# A THESIS

# FOR THE EDGREE OF MASTER OF SCIENCE

**Molecular Insights of two immune related genes via Characterization of TNFα and G-type lysozyme from Seahorse**

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**DEPARTMENT OF MARINE LIFE SCIENCES**

**GRADUATE SCHOOL**

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# Molecular Insights of Two Immune Related Genes *via* Characterization of TNFα and G-type Lysozyme from Big-belly Seahorse (*Hippocampus abdominalis*)

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A thesis submitted in partial fulfillment of the requirement for the degree of

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**DEPARTMENT OF MARINE LIFE SCIENCES**

**GRADUATE SCHOOL**

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# 요 약 문

Lysozyme은 세균의 세포벽에 존재하는 펩티도글리칸의 베타-1,4 결합을 가수분해함으로써 세포벽을 허물어 세균을 죽이는 항균성 효소이다. 동물계에는 닭에서 처음 발견한 chicken-type lysozyme (c-type lysozyme)과 거위에서 발견된 goose-type lysozyme (g-type lysozyme), 그리고 무척추동물에 존재하는 invertebrate-type lysozyme (i-type lysozyme) 등이 존재한다.

Tumor necrosis factor alpha (TNFα)는 cytokines에 속하며, 많은 세포에서 면역조절자로서 다양한 기능을 수행한다. TNFα는 주로 대식세포에서 생성되며, 병원체나 독성의 감염 시 inducible nitric oxide synthase (iNOS)나 cyclooxygenase-2 (COX-2) 등의 전염증성 인자를 발현시켜 염증 반응을 일으킴으로써 자극물질로부터 보호하는 중요한 역할을 수행한다. 염증반응을 일으키는 NO와 PGE2는 각각 iNOS와 COX-2에 의해 L-arginin과 arachidonic acid로부터 생성된다.

해마는 실고기과에 속하는 경골어류로서 주로 해안에 서식하고 있다. 그 중에 특히 빅벨리해마는 해마중에서도 가장 크고 아름다운 체색과 체형을 지니고 있어 관상용으로도 가치가 높아 국제해수관상생물 시장에서 인기가 많다. 또한 옛날부터 해마는 약용가치로서 많이 사용되어져 왔으며 이로 인해 일부 국가에서는 남획하고 있다. 뿐만 아니라 지구온난화와 연안의 환경악화로 인해 빅벨리해마가 서식할 수 있는 환경이 축소되어 개체수는 점차 감소하고 있고 호주 및 그 주변국, 중국과 한국에서 인공수정을 통한 양식이 이루어지고 있으나, 그 수가 수요에 비해 현저히 부족한 실정이다. 북해마, 갈귀해마 등에 대해 여러 분야에서 연구되고 있으나, 면역•생리학적 연구나 유전자 분석에 대한 연구는 아직 미비하다.

본 연구에서는, 이미 확립되어 있는 cDNA data를 통하여 빅벨리해마 (*Hippocampus abdominalis*)에서 LysG와 TNFα 유전자를 동정하였다. 두 유전자의 아미노산 서열은 ClustalW2 multiple sequence alignment와 EMBOSS needle pairwise sequence alignment를 사용하여 다른 척추동물의 LysG와 TNFα 서열과 비교 분석하였다. 다른 종들과의 orthologous 관계를 확립하기 위해 MEGA 5 software를 사용하여 계통수 분석을 실시하였다. 면역자극에 의해 나타나는 면역반응을 조사하기 위해 LPS, Poly I:C, 그리고 살아있는 병원체인 *Edwardsiella tarda*과 *Streptococcus iniae*를 접종하여 각 두 유전자의 시간별 mRNA 발현 양상을 quantitative real-time PCR을 통해 분석하였다. ShLysG와 ShTNFα의 분자적 특성을 조사하기 위해 두 유전자를 각각 pMAL vector에

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클로닝한 후 *Escherichia coli* cells에 형질전환하여 재조합 단백질를 추출 및 정제하였다. rShLysG의 최적활성을 위해 온도별 (10 - 60 ℃) 및 pH별 (pH3-12) 활성실험을 수행하였고, turbidimetry method를 이용하여 rShLysG의 최적 활성조건에서 다른 박테리아에 대한 항균활성 실험을 실시하였다. rShTNFα의 염증반응에 관여하지 확인하기 위해 쥐의 대식세포인 RAW 264.7 세포에 재조합단백질을 농도별로 처리하여 griess 시약을 통해 NO생성량을 측정하였으며, westen blot 분석을 통해 전염증성 매개인자의 발현 양상을 확인하였다.

ShLysG 유전자는 2개의 catalytic residues, 7개의 N-acetyl-D-glucosamine binding sites, 그리고 catalytic bacterial soluble lytic transglycosylase (SLT) domain이 잘 보존되어 있었으며, 알려진 다른 어류와 높은 상동성과 유사성을 지니고 있었다. pH 4와 20에서 가장 높은 활성을 보였고, 항균활성 결과, 3개의 *Vibrio* spp.와 *Listeria monocytogenes*, 그리고 *S.iniae*에 대해 높은 항균활성을 보이고 있었다. ShLysG는 모든 조직에서 발현하고 있으며, 특히 신장과 아가미에서 높은 발현을 보이고 있었다. 면역자극을 수행한 결과, 신장과 아가미에서 모든 면역자극에 대해 유의적으로 발현이 증가하는 양상을 보였다.

빅벨리 해마의 TNFα 유전자는 transmembrane region과 TNF domain을 가지고 있으며, 다른 어류와 비교한 결과 높은 상동성과 유사성을 가지고 있음을 확인하였다. 또한 모든 조직에서 발현하고 있으며 특히 피부와 아가미에서 가장 높게 검출되었다. 면역자극을 수행한 결과, blood과 신장에서 모든 면역자극에 대해 유의적으로 발현이 증가하는 양상을 보였다. 염증조절자로서의 활성을 실험하기 위해 염증유발인자인 NO의 생성량을 측정한 결과, 대식세포에서 농도의존적으로 NO를 생성하였고, western blot 분석 결과, iNOS와 COX-2의 단백질발현 역시 농도의존적으로 증가하였다. 이러한 결과로 미루어 볼 때, rShTNFα의 활성은 nuclear factor kB (NB-kB) 경로를 통해 전염증성 매개인자인 iNOS와 COX-2를 발현함으로써 NO와 $PGE_2$생성을 촉진시켜 염증반응을 일으키는 것이라 사료된다.

종합적으로, 본 연구에서는 빅벨리해마 (*Hippocampus abdominalis*)으로부터 두 개의 면역관련 유전자, g-type lysozyme (ShLysG)과 Tumor necrosis factor alpha (ShTNFa)를 동정하고 분자적 관점에서 연구하였으며, 더 나아가 어류의 면역학적 연구나 해마의 또 다른 유전자 분석연구에 있어 기초적인 연구자료로 사용될 수 있을 것으로 사료된다.

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## SUMMARY

Lysozyme is a bacteriolytic enzyme protecting the host from bacterial infection which catalyzes the hydrolysis of the β-1-4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan heteropolymers of the bacterial cell wall. Lysozymes are classified into three major distinct lysozyme types in the animal kingdom based on amino acid sequences, biochemical and enzymatic properties: the c-type (chicken or conventional type), the g-type(goose type) and the i-type(invertebrate type) lysozyme.

Tumor necrosis factor alpha (TNFα), also known as cachectin, is a major immunomodulator that is involved in systemic inflammation and make up the acute phase reaction. In response to stimulus such as infection or injury, this cytokine is chiefly secreted by macrophages. Increased levels of excess inflammatory mediators such as nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$) induced the production of TNFα.

Seahorse (*Hippocampus* sp.) belongs to the order Syngnathformes and family Syngnathidae resembles the appearance of a horse with a very unique look. In particular, the big-belly seahorse (*H. abdominalis*) among the seahorses is one of the largest and has ornamental value due to beautiful body color and type. Hence, big-belly seahorse is popular in global ornamental fish market. The overexploitation of seahorse for consumption as well as the exploitation of their habitats decrease the seahorse population and push to the brink of extinction. Limited research has yet been published about molecular characterization and expression profiles of big-belly seahorse's genetic elements.

In this study, TNFα and LysG genes were isolated from a previously established cDNA database of Big-belly seahorse (*Hippocampus abdominalis*), named as ShTNFα and ShLysG and characterized. The amino acid sequences of ShTNFα and ShLysG were compared with other TNFα and LysG sequences using multiple sequence alignment and EMBOSS needle pairwise sequence alignment, respectively. To investigate the relationship

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between ShTNFα and ShLysG orthologous with other species, phylogenetic analysis was performed using MEGA5 software. To examine the immune responses upon bacterial and viral stimuli, immune challenge experiments were carried out with LPS, poly I:C, live *Edwardsiella tarda* and *Streptococcus iniae*. Subsequently, mRNA expression analysis of ShTNFα and ShLysG genes was performed in a temporal manner. Two genes were cloned into pMAL vector, transformed into *Escherichia coli* cells and purified in order to use in activity assay of ShTNFα and ShLysG. Antimicrobial activity analysis of rShLysG was carried out using turbidimetry method at pH 4 and 20 ℃. To investigate whether rShTNFα is involved in inflammatory response, NO generation was detected using Griess assay and subsequent western blot analysis from RAW 2647 cells.

ShLysG was consisted with well-conserved catalytic bacterial soluble lytic transglycosylase (SLT) domain ($Ala^{48}$-$Ser^{168}$) which contained two catalytic residues ($Glu^{71}$ and $Asp^{95}$) and seven of N-acetyl-D-glucosamine binding sites ($Glu^{71}$, $Asp^{95}$, $Tyr^{98}$, $His^{99}$, $Ile^{117}$, $Tyr^{145}$ and $Asn^{146}$). Identity and similarly of ShLysG was revealed higher relationship to fish LysGs. rShLysG revealed its highest bacteriolytic activity at pH 4 and 20 ℃. Antimicrobial activity of rShLysG was strongly detected towards three *Vibrio* spp., *Listeria monocytogenes* and *S. iniae*. ShLysG was ubiquitously expressed in tissues, most notably in kidney and gill. After the immune challenges, response of ShLysG was significantly increased against all kinds of challenges in kidney and gill.

ShTNFα possessed a transmembrane region ($Ile^{31}$-$Phe^{23}$) and a TNF domain ($Ala^{82}$-$Leu^{243}$) including six receptor binding sites ($Ile^{100}$, $Asn^{101}$, $Ser^{106}$, $Thr^{158}$, $Ser^{165}$ and $Asp^{170}$) and seven polypeptide binding sites ($His^{84}$, $Phe^{128}$, $Tyr^{130}$, $Tyr^{205}$, $Phe^{210}$, $Phe^{237}$ and $Phe^{241}$). ShTNFα showed higher degree of identity and similarly with other fish TNFα orthologs. ShTNFα was expressed in all tissues, especially in skin and gill. The mRNA expression of

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ShTNFα significantly up-regulated upon live bacterial and viral challenges in blood and kidney. NO induction of mouse macrophage RAW 264.7 cells was influenced at its highest concentration (0.25 $\mu$g/mL) up to the positive control, LPS (10 ng/mL) and increased in a dose-dependent manner. Accordingly, to investigate the downstream events related to inflammatory responses, western blot assay was performed and determined the expression levels of pro-inflammatory factors; iNOS and COX-2. Both expression levels of iNOS and COX-2 were found to increase in a dose-dependent manner. This result implies that ShTNFα may mediate inflammatory responses through NF-B pathway.

In conclusion, in this study, two immune related genes; TNFα and LysG from Big-belly seahorse (*Hippocampus abdominalis*) were identified and analyzed molecularly and functionally. These genes may play an important role in the immunity of seahorse. Therefore, those genes of seahorse can be used as fundamental research materials in further studies.

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## 1. Introduction

Seahorse (*Hippocampus* sp.), belongs to the order Syngnathformes and family Syngnathidae resembles the appearance of a horse with a very unique look. They live primarily in uncontaminated sub-tropical and shallow water of coast where the body can be enough to lean on corals and algae. Around 70% of seahorse species are found in the Indo-Pacific region (LOURIE et al., 1999). In contrast, only six seahorse species could be found in Korean waters. Since seahorse is very sensitive to marginal marine environment, such as temperature changes, fungus and parasites, maintenance and artificial propagation of large seahorses in captivity is difficult (Lunn and Hall, 1998).

