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Molecular characterization and transcriptional profiling of caspase-3 and caspase-8 from black rockfish (*Sebastes schlegelii*)

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Molecular characterization and transcriptional profiling of caspase-3 and caspase-8 from black rockfish (*Sebastes schlegelii*)

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요 약 문

세포자살 (Apoptosis)는 예정 세포 사멸사 (Programed cell death)의 유형 중에 하나로 발달, 성장, 항상성 유지, 면역반응과 같은 다양한 생물학적 과정을 조절한다. 이전 연구에 따르면 세포자살은 바이러스와 같은 세포 내 병원체나 유해한 세포를 제거함에 있어 중요한 기능을 하는 것으로 알려져 있다. 또한 감염이 되면 이에 따른 병원균의 확산을 막기 위해서 세포 자살은 cell death cascades 통하여 활성화된다.

caspase는 시스테인계 아스파라산 단백질 분해효소 (cysteine aspartate specific proteinases)로서 apoptotic signaling에서 중요한 역할을 하는 효소이다. caspase 중에서 caspase-8은 extrinsic pathway에서 initiator caspase로서 세포자살을 수행하는 caspase-3를 활성화시킨다.

이 연구는 Rfcasp3과 Rfcasp8를 조피볼락 cDNA library에서 NCBI BLAST를 사용하여 동정하였다. 확인된 Rfcasp3와 Rfcasp8의 전체 cDNA 서열은 분석하였고 다른 종간에 진화적인 위치를 결정하기 위해 계통수 분석을 하였다. 발현 패턴을 조사하기 위해 Rfcasp3와 Rfcasp8을 quantitative real time PCR (qRT-PCR) 이용하여 조피볼락의 조직인 혈구, 간, 아가미, 근육, 신장, 피부, 심장, 두신, 비장, 장에서 분석하였다. 또한, 면역자극제인 poly I:C 와 LPS를 주사한 후에 Rfcasp3와 Rfcasp8의 발현을 주사 후 0, 3, 6, 12, 24, 48, 72시간에 측정하였다. PBS 처리된 집단은 control로 사용하였다.

재조합 단백질의 기질 특이적 가수분해 활성을 측정하기 위하여 pMal-c2x 벡터에 pro-domain을 제외한 ORF를 클로닝하고 *E.coli* BL21(DE3)에 형질전환하였다. IPTG 유도에 따른 재조합 Rfcasp3 단백질을 과발현시키고 pMal-c2x system을 이용하여 정제하였다. 또한 기질 특이적 가수분해 활성에 대한 온도와 농도에 따른 영향을 조사하였다.

조피볼락에서 Rfcasp3와 Rfcasp8의 전체 cDNA 서열은 각각 2224bp, 2467bp이고 ORF는 846bp와 1473bp으로 282 aa와 491aa을 암호화하고 있었다. 또한 Rfcasp3와 RFcasp8의 분자량은 각각 33.03와 55.26 kDa으로 추정되었고 등전점은 각각 5.77과 5.46으로 측정되었다.

Pairwise sequence alignment 분석 결과 Rfcasp3와 Rfcasp8은 돌돔 (*Oplegnathus*

fasciatus) 에서 높은 동일성(identity)와 유사성(similarity)를 공유하였다. Rfcasp3와 Rfcasp8은 특정적인 caspase 도메인 구조인 Large subunit과 small subunit을 포함하고 있었다. 반면에 Rfcasp3의 경우 비교적 짧은 pro-domain을 가지고 있었고 Rfcasp8의 경우에는 두개의 Death effector domain (DED)을 연결하고 있었다. 계통수 조사에서는 Rfcasp3는 유럽 농어(*Dicentrarchus labrax*) 와 가까운 것으로 나타났고 Rfcasp8의 경우 큰가시고시 (*Gasterosteus aculeatus*)와 진화적으로 유사하였다.

Rfcasp3와 Rfcasp8 유전자는 각각 모든 조직에서 발현되는 것을 확인하였고 조직 중에서도 혈액에서 매우 높은 발현량을 확인할 수 있었다. 면역자극제인 LPS와 poly I:C 를 주사하였을 때 두 유전자 발현도 혈액에서 상당히 상향 조절되었다.

정제된 Rfcasp3 단백질은 caspase-3/7 특이적 기질 (DEVD-*p*NA)과 caspase-9 특이적 기질 (LEHD-*p*NA)에 대한 기질 특이성 가수분해 활성을 조사하였다. 재조합 단백질은 DEVD-*p*NA에 매우 유의적인 활성을 가지고 있었다. 또한 단백질의 농도에 따라 기질 특이성 단백질분해 활성도 의존적으로 증가함을 알 수 있었다 다른 온도 사이에서 단백질분해 활성은 37° C 에서 가장 높았다. 반면에, 상대적으로 10° C에서 활성이 가장 낮게 측정되었다.

이 연구는 caspase-3와 caspase-8을 동정하고 분자적 특성을 분석하고 Rfcasp3의 단백질분해 활성을 이해함으로써 기능적 특성을 조사하였다. Poly I:C와 LPS에 의한 면역반응을 나타낼 때 혈액에서 발현조절을 조사하였다. 종합해보자면 우리가 이 연구에서 찾아낸 결과들은 조피볼락의 면역 반응에 관련된 Rfcasp3와 Rfcasp8의 추정되는 역할에 대한 힌트를 얻을 수 있다.

Summary

Apoptosis is one type of programed cell death and regulates the biological process including development, homeostasis and immune response. According to previous studies, apoptosis found to play important role in clearance of harmful cells or pathogens such as virus. Moreover, to protect the spread of pathogens after infection, apoptosis is activated by various immune signaling pathways through triggering cell death cascades.

Caspase as cysteine aspartate specific proteinases play an essential role in apoptotic signaling. Among the caspases, caspase-8 is an initiator caspase in extrinsic pathway and activates the caspase-3 to execute apoptosis.

In this study, Rfcasp3 and Rfcasp8 were identified from cDNA library of black rockfish using NCBI BLAST. The confirmed full-length cDNA sequences of Rfcasp3 and Rfcasp8 were analyzed and phylogenetic analysis was performed to determine the evolutionary position among other counterparts. To investigate the expression pattern, Rfcasp3 and Rfcasp8 was performed using quantitative real time PCR (qRT-PCR) from black rockfish tissues including blood cell, liver, gill, muscle, kidney, skin, heart, head kidney, spleen, intestine. Moreover, after injection of immune stimulants with poly I:C and LPS, the expression of Rfcasp3 and Rfcasp8 was measured at 0, 3, 6, 12, 24, 48, 72 h post injection. PBS treated groups was used as a control

In order to measure the substrate-specific hydrolyzing activity of recombinant Rfcasp3 protein, *Rfcasp3* open reading frame (ORF) without the coding region for pro-domain was cloned into pMal-c2x vector and transformed into *E. coli* Bl21 (DE3). Recombinant Rfcasp3 fusion protein was overexpressed by IPTG induction and purified using pMal-c2x system. Moreover, the effect of temperature and protein concentration in substrate-specific hydrolyzing activity was examined.

The full-length cDNA sequence of Rfcap3 (2224bp) and Rfcasp8 (2467bp) from black rockfish were consisted of 846bp and 1473bp ORFs, encoding 282bp and 491 amino acid

residues, respectively. Furthermore, the calculated molecular masses of Rfcasp3 and Rfcasp8 were 33.03 and 55.26 kDa, and estimated isoelectric points of Rfcasp3 and Rfcasp8 were 5.77 and 5.46, respectively.

Pairwise sequence alignment analysis revealed that Rfcasp3 and Rfcasp8 share eminent identity and similarity to relevant counterparts of rock bream (*Oplegnathus fasciatus*). Rfcasp3 and Rfcasp8 also contained characteristic caspase domain architecture including large and small subunits. On the other hand, Rfcasp3 had comparatively short pro-domain and Rfcasp8 harbored two death effector domains (DED). Phylogenetic analysis showed that Rfcasp3 was more close to european seabass (*Dicentrarchus labrax*) counterpart and Rfcasp8 has grater evolutionarily relationship to the similitude of three-spined stickleback (*Gasterosteus aculeatus*).

Rfcasp3 and Rfcasp8 demonstrated the ubiquitous expression in tissues examined, from heathy fish and the highest expression level was detected in blood cells. After injection with immune stimulant such as poly IC and LPS, the expression of both genes were significantly up-regulated in blood.

Purified recombinant Rfcasp3 fusion protein was used to investigate the Hydrolyzing activity assay against caspase-3/7 specific substrate (DEVD-*p*NA) and caspase-9 specific substrate (LEHD-*p*NA). Recombinant fusion protein exhibited a noticeable substrate-specific hydrolyzing activity toward DEVD-*p*NA. Moreover, in accordance with protein concentration, hydrolyzing activity increased in dose-dependent manner. Among the different temperature, highest hydrolyzing activity was detected at 37℃. On the other hand, least hydrolyzing activity was reported at 10℃.

This study revealed the identification and molecular characterization of caspase-3 and caspase-8 from black rockfish and investigation of functional properties of RbCasp3 through deciphering its protease activity. Their expressional modulation under immune response with poly I:C and LPS was determined in blood. Collectively, our findings in this study hints on the putative role of RfCasp3 and 8 related to immune responses of black rockfish.

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INTRODUCTION

Apoptosis in host immune defense

Apoptosis can be basically characterized by prominently detectable morphological changes in cells, such as cell shrinkage, apoptotic body formation and chromatin condensation (Broker et al., 2005). It can involve in regulation of biological functions in immune cells, including maturation and homeostasis (Krammer, 2000). Moreover, apoptosis helps to eliminate excess or damaged cells to maintain tissue homeostasis (Jacobson et al., 1997). In this regard, apoptosis can particularly involve in elimination of potentially harmful cells such as pathogen infected cells from host organisms (Sun and Shi, 2001; White and Steller, 1995), playing a significant role in cellular immunity as an immune response against pathogenic infections. Pathogen infections, particularly mounted by microbes including virus are known to trigger apoptosis through different mechanisms; such as elevation of the production of pore-forming proteins and molecules directs the endogenous death machinery in the damaged or infected cell (Everett and McFadden, 1999; Weinrauch and Zychlinsky, 1999). Apoptosis signaling can be initiated through basically two different pathways, designated as intrinsic and extrinsic pathways, from which intrinsic one is triggered by release of cytochrome c from the mitochondria whereas extrinsic one is driven by a death receptor mediated signaling cascade (Ashkenazi and Dixit, 1998; Hengartner, 2000). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin/granzyme dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A (Elmore, 2007). Apoptotic signaling is known to be a highly conserved feature among wide array of taxonomic levels comprising common signaling molecules, such as BCL family members, caspases, tumor necrosis factors (TNF) and Inhibitor of apoptosis (IAP) family

proteins, being thoroughly interconnected with other cell signaling pathways (Danial and Korsmeyer, 2004).

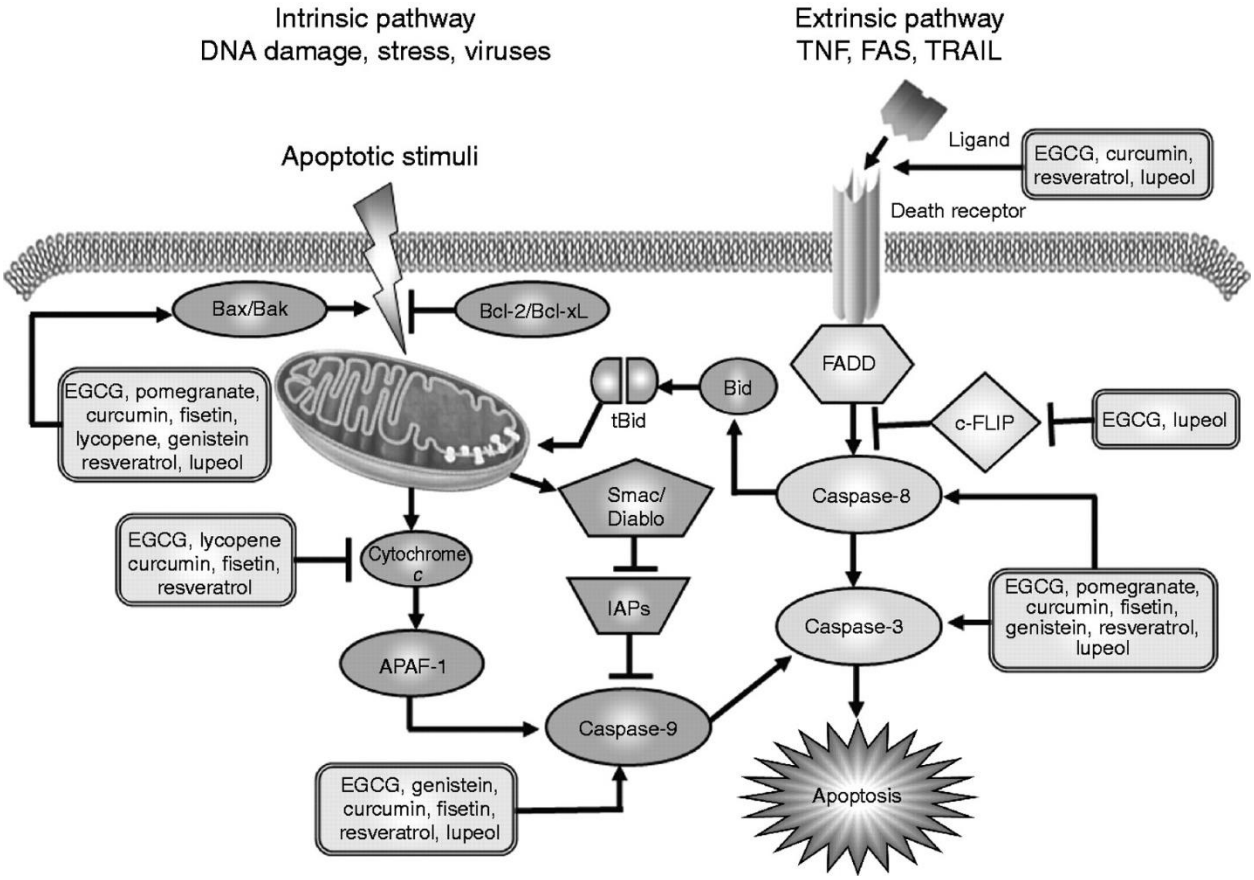

Fig.1. Illustration of two main apoptotic death cascades with their corresponding molecules partaking in.

