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Genomic characterization and expression profiles upon bacterial infection of a novel cystatin B homologue from disk abalone (*Haliotis discus discus*)

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ABSTRACT

Cystatins are a large family of cysteine proteinase inhibitors which are involved in diverse biological and pathological processes. In the present study, we identified a gene related to cystatin superfamily, AbCyt B, from disk abalone *Haliotis discus discus* by expressed sequence tag (EST) analysis and BAC library screening. The complete cDNA sequence of AbCyt B is comprised of 1967 nucleotides with a 306 bp open reading frame (ORF) encoding for 101 amino acids. The amino acid sequence consists of a single cystatin-like domain, which has a cysteine proteinase inhibitor signature, a conserved Gly in N-terminal region, QVVAG motif and a variant of PW motif. No signal peptide, disulfide bonds or carbohydrate side chains were identified. Analysis of deduced amino acid sequence revealed that AbCyt B shares up to 44.7% identity and 65.7% similarity with the cystatin B genes from other organisms. The genomic sequence of AbCyt B is approximately 8.4 Kb, consisting of three exons and two introns. Phylogenetic tree analysis showed that AbCyt B was closely related to the cystatin B from pacific oyster (*Crassostrea gigas*) under the family 1.Functional analysis of recombinant AbCyt B protein exhibited inhibitory activity against the papain, with almost 84% inhibition at a concentration of 3.5 μmol/L. In tissue expression analysis, AbCyt B transcripts were expressed abundantly in the hemocyte, gill, mantle, and digestive tract, while weakly in muscle, testis, and hepatopancreas. After the immune challenge with *Vibrio parahemolyticus*, the AbCyt B showed significant ($P < 0.05$) up-regulation of relative mRNA expression in gill and hemocytes at 24 and 6 h of post infection, respectively. These results collectively suggest that AbCyst B is a potent inhibitor of cysteine proteinases and is also potentially involved in immune responses against invading bacterial pathogens in abalone.

1. Introduction

Cysteine proteases are widespread in all living organisms, such as mammals, birds, fish, insects, plant and protozoa, and are involved in diverse biological processes (Chapman et al., 1997; Rzychon et al., 2004). Cysteine proteases play key roles in antigen presentation, apoptosis, protein processing, as well as several pathological conditions like cancer progression, inflammation and neuro-degeneration (Chapman et al., 1997; Kopitar-Jerala, 2006). A number of studies have reported that cysteine proteinases produced by bacteria and parasites play a critical role as a virulence factor in development of many diseases (Mottram et al., 2004; Rudenskaya and Pupov, 2008; Takahashi et al., 1994). The proteolytic activity of these proteases can be inhibited by proteinase inhibitors such as cystatins. Cystatins are a large family of natural tight-binding reversible inhibitors of C1 family cysteine proteinases. Like other cysteine proteinases, cystatins have been found in diverse organisms. Many cysteine proteinases including the plant-derived papain, and the mammalian cathepsin, B, H and L can interact with cystatins (Barrett, 1987;Turk and Bode, 1991). Based on the structural differences, the cystatin superfamily can be categorized into four families. Family 1 cystatins, also known as stefins, are comprised of cystatin A and B, characterized by low molecular weight (~11 kDa) and a single cystatin-like domain structure. This group of cytoplasmic proteins is composed of ~100 amino acid residues that lack of disulfide bond, signal

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peptide, and carbohydrate side chain (Barrett et al., 1986; Turk and Bode, 1991). Cystatins C, D, E, S, and SN secretory inhibitors in family 2, known by the superfamily name cystatin, possess molecular weight of ~13 kDa and contain a single cystatin-like domain, two intra-molecular disulfide bonds, whereas lack of carbohydrates (Cornwall and Hsia, 2003; Li et al., 2010). Family 3 cystatins (kininogens) exhibit high structural complexity with more than one cystatin-like domain, which are large multifunctional glycoproteins with inhibitory activities (Kellermann et al., 1987). Another family, designated as family 4, was reported recently and consists of cystatins specially from nematodes (Khaznadji et al., 2005; Li et al., 2010). All cystatins contain a papain-binding site that binds to the catalytic site of papain-like proteinases, and inhibits them reversibly. This site is created by several conserved regions of the protein, including a glycine in the N-terminal region, a central QxVxG motif in first hairpin loop of the protein, and a less conserved PW motif in the second hairpin loop in the C terminal region (Björk and Ylinenjärvi, 1989; Margis et al., 1998; Rzychon et al., 2004).

Cystatins are involved in both biological and pathological processes which cysteine proteinases participate in, including protein homeostasis, inflammatory responses, antigen processing, metastasis, immune responses, and cathepsin dependent apoptosis (Abrahamson et al., 2003; Kopitar-Jerala, 2006; Lefebvre et al., 2008; Shah and Bano, 2009; Synnes, 1998). Among cystatins, cystatin B is an intracellular cysteine proteinase inhibitor which is a tightly-binding reversible inhibitor of cathepsin B, H, and L through forming a dimer stabilized by non-covalent forces to inhibit papain and cathepsin B, H, and L (Anastasi et al., 1983; Cimerman et al., 2001; Lefebvre et al., 2008). Cystatin B was also thought to play a role in protection against the proteinases leaking from lysosomes, and in biological defense system against invaders (Lefebvre et al., 2004; Li et al., 2010; Xiao et al., 2010). Defects in cystatin B cause progressive myoclonic epilepsy type 1 (EPM1), which is an autosomal recessive disorder characterized by severe, stimulus-sensitive myoclonus, and tonic–clonic seizures in human (Pennacchio et al., 1996; Riccio et al., 2005).

Although many evidences have been reported from vertebrates and some invertebrates, the function of cystatin B in mollusk species is still poorly understood. Disk abalone (*Haliotis discus discus*) is a valuable marine gastropod species. However, significant mortality is present in disk abalone aquaculture due to many reasons, including pathogens, stressful environments, pollutants, and disease outbreaks. To investigate the role of cystatin B-like proteins in disk abalones, the present study was carried out at the molecular level. In the present study, we identified a gene encoding for cystatin B from a previously constructed cDNA library of disk abalone *H. discus discus*, and functionally analyzed the papain inhibitory activity of recombinant protein. Furthermore, to understand the role in innate immune response, the temporal mRNA expression analysis was employed after bacterial infection for the first time in mollusks species.

2. Materials and methods

2.1. Experimental animals

Healthy disk abalones were purchased from 'Youngsoo' commercial abalone farm in Jeju Island, Republic of Korea. They were maintained as 40 animals per tank in flat bottomed tanks (250 L) with sand-filtered aerated seawater at a salinity of 34‰ at 18 ± 1 °C during the experimental period at Marine and Environmental Research Institute of Jeju National University. Abalones were acclimatized for 7 days before the experiment, and they were fed with fresh sea weed, *Undaria pinnatifida*, during the acclimatization period.