Seahorse is one of the cherished ingredients in traditional medicine particularly in Southeast Asia where traditional Chinese medicine (TCM) and its derivatives (e.g. Japanese and Korean traditional medicine) are a cosmopolitanly used. Syngnathidae fishes, mainly seahorses and pipefishes are predominantly used as a medicament to treat the diseases such as asthma, heart disease and fractures. In particular, the big-belly seahorse (*H. abdominalis*) among the seahorses is one of the largest of all seahorses and has ornamental value due to possess a beautiful body color and type. Hence, big-belly seahorse is popular in global ornamental fish market.

Due to their high demand for medicines, some countries have overfished seahorses. Furthermore, habitats of seahorses are gradually reduced, due to an extension of environmental pollution and overexploitation in coastal areas. The overexploitation of seahorse for consumption as well as the exploitation of their habitats decrease the seahorse population and push to the brink of extinction. On May 15th, 2004, seahorse was announced to have 1 endangered species and 9 vulnerable species from IUCN (IUCN, 2004) and the Convention on International Trade in Endangered Species (CITES) strictly limited

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overfishing and business transaction to be protected (CITES, 2001).

Big-belly seahorse has ever been studied in the wild. Moreover, limited research has yet been published about molecular characterization and expression profile of big-belly seahorse. In this study, the molecular evidences were investigated into the immune mechanism of two genes from big-belly seahorse (*H. abdominalis*). This dissertation includes functional analysis of the two genes; g-type lysozyme (ShLysG) and NF-kB pathway as inflammation factor; tumor necrosis factor alpha (ShTNFa). The *in silico* molecular characterization, immune responsive transcriptional regulations and their functional activities were determined from above mentioned two genes.

**Fig 1. Big-belly seahorse *Hippocampus abdominalis***

The bacterial cell wall plays many roles in maintaining their size, shape and preventing them from rupture by the osmotic pressure etc. with the help with the other bacterial envelope, the plasma membrane. The bacteria are classified as gram-negative and gram-positive according to their cell wall constituents. Gram-positive bacteria are entailed with a very thick cell wall consisted of approximately 80-90 % peptidoglycan layers and an

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inner cytoplasmic cell membrane while gram-negative bacteria have a very thin cell wall containing only 10-20 % peptidoglycan layers sandwiched by an inner cytoplasmic cell membrane and an outer cell membrane containing lipopolysaccharides (LPS). The disease causing bacteria are mostly considered as saprophytic in nature. When bacteria invade the higher organisms, components of the bacterial cell wall are recognized by the host cells as pathogens and activate the defense measures. Since lipopolysaccharide (LPS) is major component of the gram-negative bacteria and it acts as immunostimulating agent constituting the bacterial cell surface, they are highly used as an immunostimulant in the immune defense mechanism studies of plants as well as animals and humans. Interestingly, LPS protects bacterial cells by blocking the toxic substance from outside invasions. LPS is combined with LPS-binding protein (LBP)/CD14 receptors in the body and activates interferon-β (IFN-β) by activating toll-like receptor 4 (TLR4). The activated IFN-β then phosphorylates STAT1 by combining cell receptor and the phosphorylated STAT1 induces the production of inducible nitric oxide synthase (iNOS). The iNOS is involved in the generation of nitric oxide (NO) which activates the myosin phosphatase and potassium channel in the wall of blood vessels that results in inflammation (Han et al., 2006). Cell wall of gram-positive bacteria is comprised of lipoteichoic acid (LTA) instead of LPS. LTA contains fatty acid including lipid part and several polysaccharides. Although the role of LTA is completely unknown, it has been suggested that LTA may cause potent immunostimulation (Morath et al., 2002; Percy and Gründling, 2014).

Lysozyme is a bacteriolytic enzyme protecting the host from bacterial infection by catalyzing the hydrolysis of the β-1-4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan heteropolymers of the bacterial cell wall. Lysozymes are classified into three major distinct lysozyme types in the animal kingdom based on amino

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acid sequences, biochemical and enzymatic properties: the c-type (chicken or conventional type), the g-type (goose type) and the i-type (invertebrate type) lysozyme (Callewaert and Michiels, 2010). Lysozymes are functionally engaged as an anti-inflammatory agent (Ogundele, 2001), an antimicrobial agent (Ibrahim et al., 2001), an anti-HIV agent (Lee-Huang et al., 2005) and possesses tumoricidal activity (LeMarbre et al., 1981). G-type lysozyme was initially identified in egg whites of the Embden goose by Canfield and McMurry (Canfield and McMurry, 1967) and since then, it have been identified in other vertebrates and invertebrates (Simpson et al., 1980; Nakano and Graf, 1991; Irwin and Gong, 2003; Nilsen et al., 2003; Zou et al., 2005; Whang et al., 2011; Bathige et al., 2013).

Inflammation is important in the immune defense responses of cells to protect body against stimulus including pathogens, damaged cells, and irritants (van Horssen et al., 2006). However, an excessive reaction of the inflammatory response can cause more damage than the pathogen and may cause diseases such as cerebral and myocardial ischaemia, rheumatoid arthritis, multiple sclerosis, atherosclerosis, Alzheimer's disease, diabetes and Crohn's disease in human (Tracey, 2002).

Tumor necrosis factor alpha (TNFα), also known as cachectin, is a major immunomodulator that involved in systemic inflammation and make up the acute phase reaction. TNFα has a wide range of roles, i.e., antitumor activity (van Horssen et al., 2006), immune modulation (Baugh and Bucala, 2001), inflammation (Bradley, 2008), anorexia (Vaisman and Hahn, 1991), cachexia (Espat et al., 1994), septic shock (Spooner et al., 1992), viral replication (Biermer et al., 2003) and hematopoiesis (Roodman et al., 1987). In response to stimulus such as infection or injury, this cytokine is chiefly secreted by macrophages. Increased levels of TNFα are produced in excess inflammatory mediators such as nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$) (Wang et al., 1999; Tsatsanis et al., 2006).

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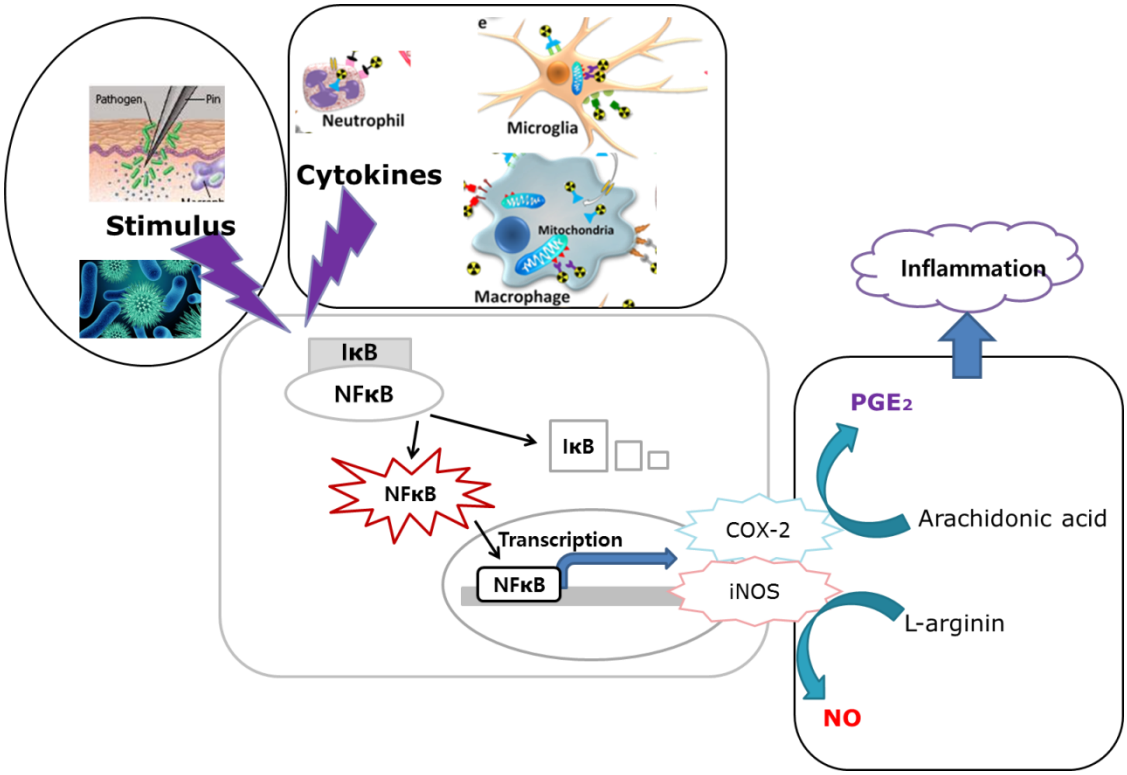


**Fig 2. Stimulated NF-kB pathway in inflammation**

NO plays important roles as a multifunctional molecule, for instance, intracellular and intercellular signaling molecule, vasodilatation, neurotoxin, host defense effector and inflammation in the immune system (Palmer et al., 1987; Nathan, 1997; Moncada and Higgs, 2000). It is synthesized by constitutive nitric oxide synthases (NOSs) via catalyzing the L-arginine form. NOSs (EC 1.14.13.39) have three distinct isoforms existing as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS were ever-present in the cell, by contrast iNOS be exposed to interferon-γ, lipopolysaccharide (LPS) and cytokine. iNOS which is closely related with the inflammatory reactions produces NO as a defense mechanism (Alderton et al., 2005) and expressed in macrophages (MacMicking et al., 1997) and other tissues such as neutrophils (Sethi and Dikshit, 2000), pulmonary epithelium (Asano et al., 1994), vasculature (Hickey et al., 2001), colonic epithelium (Perner

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et al., 2002), Kupffer cells, hepatocytes (Geller et al., 1993) and chondrocytes following stimulus such as lipopolysaccharide (LPS) and cytokine. However, over-generation of NO by iNOS induces inflammation (Nathan, 1992; McCartney-Francis et al., 1993) and tumor causing tissue damage, genetic mutation and neural damage (Stuehr et al., 1991; Weisz et al., 1996).

Prostaglandins (PGs) are produced by catalyzing of cyclooxygenase enzymes (COXs) from arachidonic acid and they act on cell growth control, regulating inflammation, and regulating hormones, etc. COXs (EC 1.14.99.1) can be divided into two isoforms, COX-1 and COX-2. COX-1 is ubiquitously expressed in most cell and assist housekeeping function, such as gastric mucosal cytoprotection, regulation of renal blood flow (RBF) and platelet aggregation (DeWitt and Smith, 1995; Surh et al., 2001). In contrast, COX-2 induces prostaglandins in inflammation through stimulus and cell proliferation. Namely, COX-2, stimulated with LPS or iterleukin-1 (IL-1), is expressed in macrophage, monocytes and inflammation sites. In addition, PGs induced by COX-2 and cause pain and febricity accompanied by inflammatory response (DeWitt and Smith, 1995; Eibl et al., 2003).

Macrophage is one of the major white blood cells of the immune system. It play important role in host defense which is related to innate and acquired immunoreaction (Elhelu, 1983; Gordon, 1998). Lee *et al*. 2004 and Mukaida *et al*. 1996 reported that LPS stimulated murine macrophage-like cell line RAW264.7 to synthesis the pro-inflammatory cytokine such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα) and interleukin-12 (IL-12) (Mukaida et al., 1996; Lee et al., 2004). Subsequently, this response induces the expressions of both iNOS and COX-2 through nuclear factor kappa B (NF-κB) and mitogen activated protein (MAP) kinase pathway (Surh et al., 2001; Aga et al., 2004).

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## Chapter I

## Transcriptional characterization and antimicrobial properties study of G-type lysozyme (ShLysG) from seahorse (*Hippocampus abdominalis*)

### 2. Materials and Methods

#### 2.1. Experimental fish

Healthy seahorses (average body size of 20 cm) were purchased from Korea Marine Ornamental Fish Breeding Center, Jeju, Korea. They were acclimated in laboratory aquarium tanks at a temperature of 20±1 ℃ and salinity of 32±1 psu for 1 week prior to the experiment.