Caspases in apoptotic death cascades

Caspases are cysteine aspartate specific proteinases; predominantly involve in cellular apoptotic cascades (Nicholson, 1999). Nearly thirteen caspases have been identified from mammals to date and categorized into two main groups, namely ‘inflammatory caspases’ (caspase 1,4,5,11,12 and 14) and ‘apoptotic caspases’ (caspase 2, 3, 6, 7, 8, 9 and 10), based on their basic function in the cells (Earnshaw et al., 1999). Caspases are synthesized as

inactive proenzymes (zymogenes) in preapoptotic cells. However, when the external or internal stimulus triggers apoptotic cascade in the cells, caspases can be activated by proteolytic cleavage on the c-terminal side of highly conserved aspartic acid residues, separating the protein into large and small subunits which in turn can form the active mature protein (Fuentes-Prior and Salvesen, 2004). Apoptotic caspases can be further divided into two classes; initiator caspases (caspase 8, 9, 10) and effector, also known as executioner caspases (caspase 3, 6 or 7). At the upstream of death cascades, initiator caspases can activate the zymogens of effector caspases to function at the downstream, eliciting apoptosis of the cell (Earnshaw et al., 1999; Schulze-Osthoff et al., 1998). However, according to the recent studies, the conventional roles of effector and initiator caspases are found to be debatable, since some effector caspases such as caspase 6 were shown to activate so called initiators (e.g. caspase 8) of the death cascade (Cowling and Downward, 2002; Monnier et al., 2011).

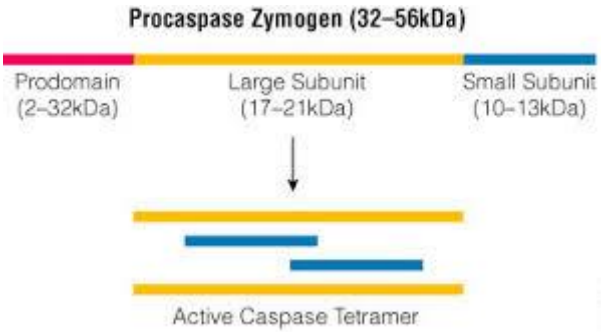

Fig.2. Caspases are expressed as inactive zymogens and activated by specific cleavage to form active tetramers

Caspase 3 and 8 in death cascades

Caspase 3, one of the effector caspases, is involved in executing the cell death signaling cascade of intrinsic and extrinsic apoptotic pathways, following its activation by caspase 8

and caspase 9, respectively (Lavrik et al., 2005). It acts as a molecular hub of caspase depended apoptosis, since each of the trigged death signaling pathways are ultimately mediated by caspase 3 to initiate its executioner phase. Activated caspase 3 mediates many of the characteristic morphological alterations of apoptosis, such as break-down of several cytoskeletal proteins, cleavage of polyadenosine dipeptide ribose polymerase (PARP) and degradation of the inhibitor of caspase-activated DNAses (ICADs), resulting in the release of CAD to cleave cell DNA and ultimately directing the cell towards death (Cohen, 1997).

Caspase 8 is an initiator caspase partake in up-stream of Fas, TNFR,1 and related TNF superfamily members driven death cascade pathways (Lawen, 2003). In general caspase-8 contains several main conservative structures including two death effector domains (DEDs) at N-terminal, P20 large subunit harboring the caspase family cysteine active site (Fan et al., 2005) and P10 small subunit (Boldin et al., 1996; Elmore, 2007; Muzio et al., 1996; Nakajima et al., 2000). Upon activation caspase 8 can activate pro-caspase 3 or 7 to execute apoptosis in cells. On the other hand, caspase 8 also involve in indirect pathway of eliciting apoptosis through cleavage and activation of BID (Luo et al., 1998). Activated BID can translocate into mitochondria and induce the release of cytochrome c to the cytosol, which in turn activate Apaf-1/caspase 9 apoptosome.

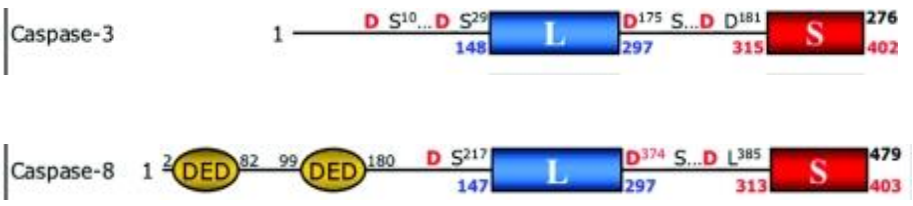

Fig. 3. Domain architecture of human caspase 3 and 7 boxes labeled as L and s represent large and small subunits, respectively.

Teleostan caspases

Different initiator as well as executioner phase caspase countparts was identified and characterized from several numbers of fish species, to date. Caspase 3 was identified and characterized at molecular level from European sea bass, bass *Dicentrarchus labrax*, zebrafish *Danio rerio*, large yellow croaker *Pseudosciaena crocea*, Atlantic salmon *Salmo salar* and Rock bream *Oplegnathus fasciatus* (Elvitigala et al., 2012; Li et al., 2011; Takle et al., 2006; Yabu et al., 2001; Yamashita et al., 2008) revealing their cysteine-aspartate specific protease activity and its activity or expressional modulation under pathogenic stress. Moreover, some homologues of vertebrate initiator caspases were also identified from teleostan origin caspase 8 from European sea bass (Reis et al., 2010) and Japanese medaka *Oryzias latipes* (Sakamaki et al., 2007); caspase 9 from large yellow croaker (Mu et al., 2010) and caspase 10 from olive flounder *Paralichthys olivaceus* (Kurobe et al., 2007) and snakehead murrel *Channa striatus* (Arockiaraj et al., 2013). Those studies provide evidence for the importance of respective caspase counterparts for the mediation of apoptotic death cascade and its expressional modulation by apoptogenic signals including pathogen infection.

Black rock fish farming in Asian mariculture industry and its challenges

Currently, mariculture farming is considered as a productive way of increasing marine fish and shellfish production in most parts of the world, including Asia. Therein, fish or shellfish species are intensively cultured with high density either in an enclosed section of ocean or in tanks, filled with seawater. Therefore, these are more susceptible for the environmental stress factors including stress arise due to pathogen infections, which can negatively affect their

survival and growth. Moreover, oxidative stress conditions in these species can suppress their immunity and in turn make them more vulnerable to the infections.

Black rockfish (*Sebastes schlegelii*) is a highly demanded delicacy in most parts of the world especially in Asia Pacific region including Korea and Japan, where fish were intensively cultured by mariculture farming. Approximately around 27000 tons are produced annually accounting for ~ 30 % of total cultured finfish in Korea. However, mass mortalities of this fish were encountered recently due to the combination of bacteria, viral and parasitic infections, resulting in an ample level of economic loss. As reported, rockfish are basically susceptible for infections mounted by Hirame Rhabdovirus (Kimura et al., 1989), as well as red sea bream iridovirus, Monogenean infestation caused by the parasites, *Microcotyle sebastis* and *Bivagina tai* (Park, 2009) along with streptococcosis caused by *Lactococcus garvieae* (Kang et al., 2004). Thus, gaining insights into its pathophysiology at molecular level is an important approach to raise them with more disease tolerant properties, using modern molecular techniques.

Aims and objectives of the study

The current study is focused on identification and molecularly characterization of two caspase counterparts (caspase 3 and caspase 8) from black rock fish, investigating their putative functional properties in apoptotic death cascade, further exploring its tentative role in host immune defense machinery through analyzing the expressional modulation under pathogen stress. We hope that our primary evidences may helpful in designing disease resistance strategies against current pathogenic threats, using molecular techniques.

Chapter I

Molecular characterization of caspase-3 from black rockfish (*Sebastes schlegelii*); protease activity and mRNA expression upon poly I:C and LPS

1. ABSTRACT

Caspase-3 plays an important role as main mediator of apoptosis in extrinsic pathway of cell death signaling. In this study, we describe the molecular characterization of caspase 3 homologue from black rockfish *Sebastes schlegelii* (Rfcasp3) with its transcriptional responses against pathogen stress and detectable protease activity. Open reading frame of Rfcasp3 encodes a 282 amino acid residues with a calculated molecular mass of 31.03 kDa and isoelectric point of 5.77. The deduced amino acid sequence contained the characteristic caspase-3 features including pro-domain, large and small subunits, active-site penta-peptide motif and protein binding domain. Pairwise sequence alignment revealed that Rfcasp3 has the greatest identity (87.3%) to *O. fasciatus* caspase-3. Transcriptional analysis showed that Rfcasp3 was ubiquitously expressed in various tissues/cells of rockfish with the greatest level in the blood cells. Significant Immune-modulation of Rfcasp3 transcription was detected in blood after injection with different stimuli by quantitative real-time PCR. Recombinant fusion protein of Rfcasp3 except pro-domain was over-expressed in *Escherichia coli* and showed the detectable hydrolyzing activity toward the mammalian caspase 3/7 specific substrate DEVD-*p*NA. Therefore, we can claim that the Rfcasp3 may act as a cysteine-aspartate protease in Black rockfish and actively involved in apoptosis triggering as an immune response.

2. MATERIALS AND METHODS

2.1. Black rockfish cDNA library construction

A black rockfish sequence data base was established using the Roche 454 genome sequencer FLX systems (GS-FLX^{TM}), a next generation DNA sequencing (NGS) technology (DNA Link, Republic of Korea). The Black rockfish cDNA sequence database was formerly constructed by principle of pyrosequencing on the Roche 454 Genome Sequencer FLX technique (GS-FLX) (Mardis, 2008). Concisely, twelve black rockfishes (~100 g) were challenged with immune stimulation on *Edwardsiella tarda* (10^7 CFU/fish), *Streptococcus iniae* (10^7 CFU/fish), *lipopolysaccharide* (LPS, 1.5 mg/fish), *polyinosinic:polycytidylic acid* (poly I:C,1.5 mg/fish). The total RNA was isolated from liver, head kidney, spleen, blood, gill and intestine using the QIAzol® (Qiagen, USA) and purified using an RNeasy Mini kit (Qiagen, USA). The purified RNA was verified by measuring the RNA integrity number (RIN) with Agilent 2100 Bioanalyzer (Agilent Technologies, Canada). The RIN was confirmed with 7.1. Thereafter, cDNA library was constructed using fragmented RNA (average size-1147bp).

2.2. Sequence characterization and phylogenetic analysis of Rfcasp3

The Rfcasp3 full-length cDNA sequence was analyzed using Basic Local Alignment Tool (BLAST) algorithm. The open reading frame (ORF) of Rfcasp3 and deduced amino acid were carried out by DNAssist (Version 2.2). Conserved domain and motifs were predicted by ExPaSy PROSITE database (http://prosite.expasy.org/). Molecular weight (MW) and isoelectric point (p*I*) were determined using ExPaSy ProtParam tool (http://web.expasy.org/protparam). The pairwise and multiple sequence alignments were performed by MatGat (2.02) and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/),

respectively. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MAGA 6.0) software (Tamura et al., 2013) and Neighbor-joining (NJ) method.