2.2. Bacterial artificial chromosome (BAC) library construction and screening

A BAC library of *H. discus discus* was constructed by Lucigen® Co. (Middleton, Wisconsin) using randomly sheared genomic DNA from gill tissue of disk abalone. Around 92,160 clones, possessing an average insert size of 120 Kb were arrayed in 240 of 384-well microtiter plates. Screening of the BAC-library was carried out with a PCR-based method (TaKaRa Bio, USA) following the manufacturer's instructions using gene-specific primers (Table 1). The identified clones were isolated from the corresponding wells and confirmed by colony PCR with gene-specific primers. After confirmation, BAC DNA from positive clone was isolated and purified using QIAGEN Large-Construct Kit, following the manufacture's protocol and was subjected to sequencing by Roche (454) Genome Sequencer FLX(GS-FLX™) system (Macrogen Inc. Korea).

2.3. Identification and sequence characterization of AbCyt B in DNA and protein level

Full length cDNA sequence of abalone cystatin B (AbCyt B) was identified by analyzing the previously constructed abalone expressed sequence tag (EST) database sequences (Munasinghe et al., 2006). BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) analysis indicated that one of the ESTs was homologous to previously identified cystatin Bs. This sequence was selected to design the BAC library screening primers and qRT-PCR primers used in the study (Table 1). The identified BAC clone sequence was used to analyze the genomic sequence.

The nucleotide and deduced amino acid sequences were analyzed using respective blastx and blastp programs at National Center for Biotechnology Information (NCBI). To determine the domains and conserved regions in AbCyt B, ExPASy PROSITE (http://www.prosite.expasy.org/) and motif scan (http://www.hits.isb-sib.ch/cgi-bin/PFSCAN) programs were used. The identity and similarity percentages of AbCyt B were detected using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/psa) at amino acid level.

2.4. Multiple sequence alignment, phylogenetic analysis and computer simulation modeling

The amino acid sequences obtained from BLAST analysis were used for the multiple alignment and phylogenetic tree construction. The amino acid sequences were aligned using ClustalW2 Multiple Sequence Alignment software with the default settings (http://www.ebi.ac.uk/Tools/msa/clustalw2). The phylogenetic tree was created using the neighbor-joining algorithm within MEGA (Molecular Evolutionary Genetic Analysis) software package (version 5.05).Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches. In order to

Table 1
List of oligonucleotides used in the study.

Name	Primer sequence (5′–3′)	Purpose
Ab-cytb F1	GTGGTGGTGCAAC CGAAGTGAAAT	Real-time PCR amplification, BAC screening
Ab-cytb R2	TGCTACCACTTGTGA ACGGAAGGA	Real-time PCR amplification, BAC screening
Ab-cytb F3	GAGAGAgaattcATGTGTGG TGGTGCAACCGAAG	Forward primer to ORF amplification, EcoRI in pMAL c2X
Ab-cytb R4	GAGAGActgcagCTACTTGGCATC AAAATAATCCAGATCTGATGCA	Reverse primer to ORF amplification, PstI in pMAL c2X
Ab-Rib F5	TCACCAACAAGGACATCATTTGTC	Real-time PCR amplification
Ab-Rib R6	CAGGAGGAGTCCAGTGCAGTATG	Real-time PCR amplification

reveal the tertiary structure of AbCyt B, computer simulation model was generated using I-TASSER (Roy et al., 2010; Zhang, 2008) online server and visualized the three dimensional (3D) structure using RasMol 2.7.5.2 software.

2.5. Genome structure and promoter region prediction

To identify the respective clone with AbCyst B in the bacterial artificial chromosome (BAC) library, the Ab-cytb F1 and Ab-cytb R2 oligonucleotides were designed based on the cDNA sequence (Table 1). The clone of the gene of interest was identified as described in Section 2.2. The genomic sequence was used to predict the transcription factor (TF) binding sites at promoter region and exon–intron structure. Exon–intron structure was determined by aligning the cDNA sequence to genomic DNA sequence using Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). TF binding sites were predicted using TFSEARCH ver 1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html) with threshold score at 90.0. Genome structures of cystatin B from other species were obtained from Ensembl Genome Browser (http://www.ensembl.org/index.html) and compared with AbCyt B genome structure.

2.6. Immune challenge experiment and tissue isolation

To study the differential expression of the AbCyt B, samples of gills, mantle, muscle, hemocytes, hepatopancreas, digestive tract and testis tissues were isolated from four healthy abalones. The abalone hemolymph was collected in a sterilized syringe from the pericardial cavities of four abalones. The hemolymph was immediately centrifuged at 3000 × *g* for 10 min at 4 °C and hemocytes were collected. All the tissues were snap-frozen in liquid nitrogen and stored at −80 °C till use for RNA extraction.

To determine the immune responses of the AbCyt B, immune stimulation/challenge experiment was devised and conducted using pathogenic Gram-negative bacterial species, *Vibrio parahemolyticus*. Animals were injected with 100 μL of 1×10^4 CFU/mL of bacteria in saline (0.9% NaCl) and the same volume of saline injected group was used as a control. Gill and hemolymph tissue samples were collected at different time points (0, 3, 6, 12, 24 and 48 h) after the infection as described previously and tissues were snap frozen and stored at −80 °C. Four replicates were used to collect the tissues at each time point.

2.7. RNA extraction and cDNA synthesis

The total RNA was isolated from tissues using the TRIzol reagent (Sigma–Aldrich) according to the manufacturer's instructions. Concentration of isolated RNA was determined at A_{260} nm in a UV-spectrophotometer (BioRad, USA). Purified RNA samples were diluted to 1 μg/μL and pooled to perform cDNA synthesis using PrimeScript™ First Strand cDNA Synthesis kit (TaKaRa Bio Inc., Japan) following the manufacturer's protocol.

2.8. Transcriptional analysis of AbCyt B by quantitative real time-PCR (qRT-PCR)

The AbCyt B mRNA expression was determined by quantitative real time PCR using the gene-specific primers (Table 1). Tissue-specific mRNA expression was analyzed in gills, mantle, muscle, digestive tract, hepatopancreas, hemocytes, and gonadal tissues. Each reaction was carried out in a 15 μL of reaction volume containing 4 μL of 1:10 diluted template cDNA, 7.5 μL of 2 × SYBR Green Mix, 0.6 μL of each primer (10 pmol/μL), and 2.3 μL of PCR-grade water using the Thermal Cycler Dice real time system Model TP800 (TaKaRa Bio Inc.). The qRT-PCR cycle program consisted of one cycle at 94 °C for 3 min, 45 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. The baseline was automatically set to maintain consistency. AbCyt B expression was determined by the $2^{-\Delta\Delta CT}$ (Livak) method (Livak and Schmittgen, 2001). To determine the expression-fold, the relative AbCyt B expression level was compared with expression level in muscle for the tissue-specific expression. To analyze the transcriptional regulation in immune challenged abalones, gill and hemocytes tissues from four abalones were used. qRT-PCR analysis was carried out as described previously. The relative mRNA expression was calculated by first normalizing to expression values of the gene codes for abalone ribosomal protein L5 (Wan et al., 2011) as the internal control (Accession No. EF103443) and then was normalizing to the expression values of the saline injected control group at each time point. All the data have been represented as means ± standard deviation and were subjected to one-way analysis of variance (ANOVA) test using the SPSS 11.5 program (Cary, NC, USA). Differences were considered statistically significant at $P < 0.05$.

