at 24 hr. As the time progressed, mRNA transcription of CLHd dropped to the control value at 48 hr post the challenge. For abalone, the demand of CLHd was significantly increased under the *Vibrio* infection.

The in vitro recombinant CLHd specifically agglutinated *V. alginolyticus* in a calcium dependent way. CLHd immobilized the invading microorganisms and inhibited its spread

in abalones. The selective binding to *V. alginolyticus* suggested that CLHd may provide a means of the self- and nonself-recognition necessary for internal defense and potentiate bacterial phagocytosis in abalones.

The characterization of CLHd enriched the knowledge of abalone immunity and provided a powerful immune molecule for abalone disease control. Since it is highly inducible by *Vibrio* infection, in future applications, CLHd can be a useful marker for the monitoring of abalone farming environment and developing the husbandry management. Being as an effective *Vibrio* agglutinin, the presence of large amount of CLHd may definitely enhance the disease resistance of abalone. Gene selection or transgenic breeding of abalones with intensive expression of CLHd will provide a promising way to save juvenile abalones from the infection of *Vibrio* disease.

The full length cDNA of pacific abalone perlucin gene consisted of 1038 bp nucleotides encoding a putative signal peptide of 22 amino acids and a mature protein of 129 amino acids, which shared 55 % identity with the homologous protein in greenlip abalone. The predicted perlucin has a molecular weight of 15 KD, and an isoelectric point of 5.7.

To make sure the success of cloning and expression, the in vitro expressed recombinant perlucin were tested in both calcium carbonate precipitation assay and calcium carbonate crystallization assay. The addition of recombinant perlucin dramatically accelerated the calcium carbonate precipitation. Since mollusk nacre growth depended on calcium carbonate accumulation and formation of aragonite crystal. The positive effect of perlucin on calcium carbonate precipitation directly reflected its promotion to the pearl formation in abalones.

In the calcium carbonate crystallization assay, the crystals became round at the acute corner firstly and was not rhombohedral any more after 24 hr perlucin treatment at 4 °C. Gradually, the calcite crystal surfaces were roughed and lost all the sharp corners. After 72 hr incubation with perlucin, an approximate hexagonal crystal was promoted

and a small rhombohedral protuberance occurred on the top of the crystal with the crystal growth. Perlucin gradually rounded calcium carbonate crystal at acute corners but not at all the corners at once. This indicated that perlucin may recognize a specific calcite crystal surface and modify the sharp edges of calcite crystal, gradually. The calcium carbonate of morphological modification was not only contributed to the final structure of pearl or shell but also the growth velocity and orientation. We proposed that perlucin promoted the growth of calcium carbonate crystals on *ab*-plane first and when calcite crystals completed every step in the four corners, the crystal growth in *ab*-plane was inhibited and then initiated the growth in *c*-axis.

In the view of molecular structure, perlucin was a typical C-type lectin, which contained one highly conserved carbohydrate recognition domain. Reverse transcription polymerase chain reaction (RT-PCR) results showed that perlucin gene was expressed not only in mantle, but also in gill and digestive tract. Since gill and digestive tract always involved in continuously water exchange and food uptake from outside environment to abalone, these tissues were more susceptible to pathogen infection and need humoral immune molecules to create barriers against the invasion pathogenic micro-organisms in ocean.

Taken all together, it should be a reasonable speculation that perlucin is a kind of defensive molecule against pathogenic microorganisms and damaging detritus. When abalones can not eject the invasion parasites or particles, perlucin will be secreted to regulate nacre entomb the offending entity as a means of inactivation the non-self materials. With the growth of nacre layer on the offending particle, a beautiful pearl is produced. Perlucin played central roles in abalone pearl formation and inner nacrein growth. However, in the view of C-type lectin, more study about the function of perlucin in abalone non-self recognition and disease resistance is promising and anticipated.

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