2.3. Experimental fish and tissue sampling

Healthy fish were obtained from aquariums at the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea, and were acclimated in 400 L laboratory aquarium tanks filled with aerated seawater at 21-22 ℃. In order to investigate the *Rfcasp3* expression, five healthy fish with average body weight of 200 g were sacrificed for tissue sampling. Blood samples (~1ml/fish) also were collected from each fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA). Blood cells were directly separated by centrifugation at 3000 x g for 10 min at 4 ℃. The tissues including head kidney, spleen, liver, gill, intestine, kidney, muscle, skin, heart were isolated. All tissue samples were snap frozen in liquid nitrogen and were stored at -80℃ until RNA isolation.

2.4. Immune challenge experiment of black rockfish

In order to analyze the immune responses of Rfcasp3 upon immune stimulants, the gram negative bacterial endotoxin LPS (*E. coli* 0127:B8; Sigma-Aldrich, USA) and double-stranded RNA viral derivative poly I:C (Sigma, St. Louis, MO, USA) were resuspended in 1 X phosphate buffered saline (PBS) prior to injection. Each black rockfish was administered a single intraperitoneal injection of 200 μL LPS (1.25 μg/μL) or poly I:C (1.5 μg/μL). Control group was injected with equal volume (200μL) of PBS. From the immune-challenge group and corresponding control group, five individuals in each group were sampled at 3, 6, 9, 12, 24, 48 and 72 h post injection, as described in Section 2.3

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated by QIAzol® (Qiagen, USA) from a pool of tissue samples (~40 mg from each fish) according to manufacturer's protocol. The concentration of RNA was determined μ-Drop Plate (Thermo Scientific, USA) and RNA quality was confirmed by 1.5% agarose gel electrophoresis. 2.5 μg of RNA were used to synthesize the first strand cDNA by the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instruction. Finally, the cDNA samples were diluted 40-fold and were stored at -80℃ until further experiments.

2.6. Rfcasp3 expression analysis by real-time PCR (qRT-PCR)

To determine the immune modulation of Rfcasp3 in challenge experiment, qRT-PCR was performed using the Dice™ Real time system thermal cycler (TP800; TaKaRa, Japan) according to MIQE guidelines (Bustin et al., 2009). Previously synthesized cDNA was used as template for qRT-PCR with gene-specific primers (Table 1). The qRT-PCR was carried out in 10 μL total volume containing 3 μL of diluted cDNA from each tissue, 5 μL of 2x TaKaRa ExTaq™ SYBR premix, 1 μL of each primer. Amplification conditions were as follows: one cycle of 95℃ for 30s, followed by 35 cycles of 95℃ for 5s, 58 ℃ for 10s, 72 ℃ for 20 s, and a final single cycle of 95 ℃ for 15 s, 60 ℃ for 30 s and 95 ℃ for 15 s. All experiments were performed in triplicate. The baseline was automatically set by the Dice™ Real Time System software (version 2.00). The black rockfish elongation factor 1α (RfEF1A) gene was used as internal reference gene (GenBank ID: KF430623). The relative expression levels of Rfcasp3 were performed by Livak ($2^{-\Delta\Delta CT}$) method. All data was presented as the mean ± standard deviation (SD). In the immune challenge experiment, the relative expression levels of Rfcasp3

were normalized to the corresponding PBS-injected controls at each time point. The relative mRNA level in un-injected control (0 h) was considered as baseline reference. Statistical analysis between the un-injected control and immune-stimulated groups was performed by a two tailed un-paired Student's t-test. *P*-values of less than 0.05 were determined to indicate the statistical significance.

2.7. Over-expression and purification of recombinant Rfcasp3 fusion protein

To purify the recombinant Rfcasp3 except pro-domain, the primer sets of Rfcasp3 were designed with restriction enzyme site for *Eco*RI and *Hin*dIII, respectively (Table 1). The Rfcasp3 sequence encoding 39-282 amino acid residues was amplified using TaKaRa thermal cycler. The PCR reaction was performed in a 50 μL volume including 50 ng of template, 20 pmol of each primer, 5 μL of 10 X Ex Taq Buffer, 4 μL of 2.5 mM dNTPs, and 5U of Ex Taq polymerase (TaKaRa, Japan). The reaction conditions were as follows: one cycle of 94 ℃ for 3min; 35 cycles of 94 ℃ for 30 s, 58 ℃ for 20 s, 72 ℃ for 40 s; and final extension at 72 ℃ for 5min. The amplified PCR product and pMal-c2x vector were digested with each restriction enzyme and ligated using mighty Mix (TaKaRa, Japan) at 16℃ for 30min followed by incubation at 4℃ overnight. The ligated pMal-c2x/Rfcasp3 product was transformed into *Escherichia coli* DH5α and sequenced. The sequence confirmed recombinant pMal-c2x/Rfcasp3 was transformed into *E. coli* BL21 (DE3). The Rfcasp3 fusion protein was overexpressed using isopropyl-β-galactopyranoside (IPTG, 0.5mM final concentration) at 20 ℃ for 8 h. The induced *E. coli* BL21 (DE3) cells were harvested by centrifugation 3500 RPM for 30min at 4 ℃. The harvested cells were resuspended in 20 mL of column buffer (20 mM Tris–HCl pH 7.4 and 200 mM NaCl) and stored at -20 ℃. After thawing and sonicating, the protein was purified using pMAL^{TM} Protein Fusion and Purification System (New

England Biolabs, UK). The purified protein was eluted with elution buffer (10 mM maltose + column buffer) and the Bradford method (Bradford, 1976) was used to measure the concentration of purified Rfcasp3 protein. The Rfcasp3 samples obtained at different purification steps was analyzed by SDS-PAGE, using 12% gel under reduced conditions, with standard molecular weight marker (Enzynomics, Korea). The 0.05% Coomassie R-250 was used to stain the gel, followed by a standard destining procedure.

2.8. Hydrolyzing activity assay of Rfcasp3

To characterize the purified Rfcasp3, hydrolyzing activity was performed using caspase-3 activity kit (Bio Vision, USA) according to manufacturer's protocol. Briefly, the 25 μL of purified protein (0.83 μg/μL) was mixed with 25 μL of column buffer, 50 μL of 2x reaction buffer and 5 μL of 4mM caspase3/7 specific substrate (DEVD-*p*NA). After incubation at 37℃ for 2 h, the color generated by para nitroaniline (*p*NA) was measured using a spectrophotometer at 405nm. In other to evaluate the substrate specific activity of Rfcasp3, caspase-3 specific substrate (DEVD-*p*NA) and caspase 8 specific substrate (LEHD-*p*NA), (Bio Vision, USA) was used. The effect of temperature and concentration in the hydrolyzing activity was also investigated. The purified protein in the concentration range of 0-391.86 μg/mL and temperature from 10℃ to 50℃ were used to analyze the activity at corresponding protein concentration or temperature. The Maltose binding protein (MBP) control was used to determine the effect of fusion protein on the activity assay of Rfcasp3. All assays both fusion protein and MBP were carried out with three replicates. The hydrolyzing activities were expressed as the mean absorbance values.

Table.1. Primer set used in this study on Rfcasp3

Name	Purpose	Sequence(5'→3')
Rfcasp3-F1	qRT-PCR of Rfcasp3	GCATTGAAGCCGACAGTGGAGATG
Rfcasp3-R1	qRT-PCR of Rfcasp3	TGCATGAACCAGGACCCAGTCATA
Rfcasp3-F2	ORF amplification of Rfcasp3 (without pro-domain)	GAGAGAGAATTCGTGATGGGCCACAGCAGCTTCAGATACA
Rfcasp3-R2	ORF amplification of Rfcasp3 (without pro-domain)	GAGAGAAAGCTTCTAAAATAATTCTCCGTACACATCCTCAGCCT
RFEF1a-F	qRT-PCR for black rockfish RF1α	AACCTGACCACTGAGGTGAAGTCTG
RFEF1a-R	qRT-PCR for black rockfish RF1α	TCCTTGACGGACACGTTCTTGATGTT

3. RESULTS

3.1. Molecular characterization of Rfcasp3

Rfcasp3 was identified and characterized from black rockfish cDNA sequence database using NCBI-BLAST program. The full-length cDNA sequence of Rfcasp3 from black rockfish consists of 2224 bp that contained an open reading frame (ORF) of 846 bp encoding 282 amino acids, a 33bp 5'-untranslated region (5'-UTR) and a 1345 bp 3'-untranslated region (3'-UTR). Furthermore, the predicted molecular mass and isoelectric point of Rfcasp3 were 31.03 kDa and 5.77, respectively. Two polyadenylation signals (^{1588}AATAAA^{1593}, ^{2207}AATAAA^{2212}) and four RNA instability motifs (^{1128}ATTTA^{1132}, ^{1551}ATTTA^{1555}, ^{1980}ATTTA^{1984}, ^{2044}ATTTA^{2048}) were identified in 3' UTR. The characteristic caspase domains were identified in predicted Rfcasp3 amino acid sequence such as a putative pro-domain (Met^{1} to Asp^{34}), a large subunit (P20, Ser^{51} to Asp^{175}) and small subunit (P10, Cys^{188} to Pro^{282}). Moreover, catalytic center (Cys^{171}, His^{129}, Gly^{130}), binding pocket (Arg^{72}, Gln^{169}, Arg^{243}, Ser^{256}), active-site penta-peptide motif (^{169}QACRG^{173}) and protein binding domain (^{217}GSWFI^{221}) were also presented within the large subunit and small subunit, respectively.

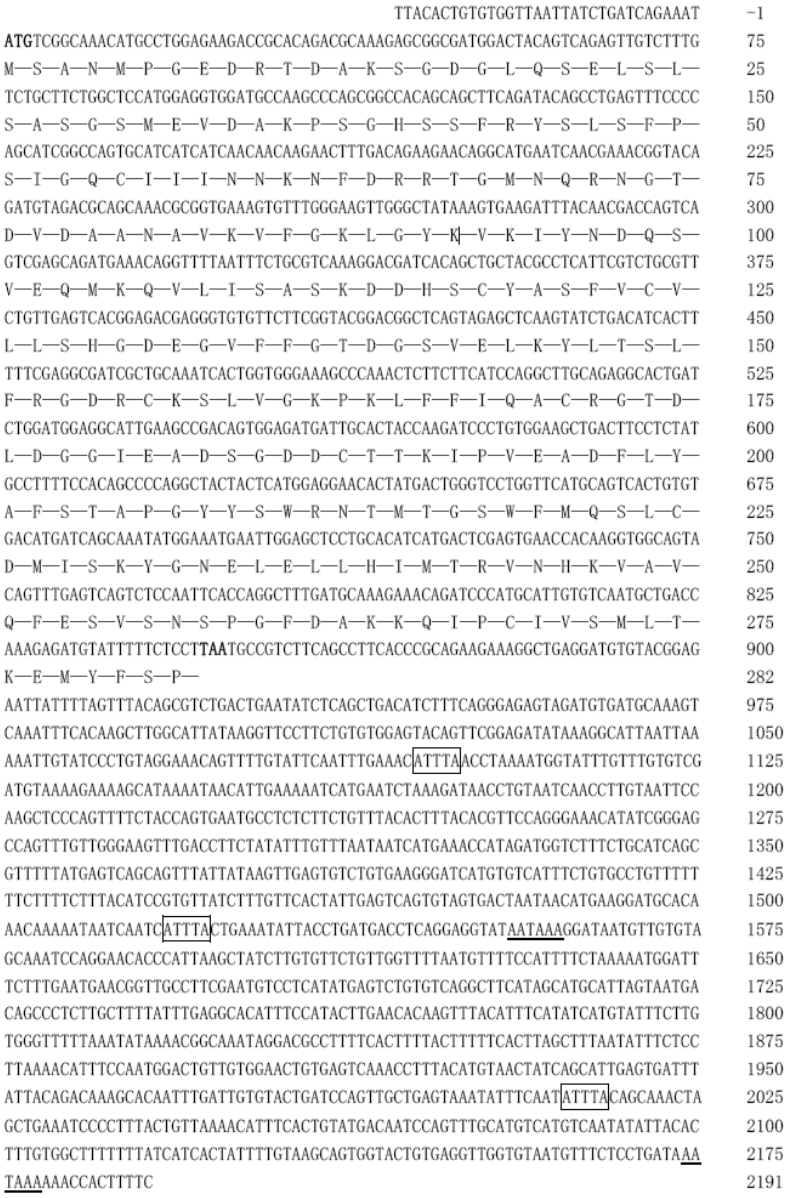

Fig.4. The full-length cDNA nucleotide sequence and deduced amino acid of Rfcasp3 from black rockfish. The start codon (ATG) and stop codon (TAA) are indicated by bold. Three RNA instability motif (ATTTA) are depicted by box. Two polyadenylation signal sequence (AATAAA) are emphasized in underline.