2.9. Cloning, expression and purification of recombinant AbCyt B fusion protein

AbCyt B ORF sequence was cloned into pMAL c2X MBP-fused expression vector using the cloning oligos (Table 1) with *EcoR I* and *Pst I* restriction sites. Cloned vector was transformed into *E. coli* BL21 cells and culture was induced with isopropyl-*β*-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. After induced at 20 °C for 8 h, cells were harvested by centrifugation and recombinant AbCyt B (rAbCyt B) fused with the maltose binding protein (MBP) was purified using the pMAL protein fusion and purification system (Maina et al., 1988).

Samples collected at different steps in expression and purification process, were analyzed by 12% SDS–PAGE with low molecular weight protein marker (BioRad, USA) and stained with 0.05% Coomassie blue R-250, followed by a standard destaining procedure. The concentration of rAbCyt B fused with the MBP was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

2.10. Cysteine protease inhibition activity assay

The cysteine protease inhibitory activity of rAbCyt B was determined using papain at the presence of azo-casein as a substrate with increasing concentrations of the inhibitor (rAbCyt B), with some modifications to the procedure described previously (Xiao et al., 2010). Briefly, papain (Sigma, P3375) was dissolved in 0.1 M potassium phosphate buffer (PPB) (pH 7.4) to reach the final concentration of 1 mg/mL. Volume of 200 μL of papain was mixed with different volumes of rAbCyt B sample to reach the final concentrations of 0.08, 0.16, 0.32, 0.64, 1.28, 2.56 and 3.5 μM/L of each reaction mixture, after adding PPB to make the total volume up to 250 μL. Subsequently the mixture was incubated at 25 °C for 10 min. After incubation, 250 μL of 0.5% azo-casein in PPB was added to each reaction mixture and incubate at 37 °C for 30 min. Subsequently, the reaction was terminated by adding 500 μL of 10% trichloroacetic acid, followed by centrifugation at 10000 × *g* for 2 min. The supernatant was collected and absorbance (A) was measured at 440 nm. The papain inhibitory activity was calculated as $100 \times [1 - (A_{440}$ of rAbCyt B/A_{440} of positive control)] (Xiao et al., 2010). All the samples were measured in triplicates, and both positive (without rAbCyt B) and negative (without papain) controls were implemented. The assays were conducted using MBP as a control to determine the effect of the fusion protein on the activity of rAbCyt B.

3. Results

3.1. Identification and sequence characterization of AbCyt B

The complete cDNA and deduced amino acid sequences are shown in Fig. 1.The complete genomic sequence was deposited in GenBank under Accession No. JQ653304. The full length cDNA sequence of 1967 bp contains a 306 bp open reading frame (ORF), a 115 bp 5′-untranslated region (UTR) and a 1546 bp 3′-UTR with a poly-adenylation signal (AATAAA). The AbCyt B is comprised of a single cystatin-like domain with a conserved glycine residue near the N-terminal, a glutamine–valine–valine–alanine–glycine (QVVAG) motif at the middle of domain and a variant of proline–tryptophan (PW) motif with tyrosine (PY) substitution at C-terminal region. However, no signal peptide or disulfide bonds were identified. The predicted molecular mass of the derived protein was shown 11 kDa and the isoelectric point was predicted as 5.49.

3.2. Genomic structure and promoter region analysis of AbCyt B

The AbCyt B genomic sequence is approximately 8.4 Kb in length, consisting of three exons and two introns. The putative promoter region and TF binding sites of the AbCyt B were shown in Fig. 2A. Two enhancer box (E-box) TF binding sites and an initiator (Inr) motif sequence (CCATTTT) were identified −36 and −253 bp upstream to the transcription initiation site, respectively. Apart from Inr motif, several TF binding sites including CdxA, AML-1A, heat shock factor (HSF), NIT 2, Sox 5, erythroid TF (GATA-1), CAAT box, ecotropic viral integration site-1 (Evi-1), and TBP related factor (TRF 1) were identified in the anticipated region. Exon–intron organization and 5′-UTR and 3′-UTR regions were mapped with sizes and the genomic sequence of the AbCyt B was compared with those of human, cattle, rat, chicken and zebrafish cystatin B genes (Fig. 2B). It was found that all genes consisted of three exons and the second exon in all species was similar in length. However, the third exon in AbCyt B was found to be longer than other species due to the extremely long 3′-UTR, while the length of 3′-UTR was more comparable with that of human cystatin B.