#### 2.2. Big belly seahorse cDNA library construction

The seahorse transcriptome database was constructed using 454 GS FLX sequencing technique (Metzker, 2010). Briefly, the total RNA was extracted from blood, liver, kidney, gill and spleen tissues of 18 seahorses. The extracted RNA was then cleaned by RNeasy Mini kit (Qiagen, USA) and assessed for quality and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), giving an RNA integration score (RIN) of 7.1. For GS FLX 454 shotgun library preparation, the RNA was fragmented into average size of 1,147 bp using the Titanium system (Roche 454 Life Science, USA). Sequencing was finally run on half a picotiter plate on a Roche 454 GS FLX DNA platform at Macrogen, Korea. The raw 454 reads were trimmed to remove adaptor and low-quality sequences, and de novo assembled into contigs using GS Assembler (Roche 454 Life Science, USA) with the default parameters.

#### 2.3. *In silico* analysis of ShLysG

The ShLysG full-length sequence was analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast). Similarities were compared with other known lysozyme sequences available in the NCBI and ENSEMBL databases. To obtain the open reading frame

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(Walsh et al.) amino acid sequence of ShLysG, DNAssist (version 2.2) was used. Characteristic domains or motifs were identified using the PROSITE profile database (Bairoch et al., 1997) and SMART proteomic database (Letunic et al., 2009). Identity, similarity and gap percentages were calculated using EMBOSS pair-wise alignment algorithms. Multiple sequence alignments and phylogenetic analysis were performed on the amino acid sequence of ShLysG versus known lysozymes, using ClustalW 2.0 (Thompson et al., 1994). The amino acid sequences were aligned using the ClustalW program and the phylogenetic tree was reconstructed using the neighbor-joining (NJ) method using MEGA 5 software package (Tamura et al., 2011) taking the bootstrapping value as 1000.

**2.4. Cloning of ShLysG coding sequence**

The open reading frame (Walsh et al.) of ShLysG was amplified, using cloning primers with corresponding restriction enzyme sites of EcoRI and HindIII (Table 1). The PCR was performed in a TaKaRa thermal cycler in a total volume of 50 μL with 5 U of Ex Taq polymerase (TaKaRa, Japan), 5 μL of 10× Ex Taq buffer, 4 μL of 2.5 mM dNTP, 50 ng of template and 10 pmol of each primer. The reaction was carried out with an initial incubation at 94 ℃ for 3 min, 35 cycles (94 ℃, 30 s; 58 ℃, 30 s; 72 ℃, 30 s), followed by a final extension at 72 ℃ for 5 min. The PCR product was analyzed on a 1% agarose gel and ethidium bromide staining. Subsequently, the amplified product was excised from a parallel gel and purified using the Accuprep$^{TM}$ gel purification kit (Bioneer Co., Korea). The PCR product and maltose binding protein (MBP)-fused expression vector pMAL-c5X (New England Biolabs Inc, USA) were digested with respective restriction enzymes and the vector was dephosphorylated with calf intestine phosphatase (NEB, USA), in accordance with the vendor's protocol. Thereafter, the vector and PCR product were purified by a 1% agarose gel using the Accuprep$^{TM}$ gel purification kit (Bioneer Co., Korea). Ligation was carried out at 16

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℃ for 30min following overnight incubation at 4 ℃ with 200 ng of pMAL-c5X vector, 50 ng of PCR product, 5 μL of Mighty Mix (TaKaRa, Japan). The ligated product, pMAL-c5X/ShLysG, was transformed into the *Escherichia coli* DH5α cells for sequencing.

**Table1. Description of primers used in this study**

| Name | Primer Sequence(5' to 3') | Objects |
|---|---|---|
| ShLysG-1F | gagaga**GAATTC**ATGGGCTACGGGGACATCATGAA | ORF amplification (*EcoR* I) |
| ShLysG-1R | gagaga**AAGCTT**CTAAAAGCCGCCTTGACTTTTGTACCAC | ORF amplification (*Hind* III) |
| ShLysG-2F | CCAGAGAGTCCAGAGCTGGAAATGTA | qPCR amplification |
| ShLysG-2R | GGAACTTGCCCTGAACCTGTTTGA | qPCR amplification |
| ShRPS7-F | GCGGGAAGCATGTGGTCTTCATT | qPCR internal reference |
| ShRPS7-R | ACTCCTGGGTCGCTTCTGCTTATT | qPCR internal reference |

F; forward and R; reverse

### 2.5. Overexpression and purification of recombinant ShLysG (rShLysG)

The successfully cloned and sequence confirmed vectors were then transferred to competent *E. coli* ER2523 cells (Novagen, Germany) in order to express the protein. The bacteria were cultured and incubated at 37 ℃ with shaking at 200 rpm until the cell count reached 0.8 at $OD_{600}$. Then the culture was induced by IPTG 0.5 mM final concentration for 8 h at 20 ℃. The induced cells were cooled on ice for 30 min, and harvested by centrifugation at 3000 rpm for 30 min at 4 ℃. Harvested cells were re-suspended with 25 mL column buffer (Tris-HCl, pH 7.4, 200 mM NaCl, 1mM EDTA) and frozen at -80 ℃ overnight. The recombinant ShLysG (rShLysG) was purified in the form of fusion protein with MBP by pMAL$^{TM}$ protein fusion and purification system (NEB, USA). After thawing, the cells were sonicated on ice. The sonicated cell suspension was centrifuged at 15000 rpm for 30 min at 4

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℃ and the resulted supernatant was considered as crude rShLysG extract. In the final purification step, amylose resin was transferred into a 1 × 5 cm size column. The crude extract was loaded onto the column and washed with 12 × volumes of the column buffer. Finally, the rShLysG fusion protein was eluted by applying a total of 4 mL elution buffer (column buffer + 10 mM maltose) in 0.5 mL aliquots. The concentration of the purified protein was determined via the Bradford method, using bovine serum albumin (BSA) as the standard (Bradford, 1976). The rShLysG samples were collected from different purification steps were subsequently analyzed on 12% SDS-PAGE with protein size markers (Enzynomics, Daejeon, Korea). The gel was stained with 0.05% Coomassie blue R-250, followed by a standard de-staining procedure. All of the activity tests performed in this study was conducted using this purified rShLysG fused with MBP.

### 2.6. Functional characterization of rShLysG

#### 2.6.1. Optimal pH and temperature

The optimal pH of ShLysG was tested at 10 different pH values (3 - 12; at 1 intervals) by turbidimetry method. As the substrate for lysozymes, *Micrococcus lysodeikticus* (Sigma) was used a standard bacterial strain. Bacteria (0.8 mg/mL) was dissolved in corresponding buffers including sodium acetate/acetic acid (pH 3-5), $K_2HPO_4/KH_2PO_4$ (pH 6-8), and boric acid/NaOH (pH 9-12). 150 μL of *M. lysodeikticus* suspension prepared in each pH buffers and mixed with 50 μL of recombinant protein (500 μg/mL). After 30min, the decrease in $OD_{450}$ was measured with a microplate reader (Multiskan EX, Thermo Scientific).

The optimal temperature assay as carried out at the range of 10 - 60 ℃ where *M. lysodeikticus* was diluted in optimal pH (pH 4). HEWL (AMRESCO, OH, USA, 500 μg/mL) and MBP (500 μg/mL) were used as positive and negative controls respectively in both of the

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assays.

### 2.6.2. Antimicrobial activity

Gram-negative and Gram-positive strains including *M. luteus* (KCTC 1056), *Vibrio salmonicida* (KCCM 41663), *V. parahemolyticus* (KCCM 11965), *V. tapetis* (KCTC 12728), *Listeria monocytogenes* (KCCM 40307), *E. tarda* (KCTC 12267), *S. iniae* (KCTC 3657) and *V. anguillarum* () were used as substrates in this study. Bacterial strains were obtained from Korean Culture Center of Microorganisms (KCCM) and Korean Collection for Type Cultures (KCTC). Each bacterial strain was prepared in pH 4 buffer and initial $OD_{450}$ adjusted to a 0.5-0.6. Fifty microliter of recombinant protein (500 μg/ml), HEWL (500 μg/ml) and MBP (500 μg/ml) were separately mixed with 100 μL of each bacterial suspension and the OD*i* at 450 nm was immediately measured. After the incubation at 20 ℃ for 30 min, the mixture measured again (OD*f*). The lytic activity (U) was calculated using the following formula: (OD*i* – Od*f*)/OD*i*.

## 2.7. Immune challenges and tissue collection

### 2.7.1. Fish tissues for the specific distribution analysis

To examine the tissue-specific expression profile of ShLysG, healthy individual big belly seahorses were carefully dissected and tissues from heart, gill, liver, spleen, kidney, intestine, stomach, skin, muscle, pouch, brain, testis and ovary were collected. Using a sterilized syringe, the blood (1-2 mL per fish) was collected from the big belly seahorse tail and immediately centrifuged at 3000 x g for 10 min at 4 ℃. The supernatant was removed and cells were harvested for RNA extraction. All tissue samples were snap-frozen in liquid nitrogen immediately after they were collected from fish, and stored at -80 ℃ until the total RNA was isolated.

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### 2.7.2 Expression pattern of ShLysG after immune challenge

For the immune challenge, big belly seahorses with average body weight of 3 g were used. LPS (1.25 μg/μL), poly I:C (1.5 μg/μL), *Edwardsiella tarda* ($5 \times 10^3$ CFU/μL), *Streptococcus iniae* ($10^5$ CFU/μL) and scuticociliate *Miamiensis avidus* ($10^3$ /μL) were prepared with PBS and injected intraperitoneally in a total volume of 100 μL. For control group, the fish were injected with 100 μL PBS. The peripheral blood cells, gill, liver and kidney were sampled from five individuals at 0, 3, 6, 12, 24, 48 and 72 hours post-injection as described in section 2.7.1. Untreated and PBS-injected animals were kept separately as the control groups.

### 2.8. RNA extraction and cDNA synthesis

The total RNA was extracted from pooled tissues (for tissue distribution 6 fish and for immune challenge 5 fish, respectively) of untreated control by RNAiso plus (Takara) followed by clean-up with RNeasy spin column (Qiagen) according to the manufacturer's protocol. Originally purified RNA was diluted to 1 mg/mL concentration prior cDNA synthesis. Twenty microliter reaction mixture containing 2.5 μg RNA was used to synthesize first-strand cDNA from each tissue using PrimeScript™ II $1^{st}$ strand cDNA Synthesis Kit (Takara). The resulting cDNA was diluted 40-fold in nuclease-free water and stored in a freezer at −80 ℃ until use.

### 2.9. Quantitative real-time PCR -based ShLysG mRNA expression analysis

Quantitative real-time PCR (qRT-PCR) was carried out to analyze the mRNA expression of ShLysG using a Thermal Cycler Dice™ TP800 (Takara), in a 10 μL reaction volume containing 3 μL of diluted cDNA from each tissue, 5 μL of 2× TaKaRa Ex Taq™ SYBR premix, 0.5 μL of each gene-specific primer (10 pmol/μL) and 1 μl $dH_2O$. The qRT-

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PCR cycle profile included 1 cycle of 95 ℃ for 30 s, followed by 45 cycles of 95 ℃ for 5 s, 58 ℃ for 10 s and 72 ℃ for 20 s, and finally, 1 cycle of 95 ℃ for 15 s, 60 ℃ for 30 s and 95 ℃ for 15 s. The primers used in this study are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the relative expression. All samples were obtained and analyzed in triplicate and the results are expressed as relative-fold change as mean ± standard deviation. The seahorse ribosomal protein S7 (accession no. KP780177) was used as the internal control gene.

**2.10. Statistical analysis**

For comparison of relative ShLysG mRNA expression and inhibition properties, statistical analysis was performed using one-way analysis with ANOVA and mean comparisons were performed by Duncan's Multiple Range Test using SPSS 11.5 at $P < 0.05$ significance level.