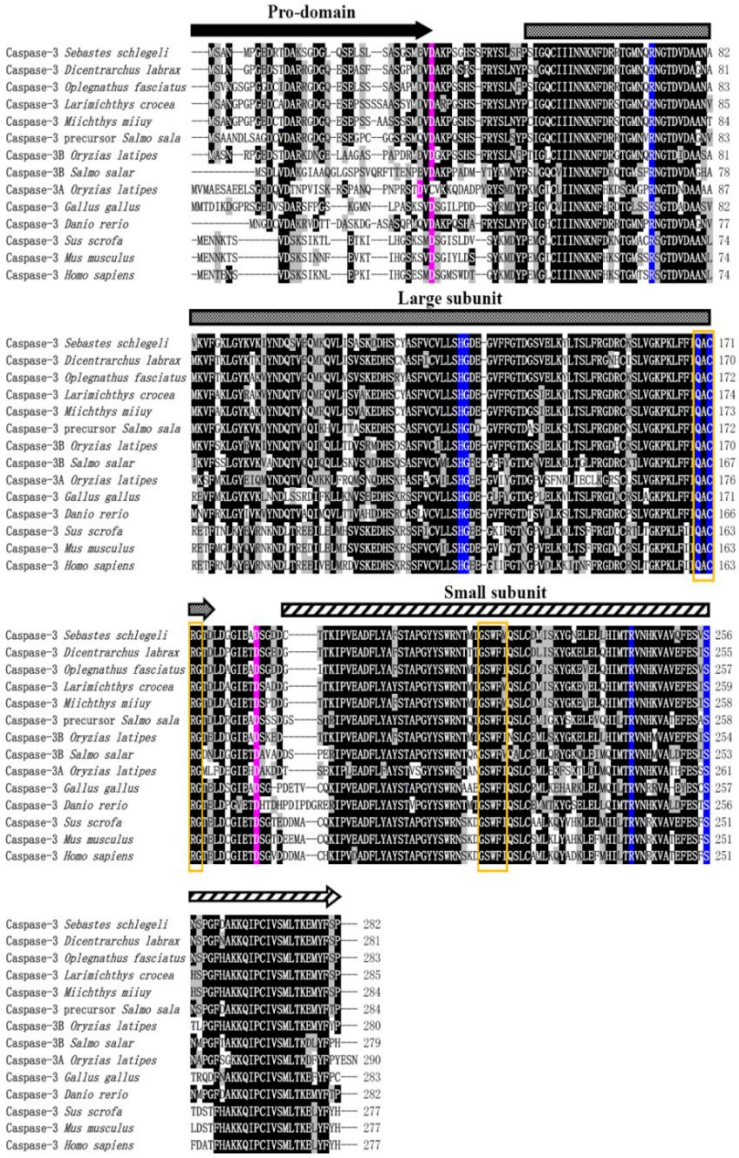

Fig.5. Multiple alignment of Rfcasp3 with other caspase-3 family member amino acid sequences by ClustalW method. The putative cleavage sites at aspartic acid residues are indicated by pink shading. Catalytic center and binding pocket are highlighted by blue shading. Active-site penta-peptide motif and protein binding domain are boxed by yellow color. Conserved and similar amino acid residues are shaded by dark and gray, respectively. The pro-domain, large and small subunits are indicated.

Pairwise sequence alignment revealed that Rfcasp3 had the degree of identity range from 87.3% to 51%. *O. fasciatus* showed the highest identity (87.3%) and similarity (91.9%). In contrast, *Xenopus laevis* had lowest identity (51%) and similarity (65.2%) with it (Table 2)

To compare the evolutionary position of Rfcasp3 with the other species, a phylogenetic tree was build using MEGA-5 software using selected counterparts from mammals, amphibians, birds and fish, picked from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The result showed that the phylogenetic tree clustered into two different subclades (fish and non-fish vertebrates) with other caspase-3 counterparts. Furthermore, Rfcasp3 shows a most evolutional relationship with *D. lavrax* (caspase-3) (Fig. 6).

Table.2. Identity and similarity of Rfcasp3 gene with other caspase-3 counterparts

Gene name	Species	Taxonomy	Genebank accession number	Identity%	Similarity%
Caspase-3	*Oplegnathus fasciatus*	Fish	AFM09714	87.3	91.9
Caspase-3	*Dicentrarchus labrax*	Fish	ABC70996	85.1	91.8
Caspase-3	*Miichthys miiuy*	Fish	AHG06618	84.2	90.5
Caspase-3	*Larimichthys crocea*	Fish	ACJ65025	82.5	89.8
Caspase-3precursor	*Salmo salar*	Fish	ACN11423	78.2	87.3
Caspase-3B	*Oryzias latipes*	Fish	NP_001098168	77.3	86.9
Caspase-3	*Danio rerio*	Fish	BAB32409	71.5	83
Caspase-3B	*Salmo salar*	Fish	AAY28972	64.3	80.9
Caspase-3	*Gallus gallus*	Aves	NP_990056	59.9	77
Caspase-3	*Sus scrofa*	Mammalia	NP_999296	58.5	74.5
Caspase-3	*Mus musculus*	Mammalia	AAH38825	58	73.4
Caspase-3	*Homo sapiens*	Mammalia	CAC88866	56.6	73.8
Caspase-3A	*Oryzias latipes*	Fish	BAC00949	54.8	70.7
Caspase-3	*Xenopus laevis*	Amphibia	NP_001081225.1	51	65.2

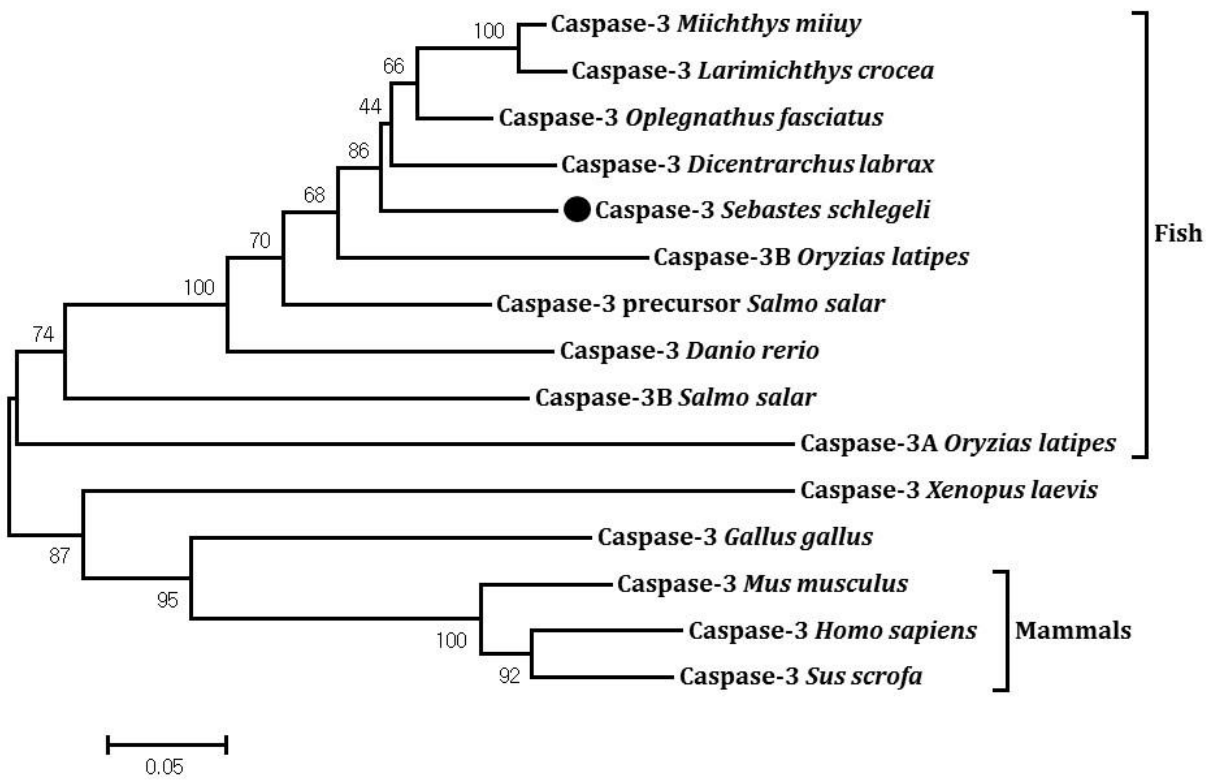

Fig.6. Phylogenetic analysis of Rfcasp3 with capase-3 sequences from other species. The phylogenetic tree was constructed by ClustalW and MEGA version 6.0 with 1,000 bootstrap repeats and neighbor-joining method. Accession numbers of species was mentioned in Table 2.

3.2. Tissue-specific expression of Rfcasp3

In other to investigate the mRNA expression of Rfcasp3 in the various tissues of healthy black rockfish, qRT-PCR was performed using gene-specific primers designed from Rfcasp3 sequence. The relative expression of Rfcasp3 in several tissues was determined using RfEF1A as a housekeeping gene and the results were compared with kidney expression level to calculate the relative tissue-specific expression profile (Fig. 7). Rfcasp3 mRNA was ubiquitously expressed in all tissues examined. Further analysis results showed that Rfcasp3 mRNA expression was higher in the blood, skin, intestine which were found to be 63-, 11.8-, 11.3-fold higher compared to that detected for kidney, respectively.

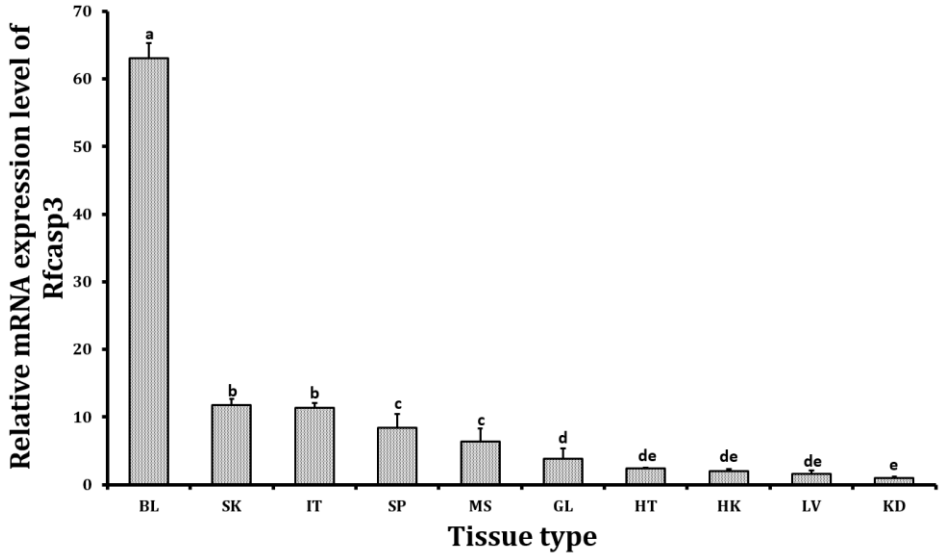

Fig.7. Tissue specific expression analysis of Rfcasp3. The relative Rfcasp3 mRNA expression in blood, skin, intestine, spleen, muscle, gill, heart, head kidney, liver and kidney was measured using RfEF1A as the reference gene compared with kidney mRNA level. Error bars represent the standard deviation (SD, n=3). The significant difference ((P<0.05)) are shown as different letters. BL, blood cell; SK, skin; IT, intestine; SP, spleen; MS, muscle; GL, gill; HT, heart; HK, head kidney; LV, liver; KD, kidney.

3.3. Transcriptional response of Rfcasp3 upon immune challenge

The expression patterns of Rfcasp3 upon the immune-stimulation such as LPS, poly I:C were determined using qRT-PCR. The mRNA expression level of detected Rfcasp3 was normalized to the RfEF1A as a reference and compared to the PBS-injected control expression in each time point (Fig. 8) The mRNA expression of Rfcasp3 in blood cells was significantly elevated after poly I:C challenge experiment. The relative mRNA expression level of Rfcasp3 was up-regulated at 6 h post treatment (p.t.) (1.6-fold) and then the expression level was gradually decreased until 72 h p.t.. No significant difference of Rfcasp3 was observed at 3 h, 12 h, 24 h, 48 h and 72 h p.t. relative to to 0 h (Fig. 8A). After the LPS challenge, the mRNA expression profile of Rfcasp3 also found to behave in similar pattern to the outcomes of poly I:C immune-stimulation in blood cells. In the early phase, the significant up-regulation of Rfcasp3 was observed at 3 h (1.5-fold), 6 h (1.6-fold) p.t. and down-regulation was measured at 72h. After that no significant difference of Rfcasp3 was found between challenge and control.