3.3. Multiple homologue and phylogenetic analysis

The deduced amino acid sequence of AbCyt B was closely coupled with cystatin B (stefin B) protein sequences from different species. The putative AbCyt B protein was 40.4–44.7% identical to the cystatin B proteins from mammals, chicken, fish, mollusks, and other invertebrates (Table 2). Multiple sequence alignment

```
   1  GAAGCAAACAAGTCA CATGAGGCGTTCCCC GCTGATTACGGAAAA CACACAGATATTTAG
  61  GAGCTGCGGTTGTTG CTGTTGTTTGCTGTG AAAGTTTGATTAAAC GAAGATGTCG  ATG
   1                                                             M
 119  TGTGGTGGTGCAACC GAAGTGAAATCTGCA ACAGAAGAAGTGCAA AAACTCTGTAATGAG
   2   C  G  G  A  T   E  V  K  S  A   T  E  E  V  Q   K  L  C  N  E
 179  GTTCGAGAGGCCTTG CAAACACAAGCAGGG AGAACGTTTGGAGCG TACAAAGCCATATCC
  22   V  R  E  A  L   Q  T  Q  A  G   R  T  F  G  A   Y  K  A  I  S
 239  TTCCGTTCACAAGTG GTAGCAGGAACCAAC TACTTTGTTAAGGTC CAGGTGGATGAAAAC
  42   F  R  S  Q  V   V  A  G  T  N   Y  F  V  K  V   Q  V  D  E  N
 299  GATGAACACTTTCAC CTGAGGATATTCGCC CCCCTCCCCTACACC AACTCCCCACCCTCC
  62   D  E  H  F  H   L  R  I  F  A   P  L  P  Y  T   N  S  P  P  S
 359  CTTGCTGGCTATCAG ACTGGACACACCGCT GCATCAGATCTGGAT TATTTTGATGCCAAG
  82   L  A  G  Y  Q   T  G  H  T  A   A  S  D  L  D   Y  F  D  A  K
 419  TAGTTGCCGTCCAAG TTTACACCATGTGCT GGACAAGCTCTTGAT AGCTTATGTTCATGG
       *
 479  GATCTTTACATAATC TCTCAGTATAACATA TCAGTAGCTAAAGCT CACGCTTCATGTGTT
 539  ATCTCCCTTGAATAT CTTCAGGAAATGATC TGTTCATAAATCATT TTGAAATAAAAAAAC
 599  AAAACAGTTATCATG ATTAATCTGGTACAT AAAAACAACATCCAT TTAGTGTATTTTAAA
 659  CAAATAAATGGATAT TTTTTACACTTTTTG TCTCGGTGGTGAGCG TCCCCTGCAGCCTGT
 719  ATCAGTTGTATTGTG TACACTCAAGAAGAA CTGCCATTAACACCA TGGAACAACATCATG
 779  GTGGTGAGATGCTTG TAAACACCAAAACAC AAGATTATGATGCAA GTATGCTCATTTCCT
 839  TCCTCAGATCAGCAT CACGTCCGCCGCACA AGGATATGTCAAATT TGTGTTTGTATTGTT
 899  TTGCTTCCCTTTGGG ACTACAGAATGTAAA AAAATGAATTTGGTT GACTATTGTCCCTAC
 959  TACAACAGGTTGTTT TCAGAAATAGTATAT TTGCTGCTGTTTAGC GTTTCATAGTTCTCA
1019  TTTTGTAACCTACCA TTTTCTATTATACTT GTTTTGTCTATCAGT ATGTAGGCTGCTGCA
1079  GGAATGCCCATATCA TGTTATGTCACTCTG TTGTGACAATGCTTC CTAGAGAGCTGCCTG
1139  TATGATCTGCTCAGA GGGGTTACAGGGATA AATATATACAGCCAG TATCTTGCTTATGTG
1199  TATTCCCCGTGCTTG TGGGTGTCACCAGTG GTACCAGAAAACGTC CGAACCTGCAACACT
1259  TTGACTTTTCCGTCC ATATTAAGCTGACAC ACCATGATATAACTG GAATACTGTTCAAAG
1319  TGTTGTTAAACCCAA CTCGCTCACTCATAT CTATACAATATAAAA ATGGTTAAAGTTGCT
1379  GAAATTACAAATTGA TGTGTGCTGTTTGAA AAGCTGAAAACTTTC TTACATTTTCATGTG
1439  AAAATGCTATAAGGC TGCATTTGCAGATTC TGACATTCTTGATTG TCCATGAAATTCTTG
1499  AATGTTCATGTAAAA TGTCAGATTTCTTTG GAAACGAGGACCAAA AGTTACAGTGCAGGT
1559  GAATTTTGATAATTT ATTATTGTCCATGAT CTCCTGATCCTTTGC AAACGAACATTATAG
1619  CAGTTTCTGCACAGT AGATACATATTTAAT ATGAAAGTTACTGAT TCAGAGGAATCTGCT
1679  CTTGGGAGTGTTTGT AGTCGGTTCCACCTG ACAGACTTATCTTAG CTGCCGTCTCCGGTT
1739  GTTTCATCTCACTGC CACTGAACCCTTCAT TTCACGTGTTGGGAT CTGTGGACTTAATTA
1799  ATCAGCAGTTCTGCA TGAAAATGCAAGACA GGAATTGTGAAACGA CAAGACCTTTTATCA
1859  GTGTCTATTTGTTGA GCCATAGATGAAATT AAGGTAACAGTTGTG ATGTTGACTTTTTTA
1919  TCTGCTGTTGAAATT TTCACATAAACAACA ATAAACCATTCTTTT TACA
```

Fig. 1. Full length cDNA and deduced amino acid sequences of AbCyt B. The start (ATG) and stop (TAG) codons are underlined. The cystatin-like domain is shaded and cystatin protease inhibitor signature sequence is in boldface. The conserved G^{3}, ^{45}QVVAG^{49} motif and $P^{74}Y^{75}$ motif are showed in italic, boxed and underlined boldface respectively. The poly (A) signal (AATAAA) is in italic boldface at the end of nucleotide sequence.

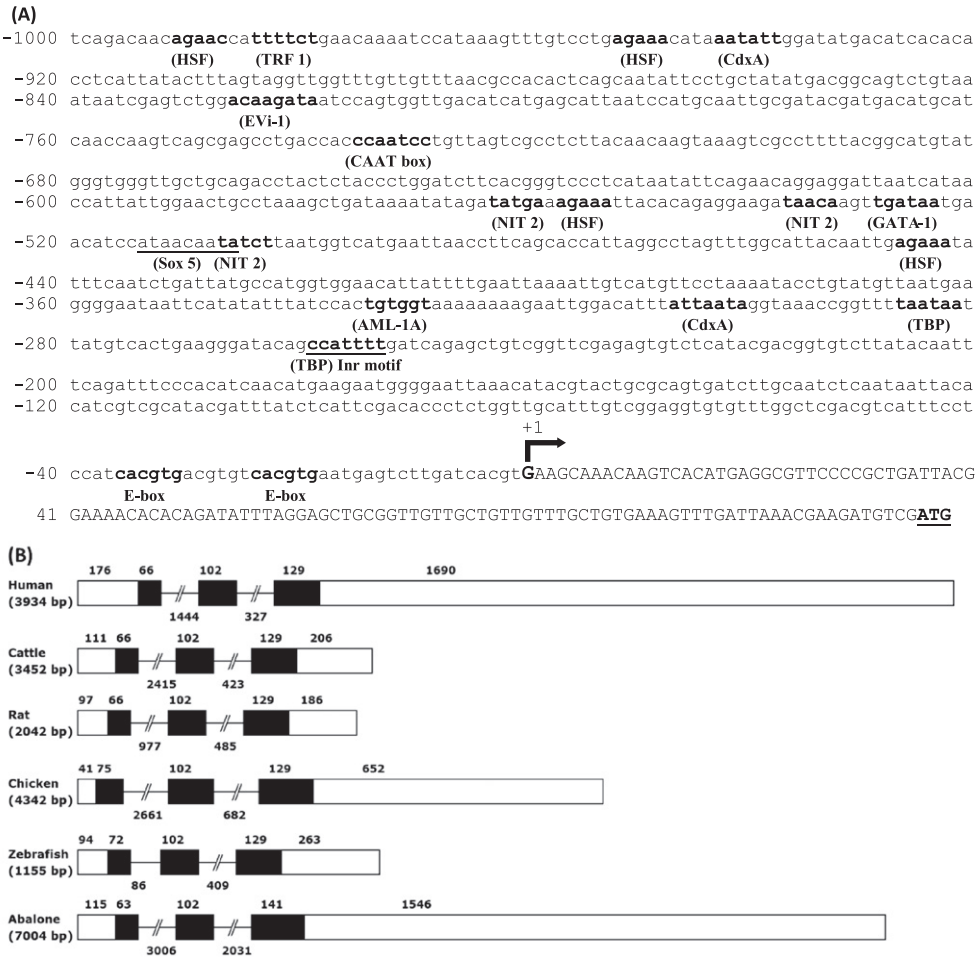

Fig. 2. AbCyt B genome sequence, (A) Putative promoter region and 5′-UTR region sequence from −1000 to +118 bp form transcription initiation site (+1) with TF binding sequences predicted by TFSEARCH online database. The 5′-UTR region is denoted by block letters and translation start cordon is underlined. (B) Schematic representation and comparison of cystatin B genomic structures with other species. UTRs, exons and introns represent by white boxes, black boxes and black lines respectively.