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## 3. Results

### 3.1 Molecular characterization of ShLysG

The nucleotide and deduced amino acid sequences of ShLysG was 123 bp comprised of a 527 bp of 5'-untranslated region (UTR), an ORF encoding 184 amino acids of 552 bp and a 3'-UTR of 134 bp with the polyadenylation signal ($^{599}$AATAAA$^{604}$ and $^{677}$AATAAA$^{682}$). The molecular mass and the predicted isoelectric point were 20 kDa and 7.5, respectively. ShLysG was neither consisted of a signal peptide sequence nor possessed any cysteine residues that generate disulfide bridges. The conserved catalytic bacterial soluble lytic transglycosylase (SLT) domain (Ala$^{48}$-Ser$^{168}$) in ShLysG contains three catalytic residues (Glu$^{71}$, Asp$^{84}$ and Asp$^{95}$) and seven N-acetyl-D-glucosamine binding sites (Glu$^{71}$, Asp$^{95}$, Tyr$^{98}$, His$^{99}$, Ile$^{117}$, Tyr$^{145}$ and Asn$^{146}$) (Fig 3). The domain is important in the hydrolysis of the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell wall.


**Fig 3. Nucleotide and deduced amino acid sequences of ShLysG.** The start codon (ATG) and stop codon (TAG) are shown by underling with bold letters. The N-acetyl-D-glucosamine binding sites are indicated by bold red letters. The catalytic residues are boxed. The SLT domain is shaded in grey.

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Pairwise and multiple sequence alignment of ShLysG with different vertebrate members by the ClustalW program are shown in Table 2 and Fig 4, respectively. The pairwise analysis of LysG counterparts with ShLysG presented higher degree of identity with Southern platyfish (*Xiphophorus maculatus*) LysG (67.4%), followed by Atlantic cod (*Gadus morhua*) (67.0%), rainbow smelt (*Osmerus mordax*) (64.5%), channel catfish (*Ictalurus punctatus*) (64.0 %), zebrafish (*Danio rerio*) (63.4%), mexican tetra (*Astyanax mexicanus*) (62.4%) and atlantic salmon (*Salmo salar*) (60.7%). Percentages of similarity were shown ranging from 10.0 to 79.3%. Multiple sequence alignment result has revealed higher conserved amino acid sequence and motif of ShLysG with other vertebrate LysG counterparts.

**Table 2. Percent identities and similarities of ShLysG with g-type lysozyme orthologs from other species.**

| Common name | Scientific name | Accession number | Identity(%) | Similarity(%) |
|---|---|---|---|---|
| Southern platyfish | *Xiphophorus maculatus* | XP_005797552.1 | 67.4 | 79.3 |
| Atlantic cod | *Gadus morhua* | AAU81661.1 | 67.0 | 78.2 |
| Rainbow smelt | *Osmerus mordax* | ACO09746.1 | 64.5 | 78.0 |
| Channel catfish | *Ictalurus punctatus* | NP_001187748.1 | 64.0 | 79.0 |
| Zebrafish | *Danio rerio* | XP_005173173.1 | 63.4 | 76.9 |
| Mexican tetra | *Astyanax mexicanus* | XP_007233085.1 | 62.4 | 75.6 |
| Atlantic salmon | *Salmo salar* | CAM35431.1 | 60.7 | 72.6 |
| Axolotl | *Ambystoma mexicanum* | AEQ98812.1 | 52.9 | 65.2 |
| Red junglefowl | *Gallus gallus* | NP_001001470.1 | 52.4 | 66.5 |
| Horse | *Equus caballus* | CDM98820.1 | 15.2 | 27.0 |
| House mouse | *Mus musculus* | CDM98781.1 | 9.4 | 15.9 |
| Human | *Homo sapiens* | CDM98741.1 | 6.5 | 10.0 |

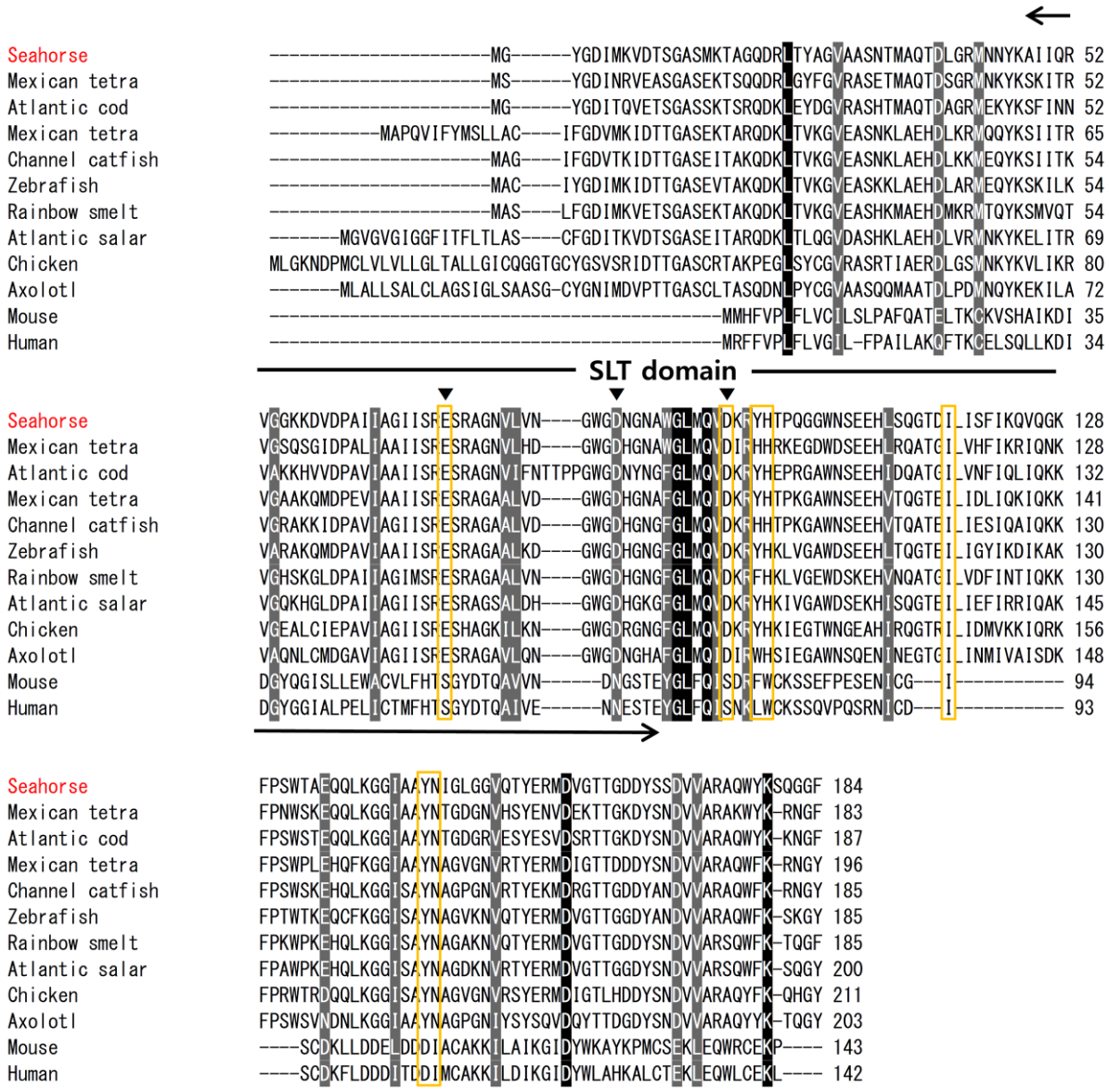


**Fig 4. Multiple sequence alignment of ShLysG with other known vertebrate LysG amino acid sequences.** Identical and similar residues are shaded with black and grey color, respectively. The N-acetyl-D-glucosamine binding sites and catalytic residues are depicted in orange color boxes and marked as ▼. The sequence alignments were obtained by the ClustalW method.

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### 3.2 Phylogenetic analysis of ShLysG

The phylogenetic tree was composed using the NJ method to analyze the evolutionary relationship with other vertebrate Lysozymes (Fig 5). Each type of lysozyme (c-type lysozyme (LysC), i-type lysozyme (LysI) and LysG) was placed in 3 distinct groups. ShLysG was positioned within teleost group in LysG clade and demonstrated closer relationships with the counterparts of *Gadus morhua*, *Lates calcarifer* and *Xiphophorus maculatus*.

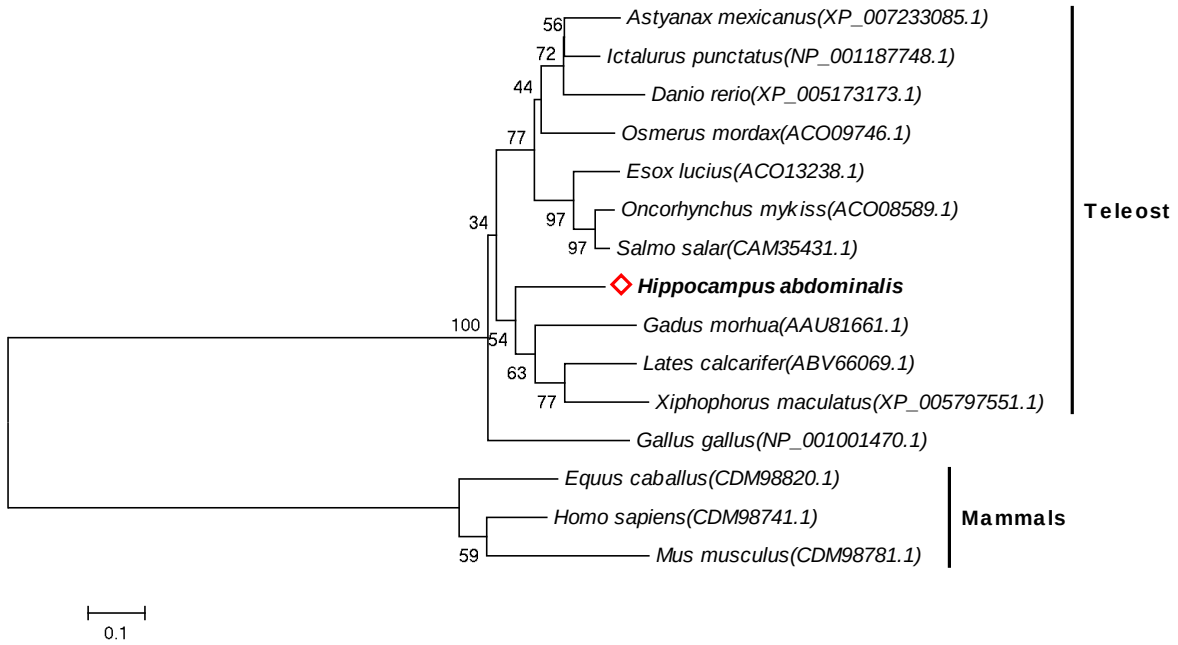


**Fig 5. Phylogenetic analysis of ShLysG with their known orthologues.** The tree is based on the alignment of the full-length amino acid sequences. Hippocampus abdominalis g-type lysozyme is indicated by ◇ and bold. The phylogram was constructed with the MEGA5.5 program by using the neighbor-joining (NJ) method. The accession numbers of the g-type lysozyme sequences are indicated in parentheses after the species names.

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### 3.3 Tissue specific expression of ShLysG mRNA

To determine the tissue specific expression of ShLysG mRNA, qRT-PCR was performed. The relative expression in 14 tissues was calculated using big-belly seahorse 40S ribosomal protein as the reference gene. ShLysG mRNA was expressed in all tissues and the highest expression level was detected in kidney (84.3-fold) followed by gill (52.3-fold), intestine (26.9-fold), testis (24.5-fold) and stomach (23.2-fold) compared to the least expressed tissue; the liver (Fig 6).