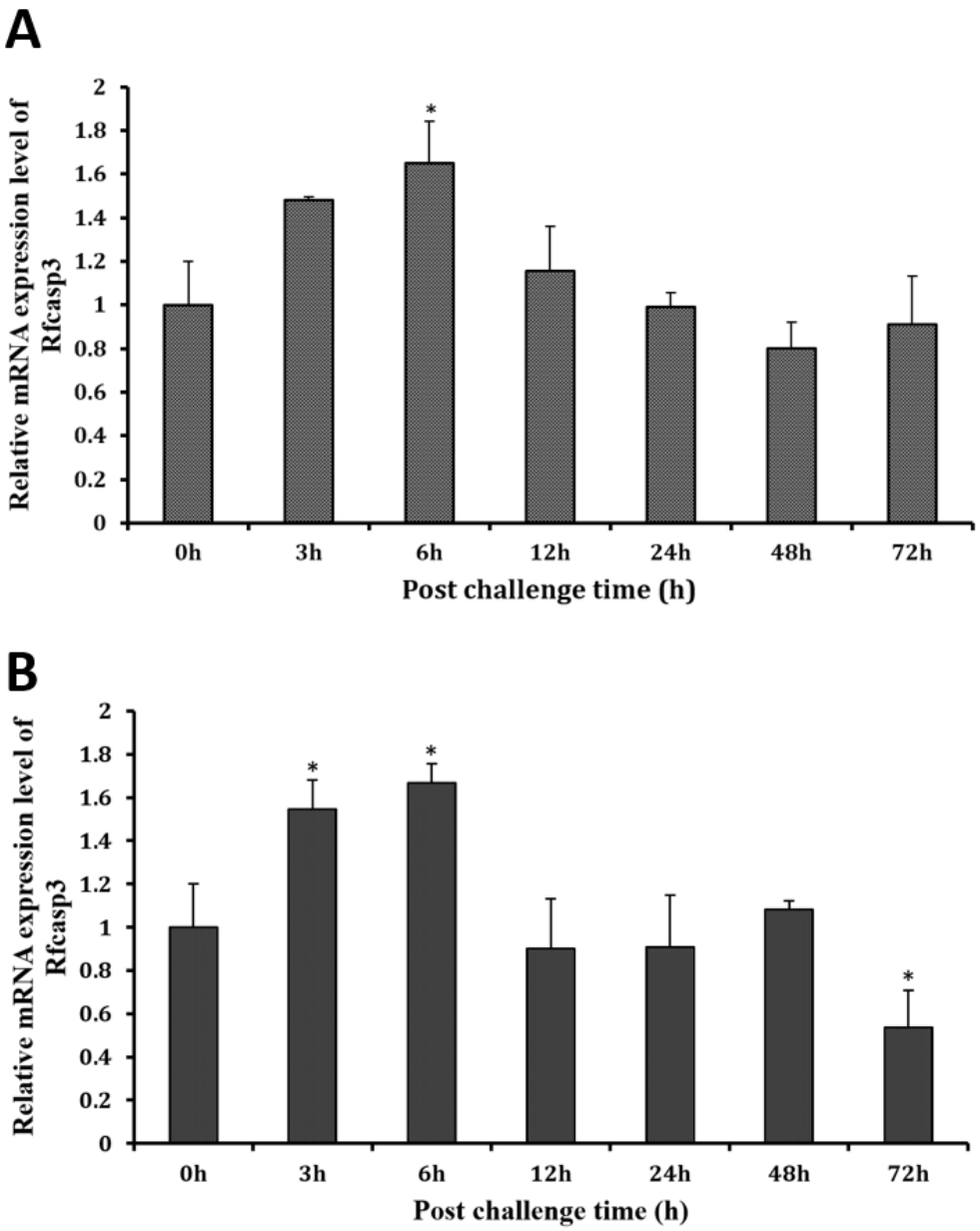

Fig.8. The mRNA expression of Rfcasp3 in blood cell upon challenge experiment with (A) poly I:C or (B) LPS. The mRNA expression of Rfcasp3 was calculated by $2^{-\Delta\Delta CT}$ method using RfEF1A as the reference gene. The relative mRNA level was compared with PBS-injected control at each time point. Error bar represent the standard deviation (SD, n=3). The differences are indicated with an asterisk (*) at $P < 0.05$.

3.4. Over-expression and purification of Rfcasp3

To investigate the hydrolyzing activity, Rfcasp3 excluding the pro-domain was cloned into the pMal-c2X vector and the fusion protein with MBP was over-expressed in *E. coli* BL21 (DE3) by IPTG induction. The purification of Rfcasp3 was performed by pMal purification system. The molecular weight of purified recombinant Rfcasp3 protein was visualized by SDS-PAGE and was ~69.6 kDa (MBP ~42.5 kDa) which agrees with deduced protein size (27.1 kDa) of Rfcasp3 (Fig. 9).

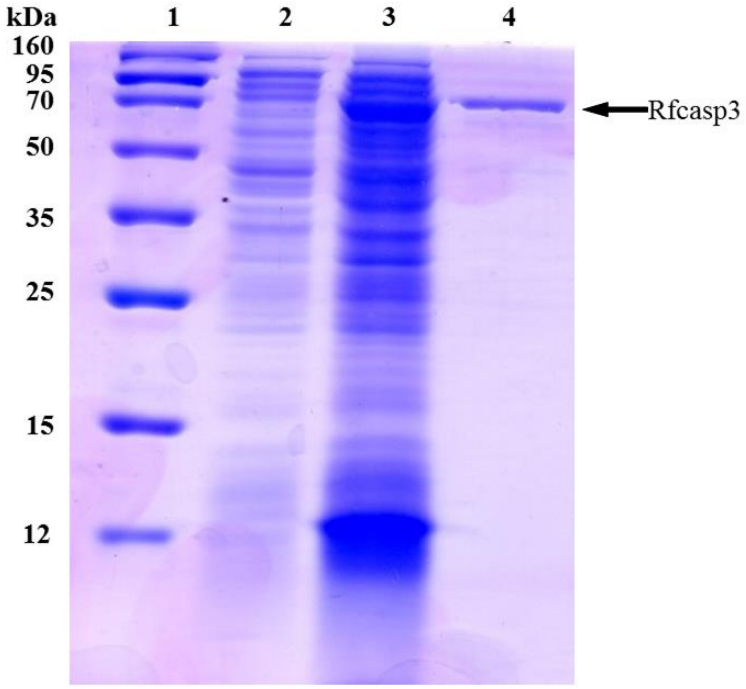

Fig.9. SDS-PAGE analysis of overexpressed and purified fusion protein of Rfcasp3. Columns represent: 1, protein marker (Enzynomics, Korea); 2, total cellular extract from *E.coli* (DE3) before IPTG induction; 3, crude protein of recombinant Rfcasp3; 4, elute recombinant Rfcasp3-MBP protein after purification.

3.5. Biochemical characterization of Rfcasp3

The specific activity of Rfcasp3 toward the caspase 3/7-specific synthetic substrate, DEVD-*p*NA was confirmed (Fig. 10). Significant hydrolyzing activity of Rfcasp3 was detected for DEVD-*p*NA (mean A400:- 0.9932). In contrast, relatively low activity of Rfcasp3 was detected against LEHD-*p*NA (mean A400:- 0.2229). To further understand the property of Rfcasp3, the assay was performed under different conditions. Rfcasp3 demonstrated a dose-dependent hydrolyzing activity which increases until 391.86 μg/mL of Rfcasp3. The activity of Rfcasp3 toward DEVD-*p*NA was gradually increased until 37 ℃ and was decreased at 50℃. The results suggest that the optimum temperature for hydrolyzing activity of Rfcasp3 is around 37 ℃.

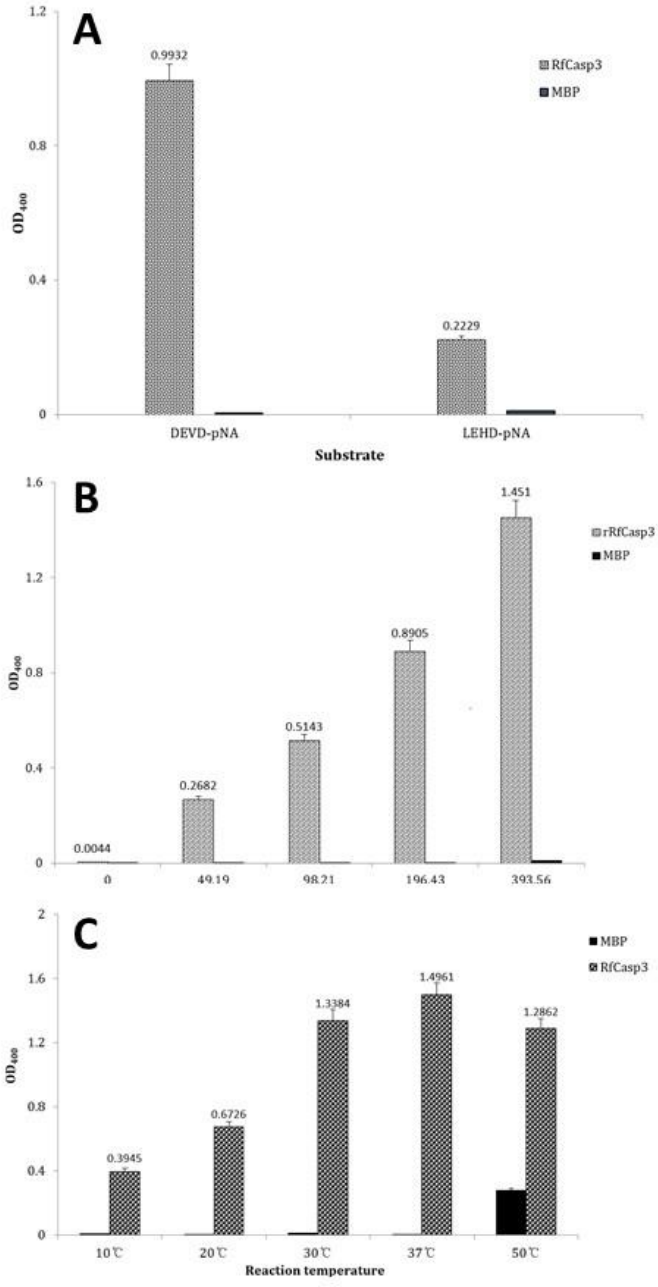

Fig.10. Hydrolyzing activity assay of Rfcasp3 under (A) specific substrate and the effect of (B) temperature and (C) protein concentration on the activity of recombinant Rfcasp3 protein. The substrate specific hydrolyzing activity against DEVD-*p*NA is measured by absorbance value at 405nm. Hydrolyzing activity was calculated at different temperature (10-50 ℃) and protein concentration (0-393.56 μg/mL). Error bars represent the standard deviation (SD, n=3)

4. Discussion

In this study, we investigated the identification and functional characterization of Rfcasp3 from black rockfish. The deduced amino acid sequence of Rfcasp3 contained the general caspase domains such as pro-domain, large subunit and small subunit. Typically, caspases are expressed as inactive pro-caspase (zymogen) and catalytically activated by proteolytic processing of three domains. Finally, large and small subunits make up an active form of caspase-3 as tetramers (Rupinder et al., 2007). The putative cleavage site ($Asp^{34}$and Asp^{183}) was also detected among pro-domain, large and small subunit. Furthermore, catalytic center and binding pocket in S1 subside that are interacted with active site of caspase-3 were identified (Li et al., 2011). Similarly, active-site penta-peptide motif and protein binding domain were completely found in large and small subunits compared with other species (Elvitigala et al., 2012).

Phylogenetic analysis of Rfcasp3 revealed that Rfcasp3 was closely located with caspase-3B isoform from *O. latipes*, rather than caspase-3A isoform from *O. latipes.* Interestingly, we can infer that cloned Rfcasp3 may be the variant B of caspase-3 in black rockfish.