Table 2
Amino acid identity and similarity of AbCyt B to other known cystatin B proteins.

Species	Accession No.	Identity (%)	Similarity (%)
Human (*Homo sapiens*)	NP000091	44.70	61.20
Norway rat (*Rattus norvegicus*)	NP036970	44.10	62.70
Chicken (*Gallus gallus*)	NP001185577	44.10	65.70
Zebrafish (*Danio rerio*)	NP001096599	40.40	59.60
Pacific Oyster (*Crassostrea gigas*)	ADI33157	44.10	65.70
Clamworm (*Perinereis cultrifera*)	AAN28679	44.10	53.90
Duck leech (*Theromyzon tessulatum*)	AAN28679	42.70	65.00

revealed that thorough conservation of N-terminal G residue, QxVxG motif as QVVAG sequence and PW motif as PY (Fig. 3).

A phylogenetic tree was constructed based on 20 different cystatin superfamily protein sequences from mammals, avian, teleost, mollusks, annelids and nematode species (Fig. 4). Different types of cystatins were clustered into 4 different phylogenetically related families, where AbCyt B was clustered with cystatin B in family 1. In the tree, AbCyt B showed closest evolutionary proximity with cystatin B from pacific oyster (*Crassostrea gigas*) (Accession. No. ADI33157).

3.4. Computer based molecular modeling

Tertiary structure of AbCyt B was determined using the computer based simulation modeling strategy of I-TASSER online server. The generated model exhibited 89 ± 7% accuracy based on TM-score value and top ten threading templates from the Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank used by the server belong to the stefin family proteinases. Moreover, the normalized *Z*-score values of the threading alignments exceeded 1, substantiating the higher degree of confidence of the predicted structure. The main body of the AbCyt B molecule comprised of five stranded anti parallel β sheet wrapped around a central five turned alpha helix but with an extension of carboxy terminus running along the back of the sheet. Furthermore, two hairpin loops, one of which contains a highly conserved amino acid sequence (QVVAG), can be prominently identified in the structure along with a conserved amino acid residue at the third position from N terminal. Altogether these features are important in forming a hydrophobic wedged shaped edge in the active site of the molecule (Fig. 5).

3.5. Tissue specific and temporal mRNA expression of AbCyt B

The mRNA expression of AbCyt B in different tissues of healthy abalones was analyzed by quantitative RT-PCR. The results showed that AbCyt B was constitutively expressed in all tissues including mantle, gill, digestive tract, hemocyte, testis, hepatopancreas, and muscle. Abundant AbCyt B expression was detected in hemocyte, gill, mantle and digestive tract while weak transcript levels were

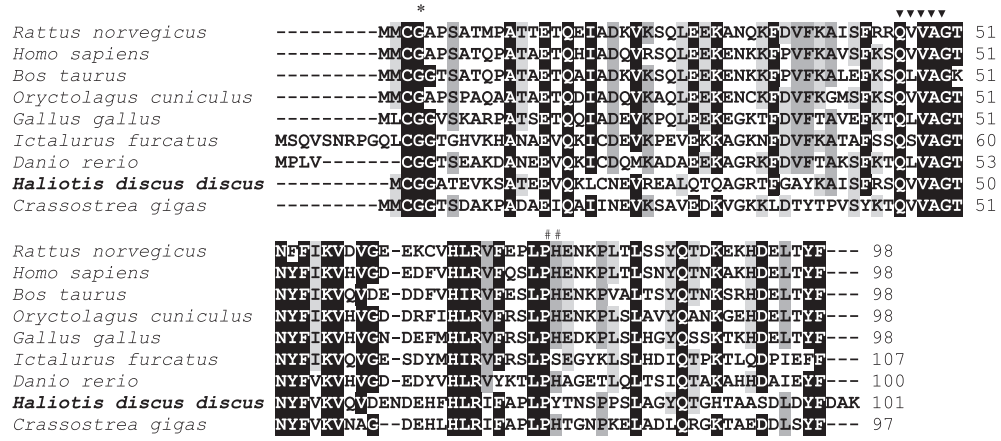

Fig. 3. Alignment of the AbCyt B amino acid sequence with selected known cystatin B sequences. The conserved G, QxVxG motif and variant of PW motif are marked with (∗), (▼) and (#) respectively. Highly conserved, strong homologies are shaded with black and weak similarities shaded in gray.

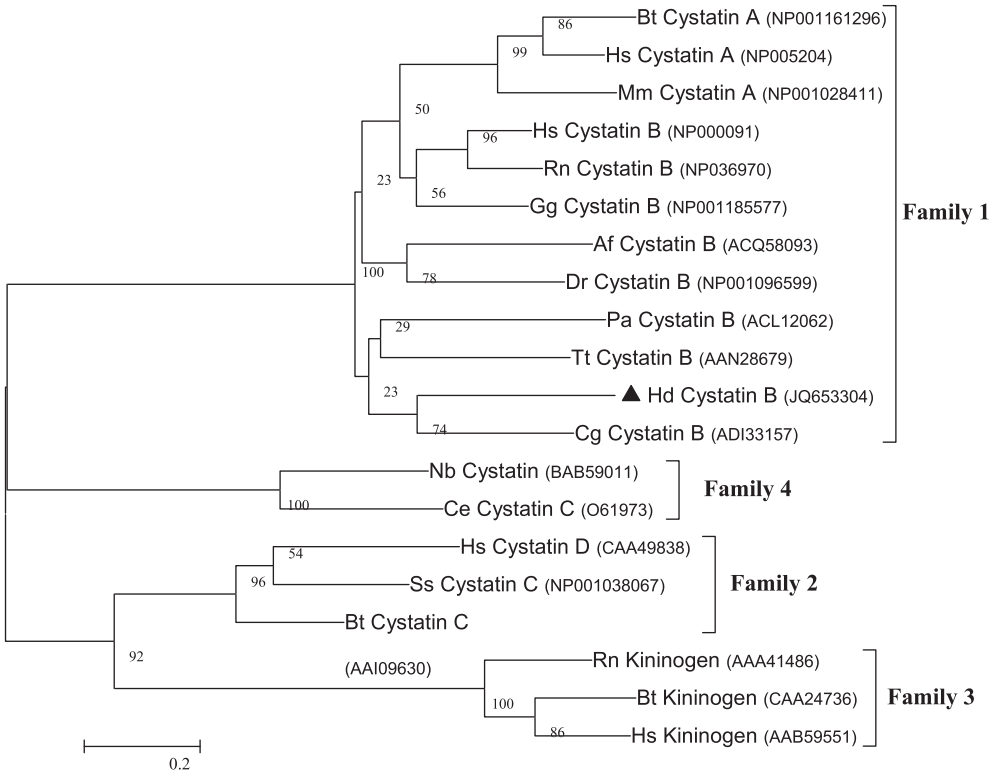

Fig. 4. Phylogenetic tree of known cystatins from different species including the AbCyt B. The tree was depicted on the overall sequences by neighbor-joining method. Bootstrap values are shown next to the branches based on 1000 replications. Bt, *Bos taurus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Gg, *Gallus gallus*; Af, *Anoplopoma fimbria*; Dr, *Danio rerio*; Pa, *Perinereis aibuhitensis*; Tt, *Theromyzon tessulatum*; Hd, *Haliotis discus discus*; Cg, *Crassostrea gigas*; Nb, *Nippostrongylus brasiliensis*; Ce, *Caenorhabditis elegans*; Ss, *Sus scrofa*.

detected in hepatopancreas and testis compared with the lowest expression level in muscle (Fig. 6).