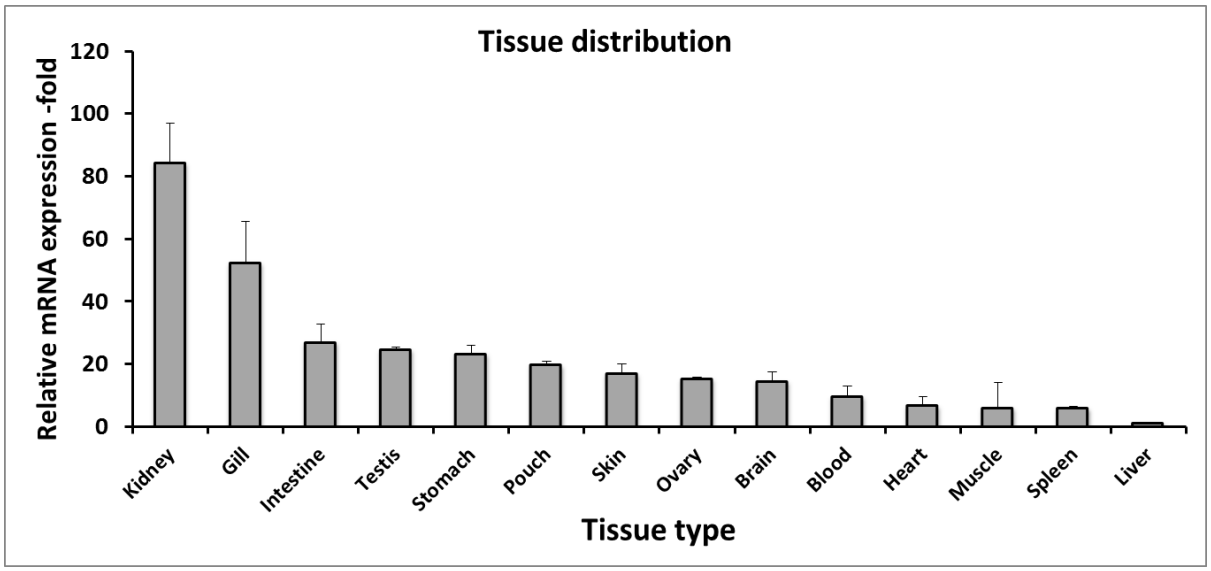


**Fig 6. Tissue-specific mRNA expression of ShLysG.** Analysis of the mRNA level was carried out by qPCR and relative expressions were calculated compared to the mRNA level detected in liver.

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### 3.4 Expression profile of ShLysG after immune stimulation

To determine the immune response of ShLysG upon pathogen infections, mRNA expression levels of kidney and gill tissues of challenged fish were assayed using qPCR. The results were compared with expression levels in PBS-injected group as control to determine the relative fold (Fig 7). In kidney tissue with all challenges, ShLysG were almost up-regulated at all the time points relative to the controls (0 h) ($P<0.05$). The highest level of ShLysG mRNA upon LPS challenge was detected at 24 h post injection (p.i.). The up-regulation pattern of both Poly I:C and *S. iniae* challenge appeared to be similar and reached its highest relative expression at 12 h p.i compared to the control. Interestingly, ShLysG transcript level has increased over time upon *E. tarda* challenge in kidney tissue and exhibited a different pattern than that of other challenges. However, no significant difference was detected upon *E. tarda* challenge in gill tissue in all the time points examined. In gill tissue, transcription level was significantly up-regulated until 12 h p.i and subsequently slightly down-regulated except in *E. tarda* challenge.

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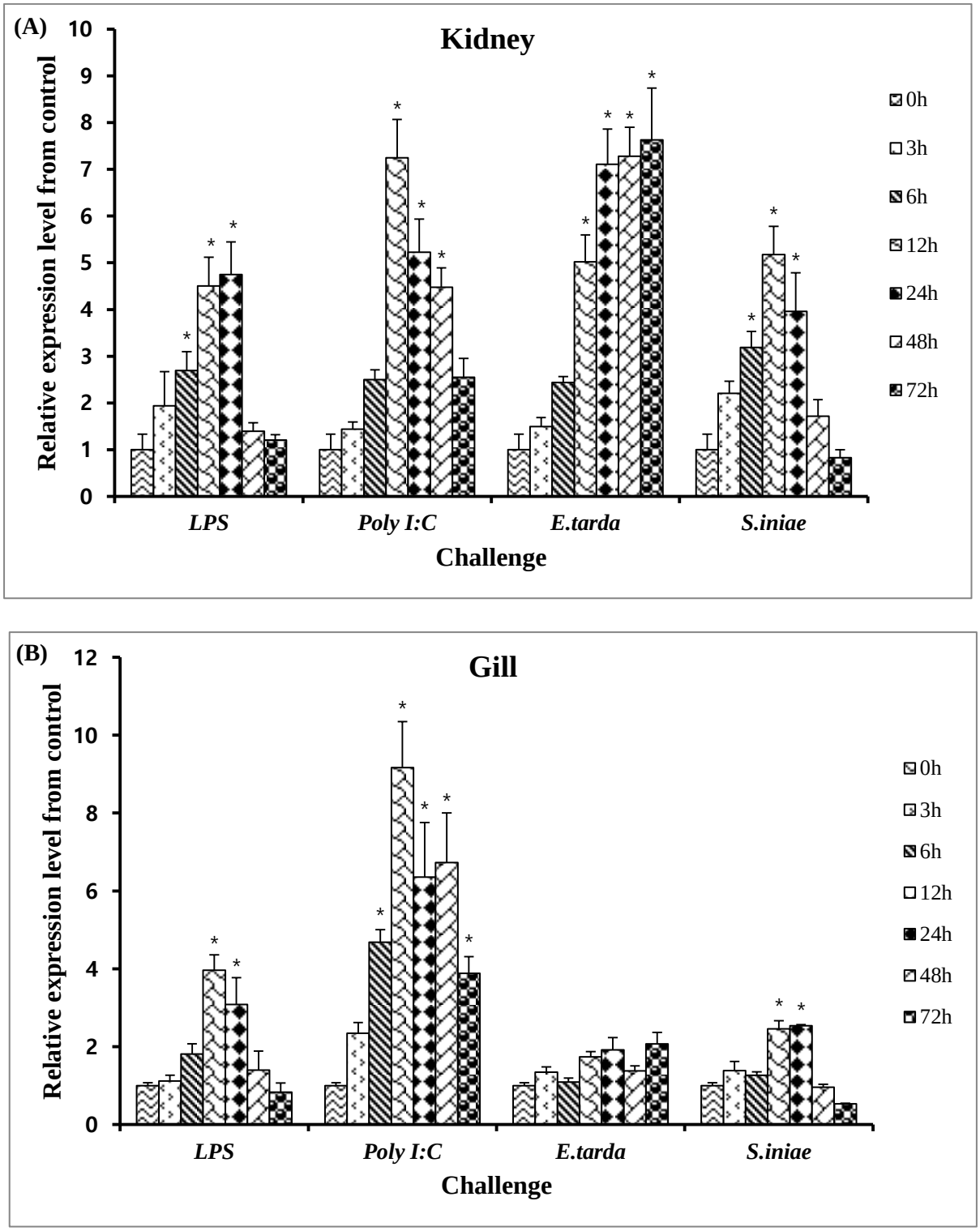


**Fig 7. The relative mRNA expression of ShLysG in kidney (A) and gill (B) tissue** from big-belly seahorse challenged with LPS, Poly I:C, E.tarda, S.iniae and PBS(control). The mRNA levels were normalized to ShRPS7 mRNA levels. Gene expression in each control group (0 h) was set at 1, and expression in each challenge group was expressed relative to the expression in the respective PBS control group. Vertical bars represent the mean ± S.D. (n=3) and significant differences are indicated with * letters at $P<0.05$

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### 3.5 Expression and purification of rShLysG

The recombinant ShLysG was overexpressed in *E. coli* ER2523 cells by IPTG induction and purified as a MBP-fusion protein. Aliquots of different fractions during the purification steps were analyzed by SDS-PAGE (Fig 8). ShLysG has been induced by IPTG (lane 2) compared to the un-induced cells (lane 1). The molecular mass of the purified recombinant ShLysG fusion protein (rShLysG; lane 5) was approximately 62.5 kDa, as determined by SDS-PAGE. Since the molecular mass of MBP is 42.5 kDa, predicted and determined molecular mass values of ShLysG appeared to be the same (20 kDa).

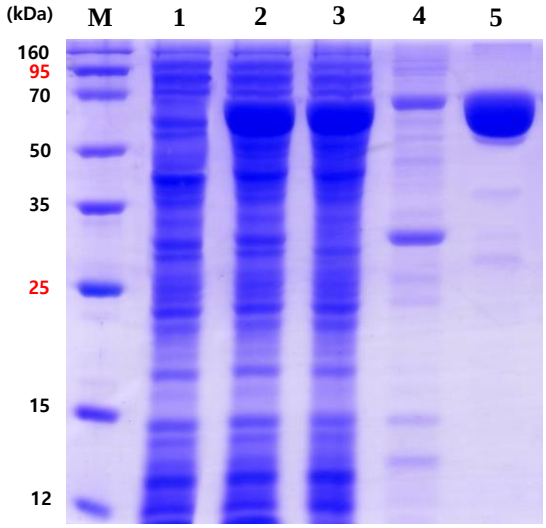


**Fig 8. SDS-PAGE analysis of recombinant ShLysG protein.** M: protein marker, lane 1: un induced, lane 2: induced protein, lane 3: supernatant after pelleted, lane 4: pellet and lane 5: purified protein elution.

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### 3.6 Enzyme activity of rShLysG

The activity of rShLysG on the hydrolysis of *M. lysodeikticus* (Sigma) as substrates by muramidase was investigated considering HEWL and MBP as positive and negative controls, respectively (Fig 9). The purified recombinant ShLysG has revealed its activity only at pH 4.0 and 20 ℃; in contrast, HEWL revealed a strong activity at a vast range of pH and temperature values (pH 6 to 10 and 30 to 50 ℃, respectively), where the optimal pH and temperature were pH 7 and 30 ~ 40 ℃, respectively. No muramidase activity was detected against *M. lysodeikticus* in the instance of MBP.

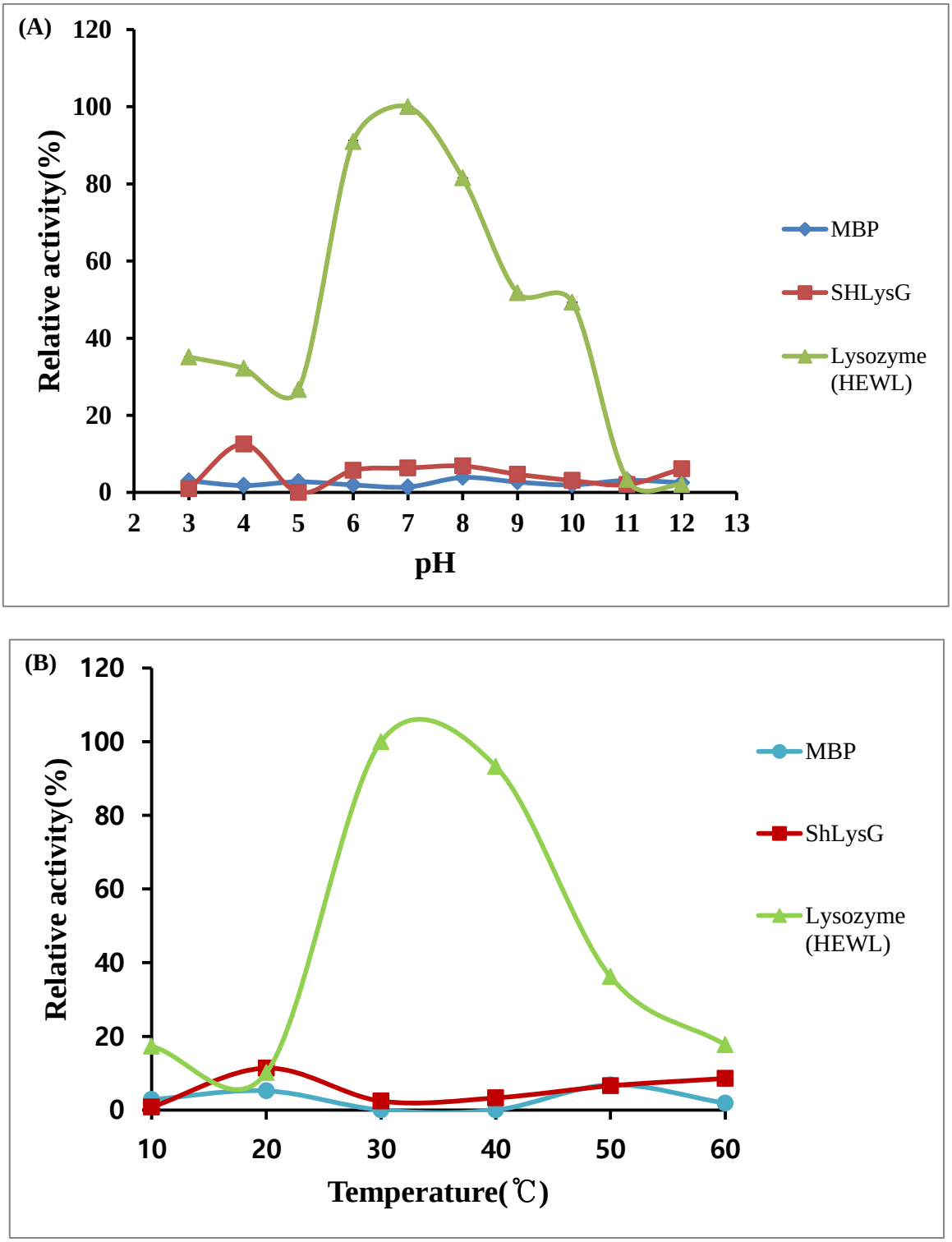


**Fig 9. Optimal pH and temperature of ShLysG.** (A) Optimal pH: Enzyme activity was assayed in various pH buffers ranging pH 3 to 12. Maximal activity was shown as 100%. (B) Optimal temperature: The effect of pH on the enzyme activity was determined under standard assay condition at temperature ranging 10 to 60 ℃.