Rfcasp3 mRNA was found to be universally expressed from healthy black rockfish, even though the expression levels of Rfcasp3 was different in different tissues examined. The previous studies indicated that caspase-3 from other species is also ubiquitously expressed. In teleost, the sea bass (*D. labrax*) caspase-3 mRNA was detected in all investigated tissues including spleen, heart, liver, intestine, head kidney, thymus (Reis et al., 2007). Rock bream (*O. fasciatus*) caspase-3 mRNA was measured in all examined tissues including blood cells, liver, brain, heart, spleen, head kidney, gill, intestine, kidney, skin, muscle (Elvitigala et al., 2012). In mammals, Rat caspase-3 mRNA was expressed in brain, heart, muscle, thymus,

spleen, liver, kidney, testis and lung (Ruest et al., 2002). Moreover, the highest expression of Rfcasp3 was mainly detected in blood cells. This result was in agreement in few fish species including Rock bream *O. fasciatus* (Elvitigala et al., 2012) large yellow croaker (Li et al., 2011). Therefore, we suggests that ubiquitous expression of Rfcasp3 may play an important role in several biological process through apoptosis

In order to understand the transcriptional pattern of Rfcasp3 against immune-stimulants with poly I:C and LPS, we investigated the mRNA expression level of Rfcasp3 from blood cells of black rockfish. The blood contains immune related cells including macrophage, neutrophil, non-specific cytotoxic cells. Among this immune related cells, non-specific cytotoxic cells undertakes the regulation of apoptosis in innate immunity (Evans et al., 2001). In accordance with poly I:C stimulation as the similar structure of double stranded RNA, Rfcasp3 exhibited significant difference of expression at 6 h post injection. This finding was in agreement with the similar pattern measured from rock bream upon the poly I:C challenge experiment. LPS was known as n endotoxin of gram negative bacteria which was reported as a powerful virulence factor of innate immunity in fish (Swain et al., 2008). The immune response to LPS challenge revealed that the significant expression of Rfcasp3 was measured at 3 and 6 h post-injection (Fig. 8). However, caspase-3 from rock bream was found to be significantly up-regulated in liver tissue in response to challenge experiment with *E. tarda* and LPS, and the highest expression of caspase-3 reached at the peak at 24 h and 48 h post-injection (Elvitigala et al., 2012). During trivalent bacterial vaccine stimulation, caspase-3 from large yellow croaker was also found to be strongly up-regulated in kidney, and reached at 48 h post injection (Li et al., 2011). This may be attributed to the difference of tissues in both experiment. Clearance of bacteria was occurred by blood stream and then bacteria was

normally collected in kidney (Ferguson et al., 1982). Therefore, these results suggest that Rfcasp3 may involve in the early phase of innate immunity against LPS and poly I:C infections.

Hydrolyzing activity assay revealed that Rfcasp3 fusion protein have significant activity relative to MBP control against the caspase 3/7 specific substrate, DEVD-*p*NA (Fig. 10). These results indicates that Rfcasp3 potentially had a biological function of caspase-3 similar to other known caspase-3 subfamily members. Furthermore, compared to caspase 8 specific substrate and caspase-9 specific substrate, Rfcasp3 exhibited an evident specific activity toward DEVD-*p*NA. The observation was found to be compatible with previous report from rock bream (Elvitigala et al., 2012), large yellow croaker (Li et al., 2011) and zebrafish (Yabu et al., 2001). We also investigated the effect of temperature and protein concentration on hydrolyzing activity of rRfcasp3. Therein, we could affirm that, hydrolyzing activity of the RfCasp3 is a function of temperature and its concentration and specific to caspase 3 substrate.

In summary, the full-length cDNA sequence of caspase-3 gene was identified from the established black rockfish cDNA database. Sequence characterization and phylogenetic analysis reveal that the putative gene and protein of Rfcasp3 have typical features of caspase compare to other members of vertebrate caspase-3. Rfcasp3 was ubiquitously detected in all tested tissues and the highest expression of Rfcasp3 was expressed in blood. After challenge experiment with immune-stimulants with LPS, poly I:C, the mRNA expression Rfcasp3 was modulated in blood. Moreover, recombinant caspase-3 fusion protein exhibited functional properties against caspase3/7 specific substrate and was determined in effect of temperature and protein concentration on cysteine protease activity. All these data indicated that Rfcasp3

may be involved in innate immunity by apoptosis signaling pathway, and may help to plan the strategy against pathogenic threat.

Chapter II

Characterization and transcriptional profiling of caspase-8 from black rockfish (*Sebastes schlegelii*) upon the immune-stimulation

1. ABSTRACT

Caspase or aspartate-specific cysteine proteases are central components of inflammation, necrosis and apoptosis. Caspase-8 was involved in upstream of the death cascades as a initiator caspase in extrinsic apoptotic pathway. In this study, we identified and characterized caspase-8 gene (Rfcasp8) from black rockfish *Sebastes schlegelii*. The complete cDNA sequence of Rfcasp8 was 2467 bp including 1473 bp open reading frame which encodes 491 amino acid peptides with theoretical molecular mas of 55.26 kDa. The two death effector domains (DED), large subunit and small subunit were conserved in the deduced amino acid residues of Rfcasp8. Moreover, phylogenetic analysis reveal that Rfcasp8 was clustered into fish caspase-8 counterparts and especially close to *Gasterosteus aculeatus*. Our quantitative real time PCR results showed that Rfcasp8 was ubiquitously detected in all tissues and highly expressed in blood. Upon injecting the different pathogen-derived molecular motifs such as poly I:C and LPS, Rfcasp8 transcript was significantly elevated in black rockfish blood cells. These findings collectively suggest that Rfcasp8 may play an essential role in antibacterial and antiviral defense.

2. MATERIALS AND METHODS

2.1. Identification of full-length cDNA sequence of Rfcasp8

Using a previously established black rockfish cDNA database based upon pyrosequencing data obtained from Roche 454 genome sequencer FLX systems (GS-FLX^{Tm}), a next generation DNA sequencing (NGS) technology (DNA Link, Republic of Korea), the full-length cDNA sequence of caspase-8 was identified using the BLAST algorithm.

2.2. Sequence analysis of Rfcasp8

The ORF sequence of Rfcasp8 was identified using DNAssist (version 2.2). The Rfcasp8 sequence was analyzed by BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To determine the conserved domain of deduced Rfcasp3 protein sequence, ExPASy-PROSITE (http://prosite.expasy.org/) was used. Multiple alignment was carried out using ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) Identity and similarity of Rfcasp8 was calculated using MatGat (Version 2.02) software and phylogenetic tree was built using the Molecular Evolutionary Genetics Analysis (MAGA 6.0) software and Neighbor-joining (NJ) method.

2.3. Experimental animals and tissue isolation

Black rockfish (average body weight 200g) was maintained in the 400 L laboratory aquarium tanks filled with aerated seawater at 22±1 ℃. Head kidney, spleen, liver, gill, intestine, kidney, muscle, skin, heart tissues of black rockfish were dissected from five heathy animals and whole blood (1 ml per fish) was isolated from five heathy fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA) and was immediately centrifuged

at 3000 x g for 10 min at 4 ℃ to remove the plasma. The collected tissues were snap-frozen in liquid nitrogen and stored in -80 ℃

2.4. Immune challenge experiment

To evaluate the immune-stimulated expression of Rfcasp3 gene, the purified endotoxin (LPS, *E. coli* 0127:B8; Sigma-Aldrich, USA) and double-stranded RNA viral poly I:C (Sigma, St. Louis, MO, USA) was used in immune challenge experiment. All immune-stimulants were dissolved in 1X PBS and were injected into intraperitoneal area of black rockfish. The concentration was described in chapter I, section 2.4. The un-injected group of fish was used as a negative control and fish were injected with equal volume (200μl) of 1X PBS was used as a injection control. Blood cells were collected from five animals in each group at 3, 6, 12, 24, 48, 72 h post-challenge. All samples were immediately frozen in liquid nitrogen and stored at -80℃

2.5. Total RNA extraction and cDNA synthesis

Total RNA extraction was performed using QIAzol® (Qiagen, USA) following manufacturer's protocol. RNA concentration was measured in μDrop Plate (Thermo Scientific, USA). First-stranded cDNA synthesis was carried out from 1 ug of total RNA using PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) according to manufacturer's protocol. The synthesized cDNA was diluted in 40-fold and stored at -80 ℃ until use as template for qRT-PCR.

2.6. Transcriptional profiling of Rfcasp8 by qRT-PCR

The Rfcasp8 mRNA expression profile was detected by quantitative Real-Time PCR (qRT-PCR) from several tissues such as head kidney, spleen, liver, gill, intestine, kidney, muscle, skin, heart tissues and blood. Moreover, to investigate expression modulation of Rfcasp8 in blood cells after the immune-stimulation with poly I:C and LPS, the relative expression level was compared to un-injected controls. The black rockfish elongation factor 1α (RfEF1A) gene was used as internal control gene (GenBank ID: KF430623). qRT-PCR was performed Dice™ Real time system thermal cycler (TP800; TaKaRa, Japan) under the following conditions: one cycle of 95 ℃ for 30 s, followed by 35 cycles of 95 ℃ for 5 s, 58 ℃ for 10 s, 72 ℃ for 20 s, and a final single cycle of 95 ℃ for 15 s, 60 ℃ for 30 s and 95 ℃ for 15 s. The baseline was set automatically by the Dice™ Real Time System software (version 2.00). The Livak ($2^{-\Delta\Delta CT}$) method was used to determine the expression level of Rfcasp8 (Livak and Schmittgen, 2001). All data were presented as the mean ± standard deviation (SD). Statistical difference between the control and groups were calculated by a two tailed un-paired student's t-test. *P*-values of less than 0.05 were determined to indicate the statistical significance.

Table.3. Primers used in this study on Rfcasp8

Name	Purpose	Sequence(5'→3')
Rfcasp8-F1	qRT-PCR of Rfcasp8	GCATTGAAGCCGACAGTGGAGATG
Rfcasp8-R1	qRT-PCR of Rfcasp8	TGCATGAACCAGGACCCAGTCATA
RFEF1a-F	qRT-PCR for black rockfish RF1α	AACCTGACCACTGAGGTGAAGTCTG
RFEF1a-R	qRT-PCR for black rockfish RF1α	TCCTTGACGGACACGTTCTTGATGTT

3. RESULTS AND DISCUSSION

3.1. Sequence characterization of Rfcasp8 cDNA

Black rockfish cDNA database was analyzed by BLAST and the complete cDNA sequence of Rfcasp8 was identified. The full-length sequence of Rfcasp8 (2467bp) was composed of a 1473 bp open reading frame (ORF), encoding 491 amino acid residues, 167 bp 5'-untranslated region (5'-UTR), and 827 bp 3'-UTR. The sequence of Rfcasp8 was submitted to NCBI Genbank database under the accession number of KT153627 and putative amino acid sequence was shown in Fig.11. The theoretical molecular weight was 55.26 kDa and the calculated isoelectronic point was 5.46. The deduced amino acid sequence of Rfcasp8 contains two death effector domains (DED, Met^{1} to Thr^{77}, Glu^{93} to Asp^{162}), large subunit (P20, Pro^{247} to Gly^{370}), small subunit (P10, Val^{405} to Leu^{488}) and active-site penta-peptide motif (Lys^{357} to Gly^{368}). The catalytic site (His^{323}, Gly^{324}, Cys^{366}) n was also identified in large subunit of Rfcasp8. In addition, the binding pocket (Arg^{226}, Gln^{364}, Arg^{425}) was observed. Catalytic site and binding pocket is known to play a essential role in active site of caspase-8 in S1 pocket (Blanchard et al., 1999). DEDs are functionally essential part in initiator caspases such as caspase-8 and -10 (Yu and Shi, 2008).