After the immune challenge with bacteria, the AbCyt B showed significant up-regulation ($P < 0.05$) of mRNA expression in gills at 24 h of post infection (p.i.) and dropped down to the basal level at 48 h of p.i. (Fig. 7A). However, in hemocytes, it was shown as relatively early response compared with the profile in gills (Fig. 7B). The significant ($P < 0.05$) induction was detected as early as 6 h of p.i., but afterward the expression was significantly down-regulated compared with the un-injected control (0 h).

3.6. Protease inhibition activity of AbCyt B

The recombinant fusion protein expressed in *E. coli* BL21 system was purified and visualized using SDS–PAGE. Purified protein exhibited a molecular mass of 53.5 kDa (42.5 kDa MBP + 11 kDa AbCyt B), comparable to the predicted molecular mass (Fig. 8). Subsequently, the protease inhibitory activity of rAbCyt B was analyzed against the activity of papain enzyme on azo-casein hydrolysis. The results showed that the rAbCyt B possess papain inhibitory activity in a concentration-dependent manner (Fig. 9). The rAbCyt

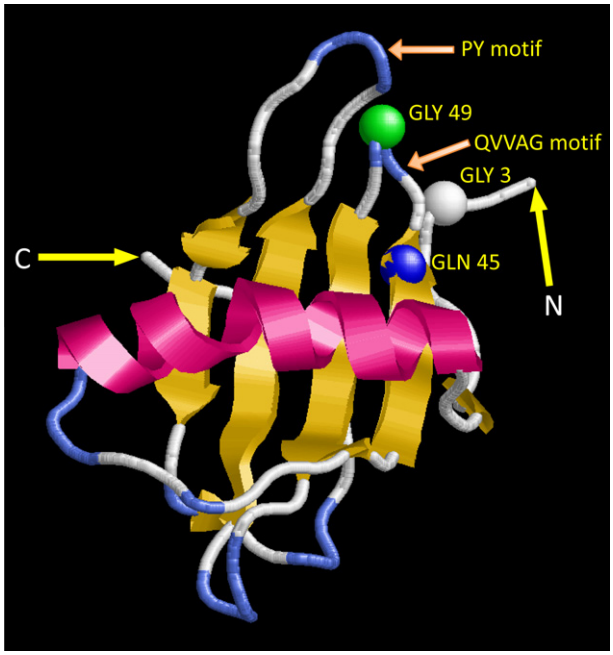

Fig. 5. Computer simulation model generated for the AbCyt B. α-Helices are represented in pink color and β strands are depicted in yellow color. Three spherical bulges denoted in green, blue and white color represents the crucial amino acid residues in the active site of the molecule. Pale orange color arrows indicate the two hairpin loops with motifs. N and C represent amino and carboxy terminals of the protein, respectively.

B was able to inhibit the papain activity by 83.8% at 3.5 μM/L of final concentration where MBP was used as the control.

4. Discussion

Cystatins function as reversible inhibitors of papain-like cysteine proteinases which constitute the largest and best described group of natural cysteine protease inhibitors (Bode et al., 1990). Interestingly, recent findings reported that cystatin B was involved in immune responses against invading bacteria in teleost, crustaceans and annelids (Lefebvre et al., 2004; Li et al., 2010; Xiao et al., 2010). In the present study, we isolated and characterized a gene related to cystatin superfamily which possesses *in vitro* cysteine protease inhibitory activity and *in vivo* immune responses against invading bacteria in disk abalone.

The AbCyt B polypeptide consists of only a single cystatin-like domain without signal peptide, disulfide bonds and carbohydrate side chains, which are characteristic features of the family 1 cystatin members (Abrahamson, 1994; Pemberton, 2006; Turk and Bode, 1991). Three conserved regions, N-terminal Gly, QxVxG motif and PW motif were found in the amino acid sequence, which are important for the cysteine protease inhibitory activity. The N-terminal Gly residue is located at 3rd position and a conserved QxVxG motif sequence is presented as QVVAG sequence, which is exactly matched with the corresponding conserved motif of number of animal species including human, rat, rabbit, and pacific oyster. However, the less conserved PW motif at second hairpin loop was substituted by PY in the AbCyt B. The PW motif was reported from other cystatin superfamily members and it was shown as PH in human stefin B (Ochieng and Chaudhuri, 2010). These conserved regions together form a hydrophobic wedge-shaped edge which is highly complementary to the active site of the papain-like cysteine proteinases in order to inhibit their protease activity (Turk and Bode, 1991). In addition to these three regions, Pol and Björk (2001) reported a free Cys 3 in N-terminal segment of the proteinase-binding region in human and bovine cystatin B that is important for tight binding of target proteinases. In the present study, cysteine at 2nd position of the amino acid sequence was found to be homologous to that of Cys 3 in human and bovine cystatin B.

The phylogenetic tree analysis showed that animal cystatin superfamily members branched into 4 families, where AbCyt B clustered under the family 1 at bootstrap value of 75 with pacific oyster cystatin B. In addition to the well described family 1–3, we also demonstrated a fourth family of cystatin, which was reported in recent studies and mostly consisted of nematode parasites (Khaznadji et al., 2005; Li et al., 2010). However, the family 1 and 3 consists of members from both vertebrate and invertebrate