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The antimicrobial activity assay was performed in pH 4 buffer and 25 ℃ using several bacterial cultures including gram-negative and gram-positive strains (Fig 10). Interestingly, rShLysG indicated stronger antimicrobial activity than that of HEWL towards 6 bacteria, i.e., *V. salmonicida*, *V. parahemolyticus*, *L. monocytogenes*, *S. iniae*, *V. anguillarum* and *M. lysodcikticus*. Neither rShLysG nor HEWL exhibited antimicrobial activity against *E. tarda* and *V. tapetis* (data not shown).

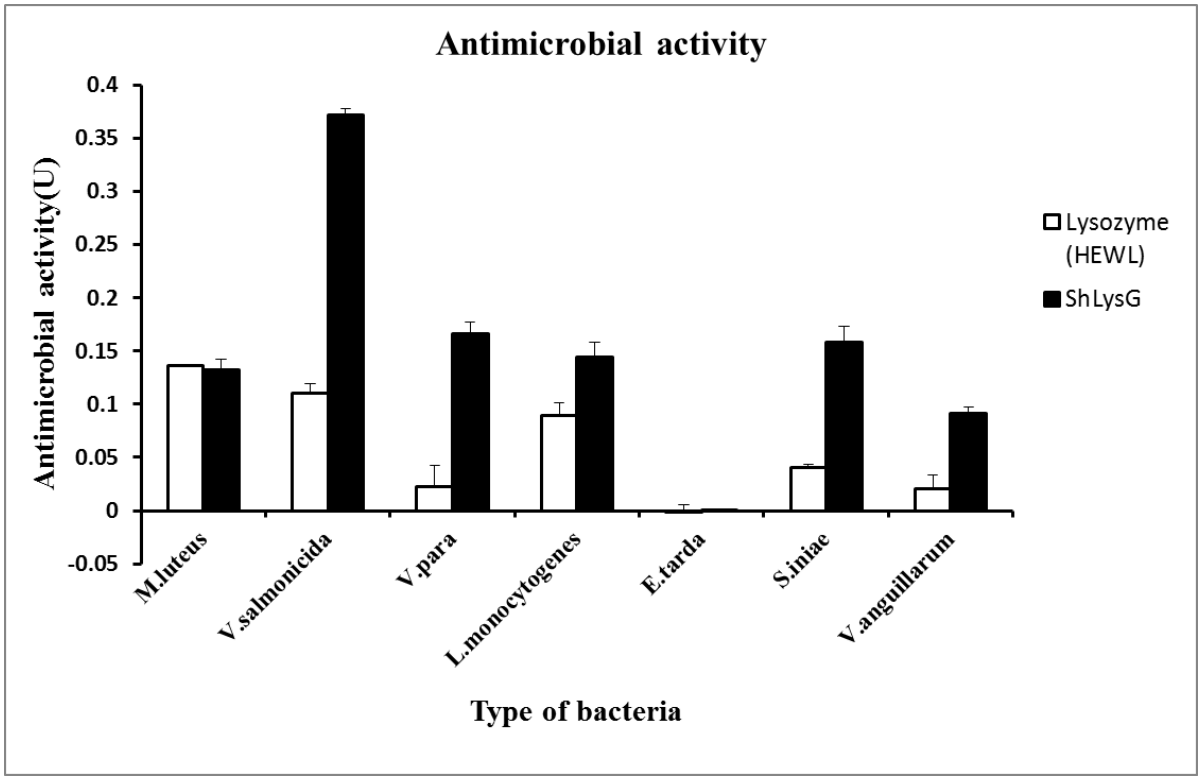


**Fig 10. Antimicrobial properties of the recombinant ShLysG.** Lytic activities are measured by turbidimetric assay and expressed as the mean U (mean ± S.D., n = 3).

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**4. Discussion**

In this study, the G-type lysozyme of big-belly seahorse (ShLysG) has been characterized. Here, the transcriptional characterization upon immune challenges and their antimicrobial properties were studied.

Mammals are subjected to countless number of diseases due to bacterial infections. Not only mammals, but also fish are affected by bacterial diseases. Lysozyme is an important effector of immune system against pathogenic bacteria which destruct them *via* bacteriolysis. Seven N-acetyl-D-glucosamine sites of lysozyme bind thru glycosidic bond within peptidoglycan in the binding site of prominent cleft. Additionally, three catalytic residues are serving as active sites which involved in C-O bond cleavage. In the SLT domain ($Ala^{48}$-$Ser^{168}$) at C-terminal of ShLysG, seven N-acetyl-D-glucosamin ($Glu^{71}$, $Asp^{95}$, $Tyr^{98}$, $His^{99}$, $Ile^{117}$, $Tyr^{145}$ and $Asn^{146}$) could be identified as binding sites while three catalytic residues; $Glu^{71}$, $Asp^{84}$ and $Asp^{95}$ were identified as activity site. The second catalytic residue ($Asp^{84}$) of ShLysG may not be conserved among LysG orthologs; for instance, $2^{nd}$ catalytic residue of common carp was replaced with $Pro^{86}$.

A conserved signal peptide sequence and four cysteine residues have been identified among the LysG of birds and mammals. Here, four cysteine residues involved in two pairs of disulfide bridges ($Cys^{4}$-$Cys^{60}$ and $Cys^{18}$-$Cys^{29}$) which is important for the stability of the enzyme structure but, not an essential factor for the catalytic activity (Kawamura et al., 2008; Ye et al., 2010). In contrast, most fish LysG orthologs do not contain any cysteine residues. Similarly, in ShLysG, neither signal peptide sequence nor active site was detected, presuming that ShLysG is an intracellular protein (Sha et al., 2012).

Pairwise sequence analysis of ShLysG was exhibited more than 60% aa sequence identity with teleost LysG sequences where higher mammal LysGs showed less than 20 %

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identity. ShLysG consisted with a SLT domain ranged in the CDS and our multiple sequence analysis results revealed its high conservation residues within the teleost LysG counterpart. Similar to mammal LysG, fish LysG has been reported to have these same features, suggesting that the LysGs of big-belly seahorse are also LysG homologs. The phylogenetic analysis amply validated that ShLysG belongs to the teleost LysG.

The ShLysG transcriptional expression was remarkably expressed in healthy kidney and gill tissues of big-belly seahorse. In fish, Lysozyme is mainly produced in kidney and intestine which are rich in leucocytes (Uribe et al., 2011) and lysozyme could be found in mucus rich tissues such as flakes, skin and gills which are first barriers to infections from external milieu (Uribe et al., 2011). Whang *et al.* 2011 reported that g-type lysozyme of rock bream was highly expressed in kidney and gill tissues as well (Whang et al., 2011). Accordingly, in this study, ShLysG mRNA levels in kidney and gill tissues were determined post-immune challenges. Here, immune responses upon bacterial and viral infections could be observed in regard to LPS, poly I:C, *E.tarda* and *S.iniae* both in kidney and gill tissues. Therefore, it could be suggested that ShLysG is important in inflammation responses of innate immune system of big-belly seahorse.

The optimal temperature for the rShLysG protein activity was detected at 20 ℃ on the same plane as positive control; HEWL. The same consequence could be observed with regards to optimum pH assay, where both proteins showed their optimal activity at pH4. The Atlantic cod, salmon and rock bream g-type lysozymes which used same substrate as the current study (*M. lysodeikticus*), showed optimum activity around at pH 4~5. Therefore, in this study, antimicrobial activity assay was carried out at 20 ℃ and pH 4.

In previous studies, fish g-type lysozymes have shown antimicrobial activities against several fish pathogenic bacteria (Hikima et al., 2001; Yin et al., 2003; Whang et al.,

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2011; Zhao et al., 2011)(). Similarly, antimicrobial activity data of this study indicated that rShLysG possesses antibacterial activities may be *via* bacteriolysis towards both Gram-positive (*M. luteus* and *S. iniae*) and Gram-negative (*V. salmonicida, V. parahemolyticus, L. monocytogenes* and *V. anguillarum*) bacteria.

In conclusion, the expression of ShLysG occurs constitutively in various tissues and is upregulated by pathogenic infections caused by bacteria and virus. ShLysG is an immune defense effector of the innate immune system of big-belly seahorse and is activated against invading fish pathogens. Additionally, the results suggest that ShLysG possesses antimicrobial activity *via* bacteriolysis with a broad spectrum of substrates including several pathogenic bacteria infecting fish.

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## Chapter II

## Transcriptional characterization & pro-inflammatory properties study of Tumor necrosis factor α (ShTNFα) from seahorse (*Hippocampus abdominalis*)

### 5. Material and Methods

#### 5.1. Experimental fish

Healthy seahorses (average body size of 20 cm) were purchased from Korea Marine Ornamental Fish Breeding Center, Jeju, Korea. They were acclimated in laboratory aquarium tanks at a temperature of 20±1 ℃ and salinity of 32±1 psu for 1 week prior the experiment.

#### 5.2. Big belly seahorse cDNA library construction

The seahorse transcriptome database was constructed using 454 GS FLX sequencing technique (Metzker, 2010). Briefly, the total RNA was extracted from blood, liver, kidney, gill and spleen tissues of 18 seahorses. The extracted RNA was then cleaned by RNeasy Mini kit (Qiagen, USA) and assessed for quality and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), giving an RNA integration score (RIN) of 7.1. For GS FLX 454 shotgun library preparation, the RNA was fragmented into average size of 1,147 bp using the Titanium system (Roche 454 Life Science, USA). Sequencing was finally run on half a picotiter plate on a Roche 454 GS FLX DNA platform at Macrogen, Korea. The raw 454 reads were trimmed to remove adaptor and low-quality sequences, and de novo assembled into contigs using GS Assembler (Roche 454 Life Science, USA) with the default parameters.

#### 5.3. *In silico* analysis of ShTNFα

The ShTNFα full-length sequence was analyzed by BLAST

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(http://blast.ncbi.nlm.nih.gov/Blast). Similarities were compared with other known TNF sequences available in the NCBI and ENSEMBL databases. To obtain the open reading frame (Walsh et al.) amino acid sequence of ShTNFα, DNAssist (version 2.2) was used. Characteristic domains or motifs were identified using the PROSITE profile database (Bairoch et al., 1997) and SMART proteomic database (Letunic et al., 2009). Identity, similarity and gap percentages were calculated using EMBOSS pair-wise alignment algorithms. Multiple sequence alignments and phylogenetic analysis were performed on the amino acid sequence of ShTNFα versus known TNFs, using ClustalW 2.0 (Thompson et al., 1994). The amino acid sequences were aligned using the ClustalW program and the phylogenetic tree was reconstructed using the neighbor-joining (NJ) method using MEGA 5 software package (Tamura et al., 2011) taking the bootstrapping value as 1000.