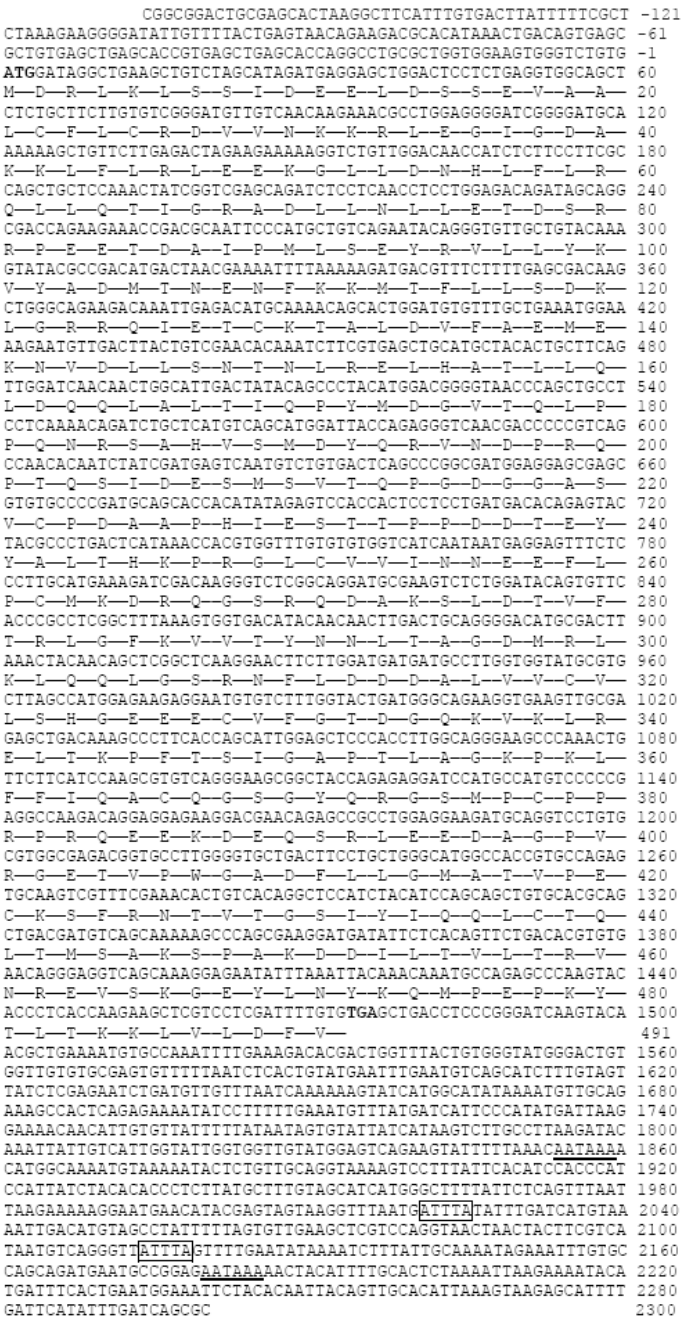

Fig.11. The complete nucleotide and putative amino acid sequences of Rfcasp8 from black rockfish. The start codon (ATG) and stop codon (TGA) was highlighted by bold. Two mRNA instability motifs (ATTTA) are boxed. Two polyadenylation signal sequence (AATAAA) was shown in underline.

3.2. Amino acid sequence alignment and phylogenetic analysis of the Rfcasp8

Multiple Sequence alignment of Rfcasp8 compared to fish and other vertebrate species indicated the relative conservation of caspase characteristic domains. Moreover, catalytic site and binding pocket, active-site penta-peptide motif were found to be completely conserved. (Fig. 12).

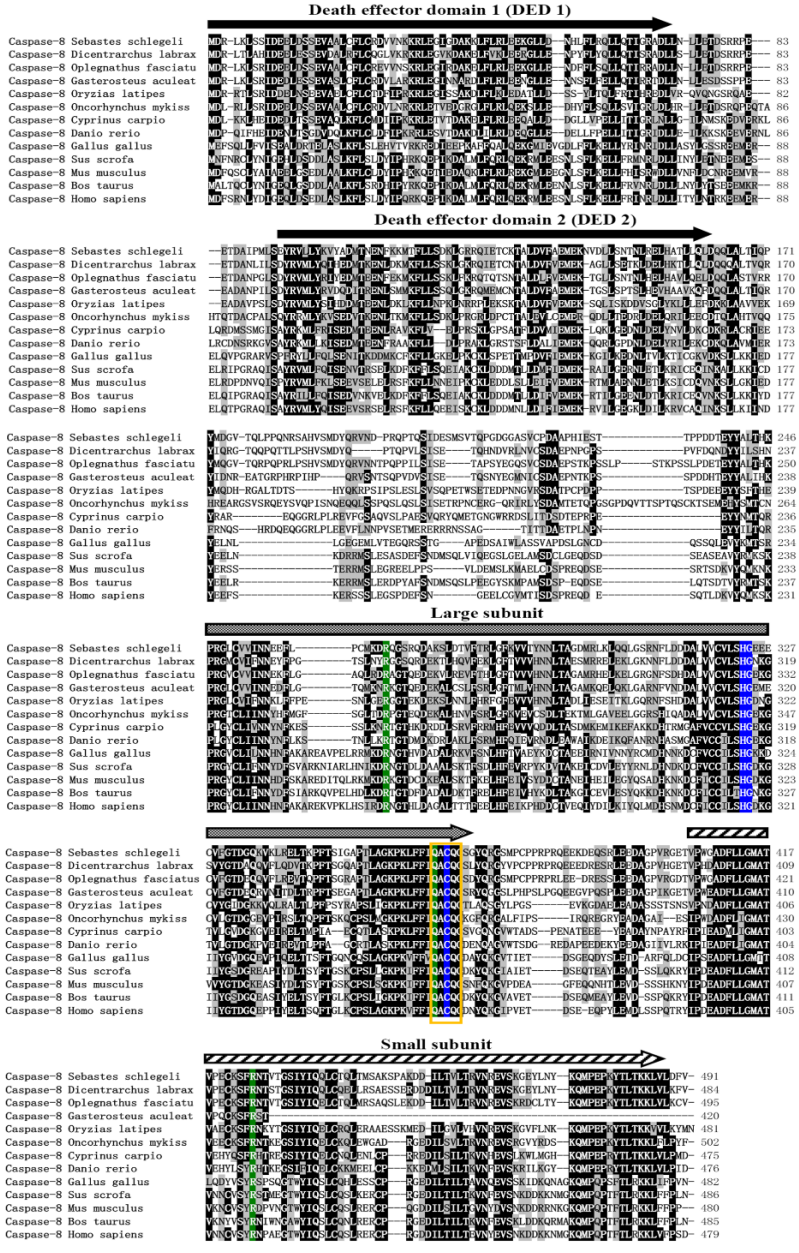

Fig.12. Multiple sequence alignment of Rfcasp8 with other species amino acid sequences. Sequence alignment results were obtained using Clustal W method. Catalytic site and binding pocket are shaded by green color and blue color, respectively. Active-site penta-peptide motif is shown as yellow color box. The completely conserved and similar residues are donated by black shading and gray shading, respectively.

The identity and similarity percentage of Rfcasp3 was significantly higher with other caspase-8 family members rather than other caapase-9 family members. The highest percentage of identity was shared with the corresponding counterpart of *O. fasciatus* (73.1%). Its identity with *D. labrax* (67.8%), *G. aculeatus* (55.7%) counterparts was also significant (Table 4). The phylogenetic tree was constructed for capase-8 using the known caspase-8 and -9 family members from other species such as fish, amphibian, aves and mammals. As shown in Fig. 13, the caspase-8 and -9 were clustered closely but separately in phylogenetic tree. Each clade diverged into vertebrate subclasses (Fish and non-fish vertebrates) and Rfcasp8 lies on the fish caspase-8 subclass.

Table.4. Percent identity and similarity of Rfcasp8 gene with other species

Gene name	Species	Taxonomy	Genebank accession	Identity%	Similarity%
Caspase-8	*Oplegnathus fasciatus*	Fish	AHH30803	73.1	82
Caspase-8	*Dicentrarchus labrax*	Fish	ACO53629	67.8	78.6
Caspase-8	*Gasterosteus aculeatus*	Fish	NP_001254591	55.7	68.8
Caspase-8	*Oncorhynchus mykiss*	Fish	NP_001268251	52.4	68.5
Caspase-8	*Oryzias latipes*	Fish	NP_001098258	52.3	69.2
Caspase-8	*Cyprinus carpio*	Fish	AGQ03809	42.7	59.9
Caspase-8	*Danio rerio*	Fish	NP_571585	40.9	59.5
Caspase-8	*Mus musculus*	Mammalia	CAA07677	37.1	59.7
Caspase-8	*Bos taurus*	Mammalia	NP_001039435	36.9	56.4
Caspase-8	*Sus scrofa*	Mammalia	NP_001026949	36.3	56.8
Caspase-8	*Homo sapiens*	Mammalia	AAD24962	35.9	55.8
Caspase-8	*Gallus gallus*	Aves	AAL23700	35.1	56
Caspase-8	*Xenopus laevis*	Amphibia	BAA94749	31.8	51.4
Caspase-9	*Mus musculus*	Mammalia	AAH56447	28	43.4
Caspase-9	*Cyprinus carpio*	Fish	AGM34043	27.3	42.8
Caspase-9	*Homo sapiens*	Mammalia	BAA82697	26.9	43.6
Caspase-9	*Bos taurus*	Mammalia	NP_001192433	26.6	40.7
Caspase-9	*Dicentrarchus labrax*	Fish	ABC70999	26	42.8
Caspase-9	*Oncorhynchus mykiss*	Fish	NP_001118119	25.9	40.7
Caspase-9	*Cuculus canorus*	Aves	KFO77303	24.9	40.3
Caspase-9	*Xenopus laevis*	Amphibia	BAA94750	24.4	40.1
Caspase-9	*Danio rerio*	Fish	NP_001007405	23.9	42

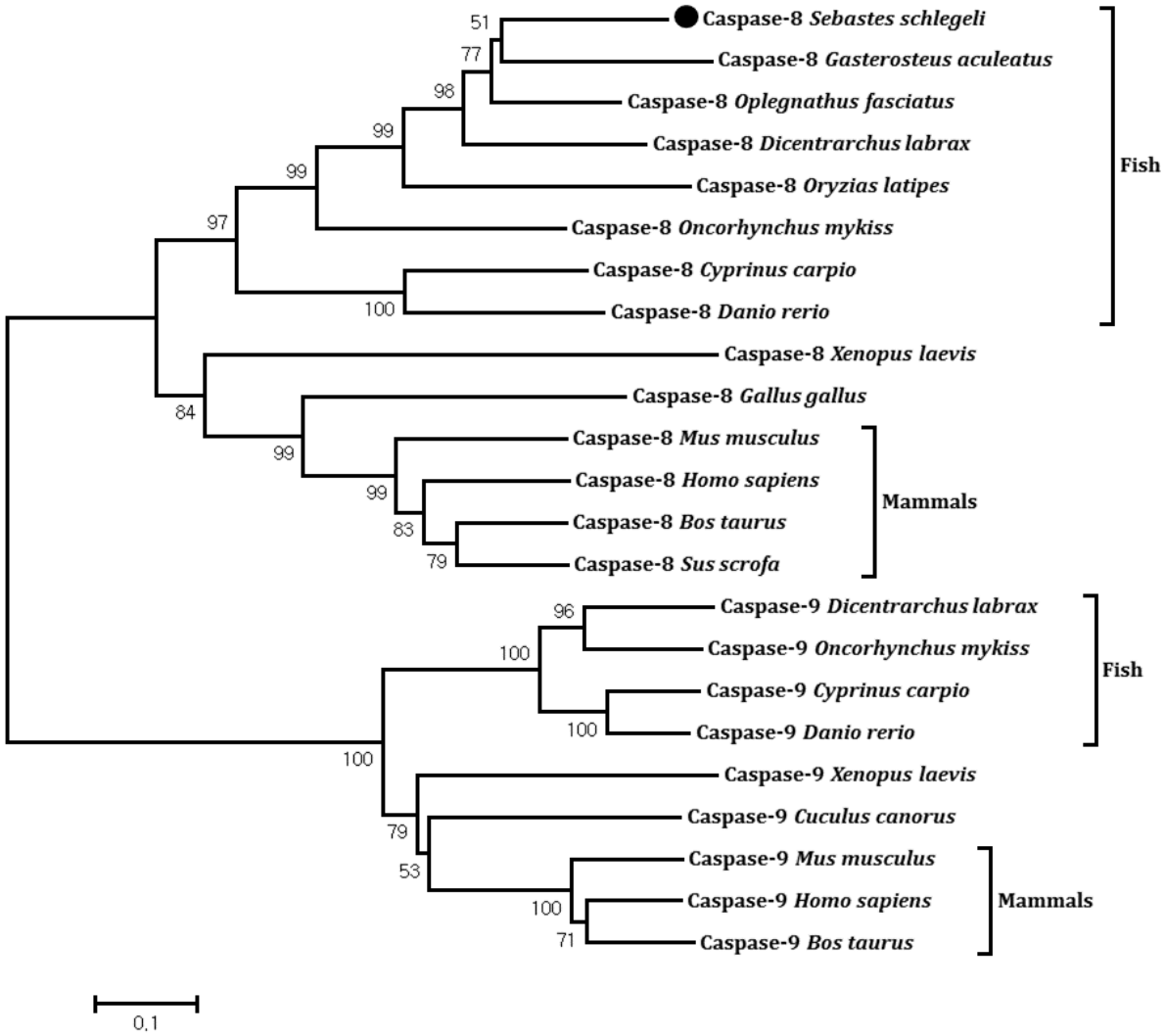

Fig.13. Phylogenetic tree of Rfcasp8 with other selected species caspase-8 and caspase-9 family members. The tree was constructed by neighbor-joining method and MEGA version 6.0 using full-length amino acid sequence. The numbers at the branch indicate the bootstrap value derived from 1000 replicates. The accession numbers of amino acid sequences are denoted in Table 3.