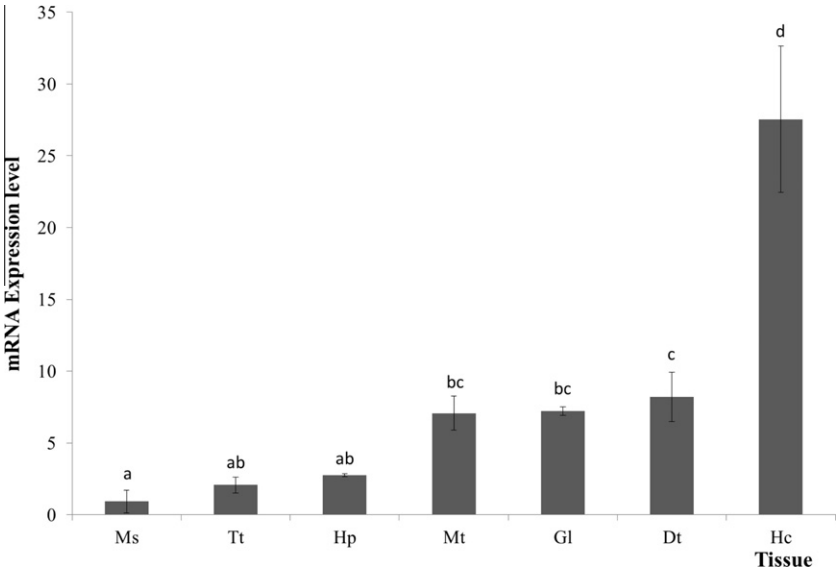

Fig. 6. Tissue specific mRNA expression of AbCyt B in different tissues determined by quantitative real-time PCR. AbCyt B mRNA expression of each tissue was calculated by the $2^{-\Delta\Delta CT}$ method using abalone ribosomal protein L5 as the internal control (Accession No. EF103443). The mRNA level was compared with expression level in muscle to determine the relative fold change. Ms, muscle; Mt, mantle; Gl, gill; Dt, digestive tract; Hc, hemocyte; Hp, hepatopancreas; and Tt, testis. Statistical analysis was performed by one-way ANOVA test. Data with different letters are significantly different ($P < 0.05$) among different tissues. Vertical bars represent the standard deviation ($n = 3$).

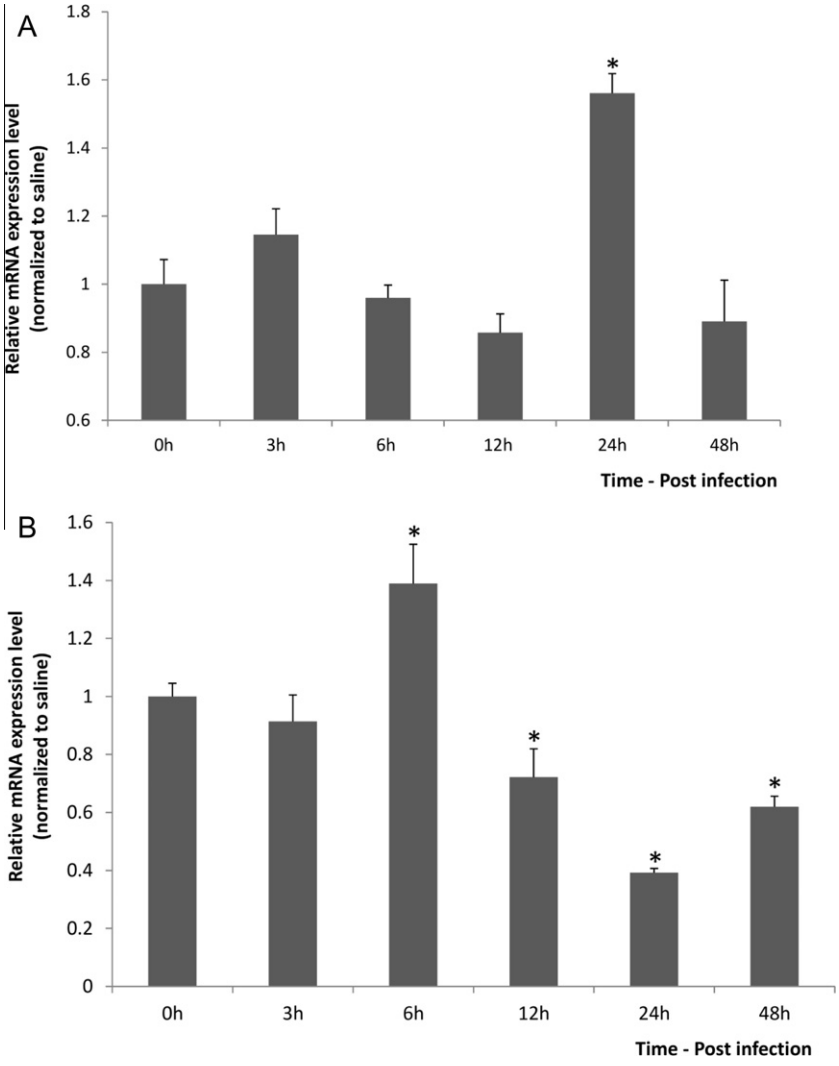

Fig. 7. AbCyt B mRNA expression after bacterial challenge in gill (A) and hemocytes (B).The relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method using abalone ribosomal protein L5 as the internal control and normalizing to the saline injected control at each time point. Statistical analysis was performed by one-way ANOVA test. Asterisk indicates significant differences ($P < 0.05$) to the 0 h (non-injected control). Error bars represent the standard deviation ($n = 3$).

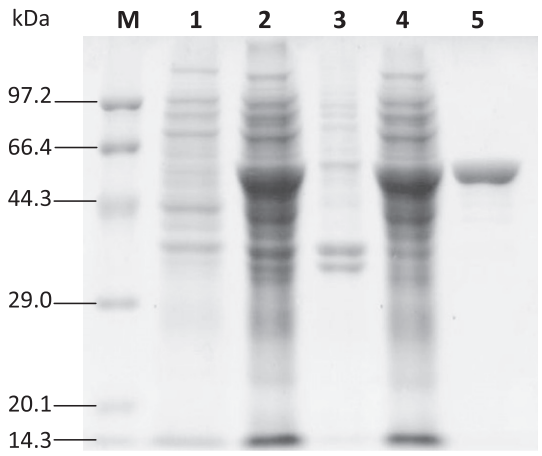

Fig. 8. SDS–PAGE analysis of rAbCyt B on *E.coli* BL21. Lanes M, protein molecular mass marker (kDa); 1, negative control for rAbCyt B (without induction); 2, IPTG induced rAbCyt B; 3, pellet of broken cell lysate; 4, supernatant of broken cell lysate; 5, purified rAbCyt B protein.

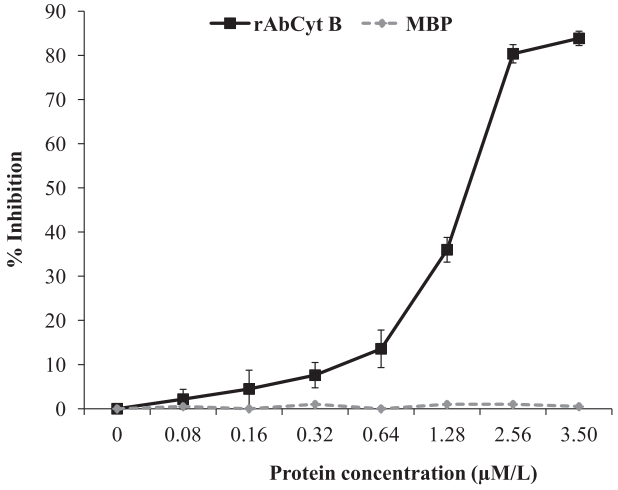

Fig. 9. *In vitro* protease inhibitory activity assay profile against the activity of papain where azo-casein was used as the substrate. Maltose binding protein (MBP) was used as the control. Error bars represent the standard deviation ($n = 3$).

species and our interested member also clustered with the family 1 members.