### 5.4. Cloning of ShTNFα coding sequence

The open reading frame (Walsh et al.) of ShTNFα was amplified, using cloning primers with corresponding restriction enzyme sites of PstI and EcoRI (Table 1). The PCR was performed in a TaKaRa thermal cycler in a total volume of 50 µL with 5 U of Ex Taq polymerase (TaKaRa, Japan), 5 µL of 10× Ex Taq buffer, 4 µL of 2.5 mM dNTP, 50 ng of template and 10 pmol of each primer. The reaction was carried out with an initial incubation at 94 ℃ for 3 min, 35 cycles (94 ℃, 30 s; 56 ℃, 30 s; 72 ℃, 60 s), followed by a final extension at 72 ℃ for 5 min. The PCR product was analyzed on a 1% agarose gel and ethidium bromide staining. Subsequently, the amplified product was excised from a parallel gel and purified using the Accuprep$^{TM}$ gel purification kit (Bioneer Co., Korea). The PCR product and maltose binding protein (MBP)-fused expression vector pMAL-c2X (New England Biolabs Inc, USA) were digested with respective restriction enzymes and the vector was dephosphorylated with calf intestine phosphatase (NEB, USA), in accordance with the

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vendor's protocol. Thereafter, the vector and PCR product were purified by a 1% agarose gel using the Accuprep$^{TM}$ gel purification kit (Bioneer Co., Korea). Ligation was carried out at 16 ℃ for 30min following overnight incubation at 4 ℃ with 200 ng of pMAL-c2X vector, 50 ng of PCR product, 5 μL of Mighty Mix (TaKaRa, Japan). The ligated product, pMAL-c2X/ShTNFα, was transformed into the *Escherichia coli* DH5α cells for sequencing.

**Table 3. Description of primers used in this study**

| Name | Primer Sequence(5' to 3') | Objects |
|---|---|---|
| ShTNFα-1F | gagaga**CTGCAG**ATGGAAGGTGACTGTCAAGTGAATCTTTTC | ORF amplification (*Pst* I) |
| ShTNFα-1R | gagaga**GAATTC**TCACAAGGCAAAGACCCCAAAGAA | ORF amplification (*EcoR* I) |
| ShTNFα-2F | ACGTCAGAGCTGCCATTCACTTAACA | qPCR amplification |
| ShTNFα-2R | CCGCGACAACTGACCCGAAA | qPCR amplification |
| ShRPS7-F | GCGGGAAGCATGTGGTCTTCATT | qPCR internal reference |
| ShRPS7-R | ACTCCTGGGTCGCTTCTGCTTATT | qPCR internal reference |

F; forward and R; reverse

### 5.5. Overexpression and purification of recombinant ShTNFα (rShTNFα)

The successfully cloned and sequence confirmed vectors were then transferred to competent *E. coli* BL21 (ED3) cells (Novagen, Germany) in order to express the protein. The bacteria were cultured and incubated at 37 ℃ with shaking at 200 rpm until the cell count reached 0.8 at $OD_{600}$. Then the culture was induced by IPTG 0.5 mM final concentration for 5 h at 30 ℃. The induced cells were cooled on ice for 30 min, and harvested by centrifugation at 3000 rpm for 30 min at 4 ℃. Harvested cells were re-suspended with 25 mL column buffer (Tris-HCl, pH 7.4, 200 mM NaCl) and frozen at -80 ℃ overnight. The

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recombinant ShTNFα (rShTNFα) was purified in the form of fusion protein with MBP by pMAL™ protein fusion and purification system (NEB, USA). After thawing, the cells were sonicated on ice. The sonicated cell suspension was centrifuged at 15000 rpm for 30 min at 4 ℃ and the resulted supernatant was considered as crude rShTNFα extract. In the final purification step, amylose resin was transferred into a 1 × 5 cm size column. The crude extract was loaded onto the column and washed with 12 × volumes of the column buffer. Finally, the rShTNFα fusion protein was eluted by applying a total of 3 mL elution buffer (column buffer + 10 mM maltose) in 0.5 mL aliquots. The concentration of the purified protein was determined via the Bradford method, using bovine serum albumin (BSA) as the standard (Bradford, 1976). The rShTNFα samples were collected from different purification steps were subsequently analyzed on 12% SDS-PAGE with protein size markers (Enzynomics, Daejeon, Korea). The gel was stained with 0.05% Coomassie blue R-250, followed by a standard de-staining procedure. All of the activity tests performed in this study was conducted using this purified rShTNFα fused with MBP.

### 5.6. Functional characterization of rShTNFα

#### 5.6.1. Cells culture

The murine macrophage cell line RAW264.7 cells were obtained from the American Type Culture Collection (ATTC). RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) from Gibco-BRL (Grand Island, NY) containing 1 % penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum and maintained in a 37℃ humidified incubator containing 5% $CO_2$.

#### 5.6.2. NO production assay

NO production in the medium was assessed by measuring nitrite/nitrate, the stable

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degradation products of NO, as follows: The cells were treated with 10 μL LPS (10 ng/mL) to generate NO (positive control). The Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) was added to the medium. The absorbance of the mixture at 540 nm was determined using a microplate reader, and nitrite concentration was determined using a dilution of sodium nitrite as a standard.

#### 5.6.3. Western blot analysis

Cells were lysed with lysis buffer containing 1 X RIPA (Upstate Biotechnology, Temecula, CA, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM $Na_3VO_4$, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL pepstatin, 1 μg/mL leupeptin and incubated on ice for 1 h. The cell debris was removed by centrifugation and the protein concentration in lysates was measured using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were subjected to electrophoresis in an 8% SDS-polyacrylamide gel for 90 min at 100 V and then transferred to polyvinylidene difluoride membranes. The membranes were incubated for 1 h at room temperature with 5% dried milk or 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% (v/v) Tween 20. The membranes incubated overnight at 4 ℃ in the presence of primary antibody. The membranes were washed extensively, and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. The membranes were again washed extensively, and the protein bands were visualized using a WEST-ZOL Western blot detection system (Intron Biotechnology, Sungnam, Kyungki, Korea).

### 5.7. Immune challenges and tissue collection

#### 5.7.1. Fish tissues for the specific distribution analysis

To examine the tissue-specific expression profile of ShTNFα, healthy individual big

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belly seahorses were carefully dissected and tissues from heart, gill, liver, spleen, kidney, intestine, stomach, skin, muscle, pouch, brain, testis and ovary were collected. Using a sterilized syringe, the blood (1-2 mL per fish) was collected from the big belly seahorse tail and immediately centrifuged at 3000 x g for 10 min at 4 ℃. The supernatant was removed and cells were harvested for RNA extraction. All tissue samples were snap-frozen in liquid nitrogen immediately after they were collected from fish, and stored at -80 ℃ until the total RNA was isolated.

### 5.7.2 Expression pattern of ShTNFα after immune challenge

For the immune challenge, big belly sea horses with average body weight of 3 g were used. LPS (1.25 μg/μL), poly I:C (1.5 μg/μL), *Edwardsiella tarda* ($5 \times 10^3$ CFU/μL), *Streptococcus iniae* ($10^5$ CFU/μL) and scuticociliate *Miamiensis avidus* ($10^3$/μL) were prepared with PBS and injected intraperitoneally in a total volume of 100 μL. For control group, the fish were injected with 100 μL PBS. The peripheral blood cells, gill, liver and kidney were sampled from five individuals at 0, 3, 6, 12, 24, 48 and 72 hours post-injection as described in section 5.7.1. Untreated and PBS-injected animals were kept separately as the control groups.

## 5.8. RNA extraction and cDNA synthesis

The total RNA was extracted from pooled tissues (for tissue distribution 6 fish and for immune challenge 5 fish, respectively) of untreated control by RNAiso plus (Takara) followed by clean-up with RNeasy spin column (Qiagen) according to the manufacturer's protocol. Originally purified RNA was diluted to 1 mg/mL concentration prior cDNA synthesis. Twenty microliter reaction mixture containing 2.5 μg RNA was used to synthesize first-strand cDNA from each tissue using PrimeScript™ II 1st strand cDNA Synthesis Kit

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(Takara). The resulting cDNA was diluted 40-fold in nuclease-free water and stored in a freezer at −80 ℃ until use.

### 5.9. Quantitative real-time PCR-based ShTNFa mRNA expression analysis

Quantitative real-time PCR (qRT-PCR) was carried out to analyze the mRNA expression of ShTNFα using a Thermal Cycler Dice™ TP800 (Takara), in a 10 μL reaction volume containing 3 μL of diluted cDNA from each tissue, 5 μL of 2× TaKaRa Ex Taq$^{TM}$ SYBR premix, 0.5 μL of each gene-specific primer (10 pmol/μL) and 1 μL $dH_2O$. The qRT-PCR cycle profile included 1 cycle of 95 ℃ for 30 s, followed by 45 cycles of 95 ℃ for 5 s, 58 ℃ for 10 s and 72 ℃ for 20 s, and finally, 1 cycle of 95 ℃ for 15 s, 60 ℃ for 30 s and 95 ℃ for 15 s. The primers used in this study are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to calculate the relative expression. All samples were obtained and analyzed in triplicate and the results are expressed as relative-fold change as mean ± standard deviation. The seahorse ribosomal protein S7 (accession no. KP780177) was used as the internal control gene.

### 5.10. Statistical analysis

For comparison of relative ShTNFα mRNA expression and inhibition properties, statistical analysis was performed using one-way analysis with ANOVA and mean comparisons were performed by Duncan's Multiple Range Test using SPSS 11.5 at $P < 0.05$ significance level.

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## 6. Results

### 6.1. Molecular characterization of ShTNFα

The complete cDNA sequence of seahorse TNFα was consisted of 729 bp, with an open reading frame (Walsh et al.) that encoded 243 amino acid (aa). The putative protein had a molecular mass of 26 kDa and a 5.6 of isoelectric point. Conserved tumor necrosis factor (TNF) domain ($Ala^{82}$-$Leu^{243}$) of ShTNFα is consisted six receptor binding sites ($Ile^{100}$, $Asn^{101}$, $Ser^{106}$, $Thr^{158}$, $Ser^{165}$ and $Asp^{170}$) and seven polypeptide binding sites ($His^{84}$, $Phe^{128}$, $Tyr^{130}$, $Tyr^{205}$, $Phe^{210}$, $Phe^{237}$ and $Phe^{241}$) (Fig 11).


**Fig 11. Nucleotide and deduced amino acid sequence of big-belly seahorse TNFα.** The start codon (ATG) and stop codon (TGA) are shown by underling with bold letters. The transmembrane region is indicated in blue color. The TNF domain is shaded in grey. The receptor binding sites are indicated by bold red letters. The polypeptide binding sites are boxed and bold.

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Pairwise and multiple sequence alignments of ShTNFα with different vertebrate members by the ClustalW program are shown in Table 4 and Fig 12. The pairwise analysis of TNFα counterparts with ShTNFα presented higher degree of identity with Zebra mbuna (*Maylandia zebra*) TNFα (59.4 %), Brichardi (*Neolamprologus brichardi*) TNFα (59.4 %), Pacific bluefin tuna (*Thunnus orientalis*) TNFα (59.1 %), bicolor damselfish (*Stegastes partitus*) TNFα (58.6 %), orange-spotted grouper (*Epinephelus coioides*) TNFα (58.1 %) and Atlantic salmon (*Salmo salar*) TNFα (46.5 %). High percentages of similarity were shown ranging from 42.3 to 73.4 %. Multiple sequence analysis result has revealed higher conserved amino acid sequence and motif of ShTNFα with other vertebrate TNFα sequences.