3.3. Tissue-specific expression profile of Rfcasp8

To investigate the constitutive expression pattern of Rfcasp8 in different tissues of black rockfish, the qRT-PCR was carried out using Dice™ Real time system thermal cycler (TP800; TaKaRa, Japan). The dissociation curves for Rfcasp8 were confirmed as single peaks. Rfcasp8 mRNA was expressed in all tissues examined but the mRNA expression level of Rfcasp8 from each tissue was varied (Fig. 14). According to mRNA expression level of different tissues, Rfcasp8 mRNA was significantly detected in blood (128.4-fold), followed by spleen (74.8-fold), skin (62.2-fold) and head kidney (31.87-fold) corresponding to muscle tissue, respectively. This results was in agreement with previous reports from other species. In human caspase 8 showed a very similar pattern of mRNA expression level from different tissues, the highest mRNA expression level was measured in blood leukocytes, spleen, thymus and liver (Eckhart et al., 2001). Although, blood does not show a detectable expression , mouse had similar pattern of expression level from tissues such as spleen, thymus and liver (Sakamaki et al., 1998). In the fish, sea bass was also observed in tested tissues including spleen, heart, liver, head kidney, thymus (Reis et al., 2010). Therefore, we could suggest that blood is essential tissue to study the transcriptional level of caspase-8.

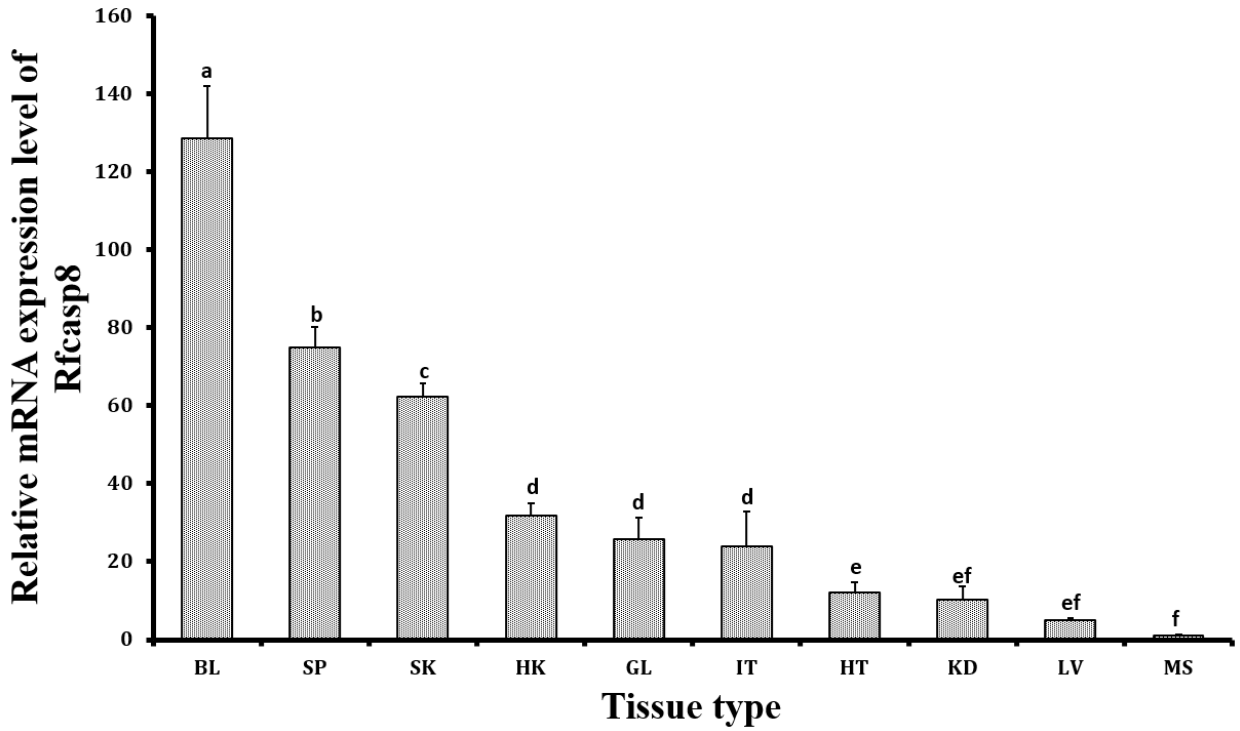

Fig.14. Tissue distribution analysis of Rfcasp8. Data above bars represent as mean values (n=3) and error bars denote the standard deviation (SD). Data shown with different letters indicate the significant different expression levels at $P < 0.05$. BL, blood cell; SK, skin; IT, intestine; SP, spleen; MS, muscle; GL, gill; HT, heart; HK, head kidney; LV, liver; KD, kidney.

3.4. Rfcasp8 transcriptional profiling using qRT-PCR

The caspase-8 gene is known to be initiator caspase in extrinsic pathway and was highly inducible with a several external stimuli like LPS, poly I:C (Maelfait et al., 2008). The significant basal expression of *Rfcasp8* in blood was considered in transcriptional expression analysis. Moreover, blood was pivotal elements of innate immunity in vertebrate and invertebrate. Therefore, we investigate the transcriptional modulation of *Rfcasp8* in blood obtained from challenged fish after immune-stimulation with poly I:C and LPS (Fig. 15).

The expression of Rfcasp8 was up-regulated at 6 h post injection and then down-regulated at 24 h and 48 h post injection after poly I:C challenge, which indicated early phase of immune response. This observation was in an agreement with other species. In invertebrate, disk abalone caspase-8 mRNA was detected at 6 h and 12 h post injection after VHSV challenge (Lee et al., 2011). Our data suggest that Rfcasp8 might be involved in defense mechanism of virus infection from black rockfish.

The endotoxin LPS which was found on cell wall of gram negative bacteria known to be an immune-modulator of mammals (Rietschel et al., 1994). The Rfcasp8 was significantly expressed in LPS stimulated blood relative to PBS-injected control. Moreover, mRNA level was slightly up-regulated at 6 h post treatment (p.t.) and then significantly expressed at 48 h post injection. Similarly, in fish, sea bass caspase-8 mRNA level in spleen was detected between 6 and 24 h p.t. after LPS challenge (Reis et al., 2010). In vertebrate, Disk abalone caspase-8 mRNA level was also detected at early phase (24 h p.t.) immune response after bacteria challenge. In fact, LPS was known to induce the apoptosis in macrophages by TNF-α which is main death factor in extrinsic pathway (Xaus et al., 2000). We suggest that Rfcasp8 might paly essential role in antibacterial mechanism in black rockfish.

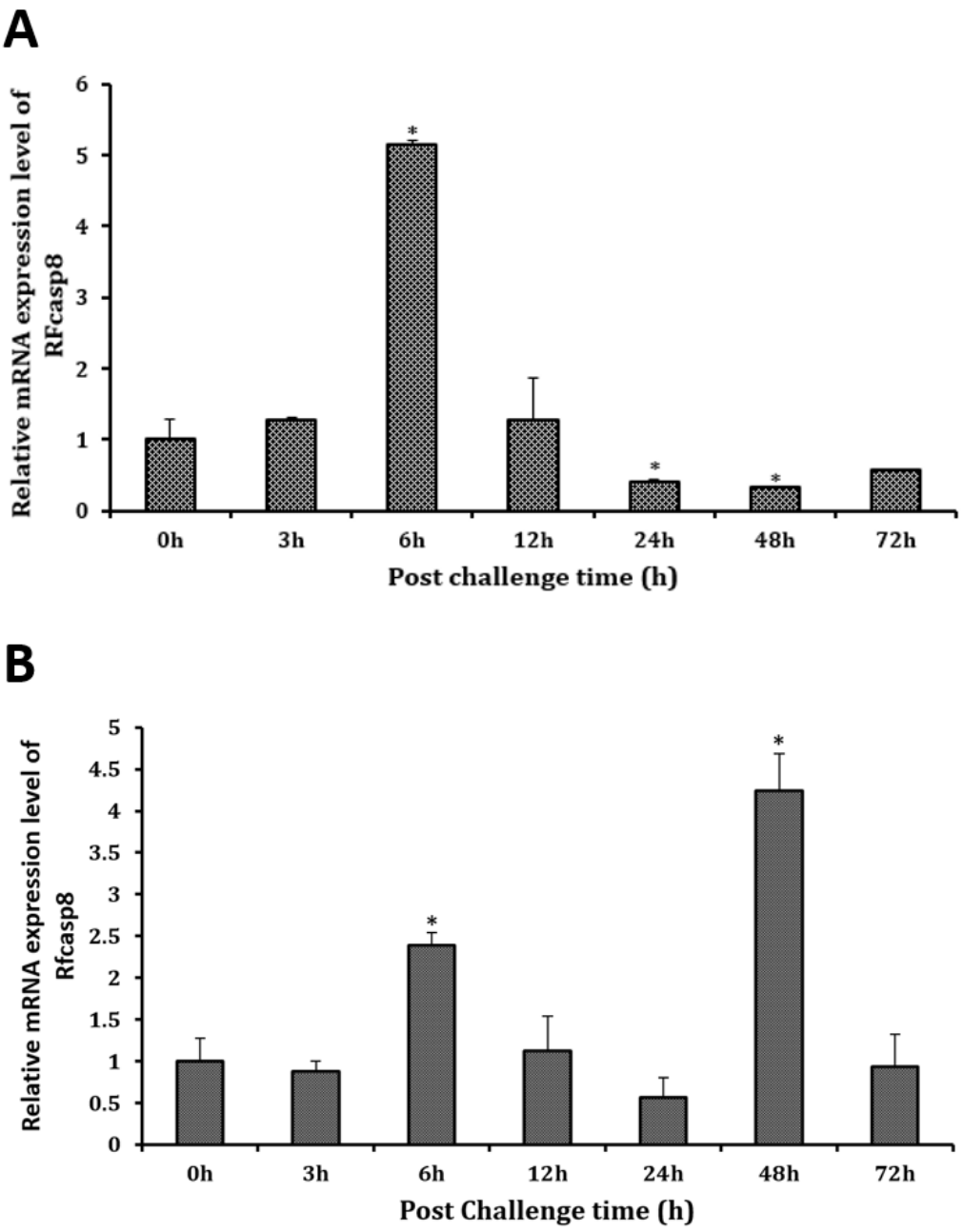

Fig.15. Rfcasp8 mRNA expression analysis upon the immune stimulation with (A) poly I:C or (B) LPS. Relative mRNA expression was calculated by $2^{-\Delta\Delta CT}$ method compared with PBS-injected controls and normalized with RfEF1A as the reference gene. Data above bars represent as mean values (n=3) and error bars denote the standard deviation (SD). The differences are indicated with an asterisk (*) at $P < 0.05$.

In conclusion, the full-length cDNA of Rfcasp8 was identified from black rockfish cDNA library database. *In silico* analysis of putative amino acid sequence showed that typical caspase feature including two DEDs, large and small subunits. Rfcasp8 transcript level was universally detected in all examined tissues and highly expressed in blood. The transcriptional modulation of Rfcasp8 mRNA level upon the challenge experiment with LPS and poly I:C was indicated as an evidence of antibacterial and antiviral defense in black rockfish.

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감사의 글

학부부터 석사과정거치면서 무사히 마칠 수 있도록 도와주시고 많은 가르침을 주셨던 분들께 감사의 말씀을 전하고 싶습니다.

먼저 학자의 길로 한 발짝 더 나아 갈 수 있도록 많은 가르침을 주시고 저를 믿고 이끌어 주신 이제희 교수님께 감사 드립니다. 그리고 바쁘신 가운데도 부족한 저의 논문을 심사해주신 임봉수 박사님, 정형복 박사님 감사 드립니다. 또한 학부 시절부터 흥미롭고 많은 학문을 가르쳐 주셨던 송춘복교수님, 최광식교수님, 전유진교수님, 여인규교수님, 김기영교수님, 정준범교수님, 이승헌교수님, 허문수 교수님께 감사 드립니다.

처음 실험실에 들어왔을 때 많은 호기심과 많은 질문에도 답해주시던 철홍이형, 제가 실험실 생활을 하며 잘 할 수 있도록 도와준 영득이형, 가족처럼 걱정해주고 옆에서 응원해준 숙경이 누나, 민영이 누나, 유철이 형, 논문을 쓰며 많은 충고와 도움을 준 Anushka, 같이 실험실 생활을 하며 힘이 되어 준 지연이, 은영이도 고맙다는 말을 전하고 싶습니다.

학부 시절부터 힘들 때 같이 걱정해주고 좋은 일이 있을 때 같이 좋아해주던 태수, 준영이, 민기, 진수, 혜나에게도 고맙고 열심히 해보자!

마지막으로 뒤에서 묵묵히 버팀목처럼 저를 지원해주신 부모님께 진심으로 감사 드립니다.