According to our approach in generating a 3D structural model of AbCyt B to determine its tertiary structure, in order to correlate with its function, demonstrated the typical features of the stefin family proteinases as described in the result section. The anticipated 3D model encompasses the characteristic features of stefin family protease inhibitors, especially related to the human stefin/cystatin B (Stubbs et al., 1990). Importantly, the penta peptide conserved motif (QVVAG) in the first hairpin loop, PY motif, and the glycine residue located close to the N-terminal of the molecule manifest that the potential of forming a wedged-shaped edge which is highly compatible to the active site of the papain-like cysteine proteases, substantiating the structural and functional relationship of novel AbCyt B.

In most of the protein coding genes, the TATA box facilitate transcription factor (TF) II D (TBP) binding to the promoter region and is found in the 25–30 bp upstream of the transcription start site within the core promoter region, where core promoter can be extended ~35 bp upstream/downstream to the transcription initiation site (Smale and Kadonaga, 2003). However most of the human core promoters contain the consensus Inr motif (YYAN(T/A)YY) and considerable number of promoters were Inr motif containing TATA-less genes (Yang et al., 2007). Similarly to the TATA box, the Inr motif facilitates the binding of TF II D (TBP) (Xi et al., 2007). In our study we also identified Inr motif at −259 to −253 bp upstream to the initiation site. Additionally, two E-boxes were identified within first −36 bp region, which are able to enhance the transcription of AbCyt B (Chaudhary and Skinner, 1999). Another TBP binding sequence was predicted at −287 bp upstream and CAAT box sequence was identified at −735 bp, which functions as a regulatory element for transcription initiation (Xi et al., 2007). Presence of HSF, NIT 2, GATA-1 and Evi-1 TF binding sites in promoter region suggest that AbCyt B may involve in physiological processes (Bard-Chapeau et al., 2012; Sorger, 1991).

Family 1 cystatins were reported to be distributed in vast types of tissues and cells in different species (Pemberton, 2006; Turk and Bode, 1991), since it has been involved in many physiological and pathological processors including inflammation, apoptosis, immunomodulation, cellular matrix remodeling etc. (Abrahamson et al., 2003; Lefebvre et al., 2008; Synnes, 1998). In the present study, we observed that AbCyt B was universally expressed in seven different tissues examined. However, significantly higher expression was observed in hemocytes, gills, mantle and digestive tract. Similar high expression of cystatin B in hemolymph was observed in Horseshoe crab (*Tachypleus tridentatus* L-Cystatin) and Chinese mitten crab EsCystatin (Agarwala et al., 1996; Li et al., 2010). In the present study, AbCyt B showed the lowest expression in muscle tissue; however, a contradictory result for the mRNA expression in muscle was reported in EsCystatin in Chinese mitten crab where it was the highest expression. Hemolymph, gill and mantle are important tissues involved in innate immune functions in abalones. Cellular responses in first line innate immunity of mollusks are carried out by circulating hemocytes which can kill microbes by phagocytosis and cytotoxic reactions (Pruzzo et al., 2005). Therefore, we suggested that AbCyt B might play a critical role associated with hemocytes. The gill tissue in mollusks is a key respiratory organ whose surface is exposed to the external environment and frequently in contact with pathogenic microbes, and it was believed to be involved with the mollusks immune system (Chakraborty et al., 2010). In addition to the proteolytic activity, the cystatin was reported to be involved in immune responses against invading pathogens in Chinese mitten crab and leech (Lefebvre et al., 2004; Li et al., 2010). In the present study, the relative mRNA expression level was significantly up-regulated ($P < 0.05$) in gill tissue at 24 h of p.i. and in hemocytes at 6 h of p.i. and subsequently showed down-regulation in hemocytes as a response to the pathogenic bacteria. Similar results were reported in EsCystatin against bacteria as down-regulation at 3 h p.i. and up-regulation of mRNA expression at 24 h p.i. in hemolymph (Li et al., 2010). Cathepsins, well-known cysteine proteases, are reported to be involved in immune responses through the toll like receptor signaling pathway (Creasy and McCoy, 2011). A previous study in *Drosophila melanogaster* showed that bacterial infections up-regulated the expression of cathepsins (De Gregorio et al., 2001). As lysosomal cysteine proteases play major role in MHC class II-mediated antigen presentation by processing and degradation of antigens to peptides are known to be protease activity-dependent, a considerable down-regulation of cystatin gene expression thereby can be expected during the bacterial infection (Turk et al., 2000). However, due to scanty information on cysteine proteases and cystatin B-like genes from mollusks, it is unclear about the functional relationship between cysteine proteases and their inhibitors in immune responses. Further studies might elucidate the link between cysteine protease inhibitor and their role in bacterial infections. We report these results for the first time in mollusks species, and these results suggest that AbCyt B is immune responsive and inducible by bacterial infection.

The rAbCyt B showed remarkable protease inhibitory activity against the proteolytic activity of papain to the azo-casein suggesting AbCyt B biologically active protein. The papain activity was 84% inhibited by rAbCyt B at 3.5 μmol/L of final concentration. rEsCystatin in Chinese mitten crab and Limulus cystatin in Horseshoe crab reported 89% and 90% inhibition of papain activity at 300 $\mu g\ mL^{-1}$rEsCystatin and at 1:1 ratio of papain to Limulus cystatin, respectively (Agarwala et al., 1996; Li et al., 2010). Cystatins do not form a covalent bond with cysteine proteinases, but instead cover the active site cleft blocking access to the active site (Calkins and Sloane, 1995). Previous studies reported that cathepsin B expressed in extra-cellularly on the surface of tumor cells, plays a key role in tumor cell invasion, and anti-protease activity of cystatin possesses a critical role at tumor cell invasion by inhibiting the activity of cathepsins (Ochieng and Chaudhuri, 2010). Moreover, in microbes, especially bacteria, cysteine proteases have elastin degradation properties, which may lead to a pathogenesis. For example, Arg-gingipain (RGP) and Lys-gingipain (KGP) produced by bacteria play a major role in pathogenesis and act as a virulence factor (Otto and Schirmeister, 1997). Therefore, it is clear that inhibitory activity of cystatin plays an essential role in host immune defense system. However, further studies are needed for detailed clarification of the exact role of cystatin B in mollusks immunity.

In conclusion, the present study results suggest that AbCyt B is a member of family 1 cystatin (stefin) and is more homological with the cystatin B counterparts of other organisms. AbCytB can exert cysteine protease inhibitory activity against proteases like papain and is potentially involved in immune defense system against invading bacteria in disk abalone.

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