**Table 4. Percent identities and similarities of ShTNFα with TNFα orthologs from other species.**

| Common name | Scientific name | Accession number | Identity(%) | Similarity(%) |
|---|---|---|---|---|
| Orange-spotted Grouper | *Epinephelus coioides* | AEH59795.1 | 58.1 | 73.4 |
| Pacific Bluefin Tuna | *Thunnus orientalis* | BAG72142.1 | 59.1 | 71.4 |
| Atlantic Salmon | *Salmo salar* | ABO14978.1 | 46.5 | 61.5 |
| Zebra Mbuna | *Maylandia zebra* | XP_004560988.1 | 59.4 | 70.5 |
| Brichardi | *Neolamprologus brichardi* | XP_006803448.1 | 59.4 | 70.1 |
| Bicolor damselfish | *Stegastes partitus* | XP_008279527.1 | 58.6 | 71.9 |
| Frog | *Xenopus (Silurana) tropicalis* | NP_001107143.1 | 29.6 | 42.3 |
| Mouse | *Mus musculus* | NP_038721.1 | 28.6 | 45 |
| Human | *Homo sapiens* | CAA78745.1 | 28 | 47.7 |

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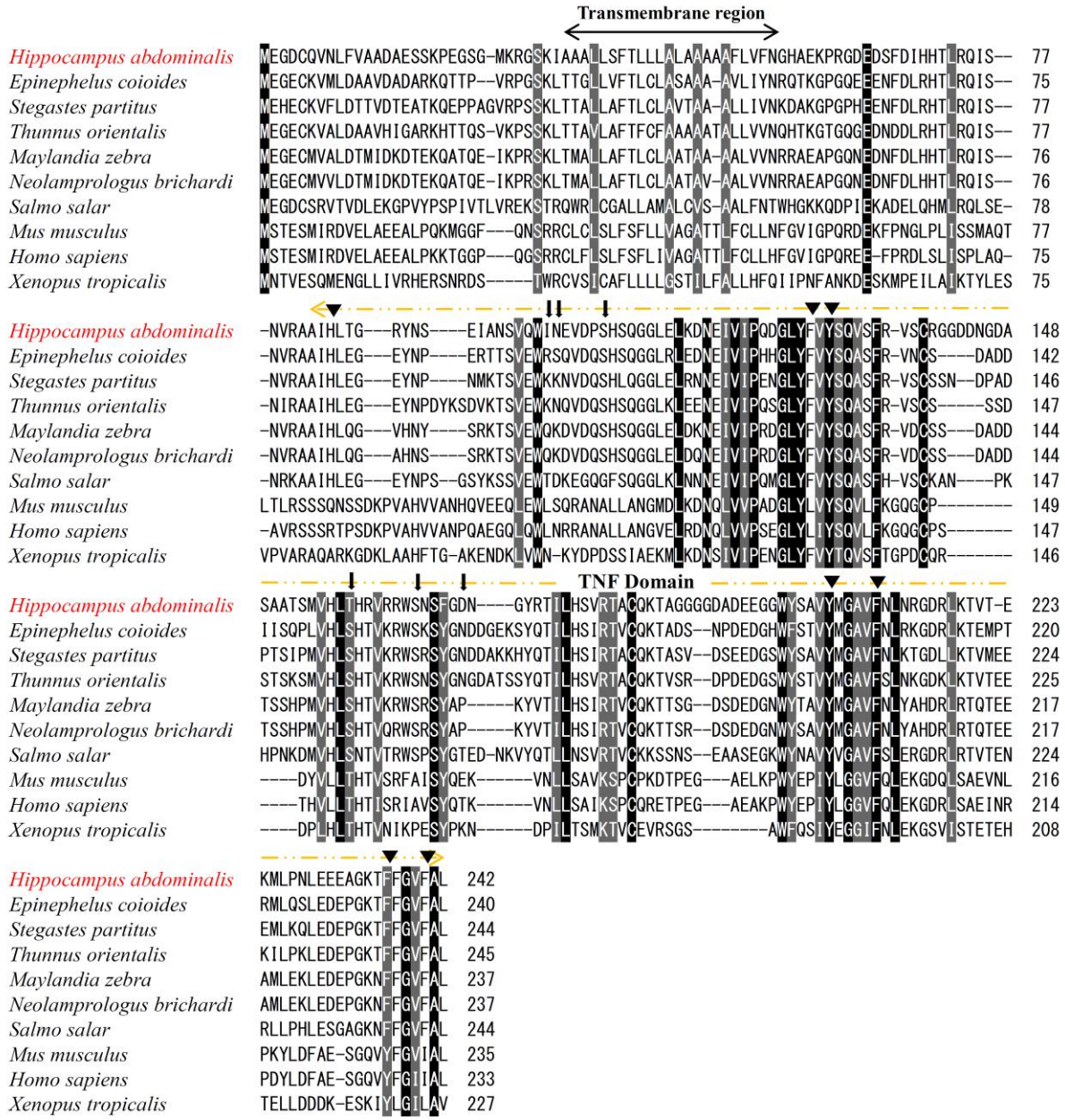


**Fig 12. Multiple sequence alignment of vertebrate TNFα.** Identical and similar residues are shaded with black and grey color, respectively. The transmembrane region and TNF domain are indicated the arrow and orange dash. The polypeptide binding sites and receptor binding sites are marked as ▼ and ⬇. Sequence alignment was obtained by the ClustalW method.

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### 6.2. Phylogenetic analysis of ShTNFα

Evolutionary relationship of ShTNFα was analyzed with other vertebrates developing a gene tree with the help of MEGA 6 (Fig 13). Here, Neighbor Joining method with the bootstrap support of 1000 replicates was used to develop the tree. TNF counterparts were clearly and distinctly formed two main clusters where TNFα and TNFβ counterparts were lied. The vertebrate TNFα members were divided into two sub clusters where amphibian TNFα was lied as an outlier of the teleost group. ShTNFα was clustered within the teleost TNFα counterparts which showed higher evolutionary relationship to *Maylandia zebra*, *Neolamprologus brichardi* and *Salmo salar* TNFa counterparts.

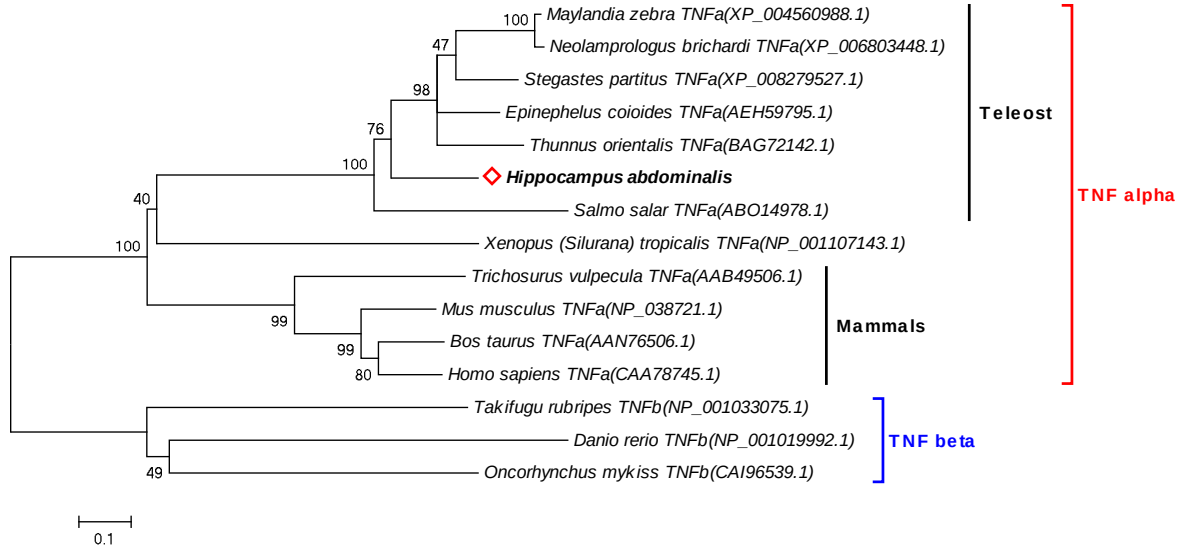


**Fig 13. Phylogenetic tree of various TNFα with ShTNFα.** Hippocampus abdominalis TNFα is indicated by ◇ and bold. The phylogram was constructed with the MEGA5.5 program by using the neighbor-joining (NJ) method. The accession numbers of the TNFα sequences are indicated in parentheses after the species names.

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### 6.3. Tissue specific expression of ShTNFα mRNA

The tissue specific expression of ShTNFα mRNA, was analyzed using qRT-PCR. The relative expression of ShTNFα in14 tissues was calculated taking big-belly seahorse 40S ribosomal protein as the reference gene (Fig 14). ShTNFα mRNA was expressed in all tissues in different magnitudes, where skin (511-fold) scored the highest followed by gill (33-fold), pouch (24-fold), muscle (22-fold) and intestine (22-fold) in comparison to the liver.

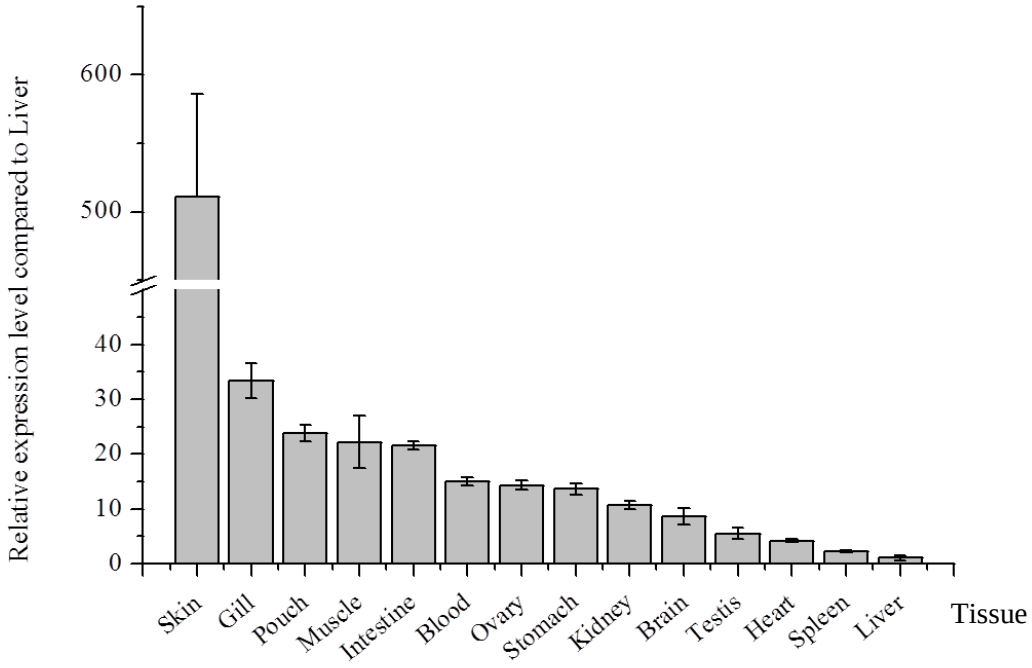


**Fig 14. Tissue-specific mRNA expression of ShTNFα.** Analysis of the mRNA level was carried out by qPCR and relative expressions were calculated compared to the mRNA level detected in liver.

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### 6.4. Expression profile of ShTNFα after immune stimulation

To analyze the immune response of challenged fish, ShTNFα mRNA expression levels were determined in blood and kidney (Fig 15). The results were compared with mRNA expression levels of PBS-injected fish as control, to determine the relative fold. In all the challenge experiments, ShTNFα was up-regulated at all the time points in both tissues, relative to the controls (0 h) ($P<0.05$). However, the expression levels were significantly very high in kidney than that of blood in all the instances. In both tissues, the up-regulation pattern was appeared to be similar in LPS and poly I:C challenges where it reached the highest relative expression at 3 h p.i compared to the control (60.4-fold and 12.7-fold in blood tissue; and 410.6-fold and 232.4-fold in kidney tissue, respectively). Subsequently, the transcript level has decreased over time after 3 h p.i. Otherwise, mRNA expression upon LPS challenge was again up-regulated at 48 h p.i in blood tissue (27.3-fold). Upon *E. tarda* challenge, the blood tissue showed significant up-regulation of ShTNFα transcripts at 12 h and 48 h p.i (29.3- and 23.6-fold), whereas kidney tissue expressed the ShTNFα transcripts at 6 h and 24 h p.i (357.4- and 319.3-fold). The highest level of ShTNFα mRNA upon *S. iniae* challenge was outstandingly detected at 48 h p.i in blood (45.6-fold) and 6 h p.i in kidney (1089.7-fold).

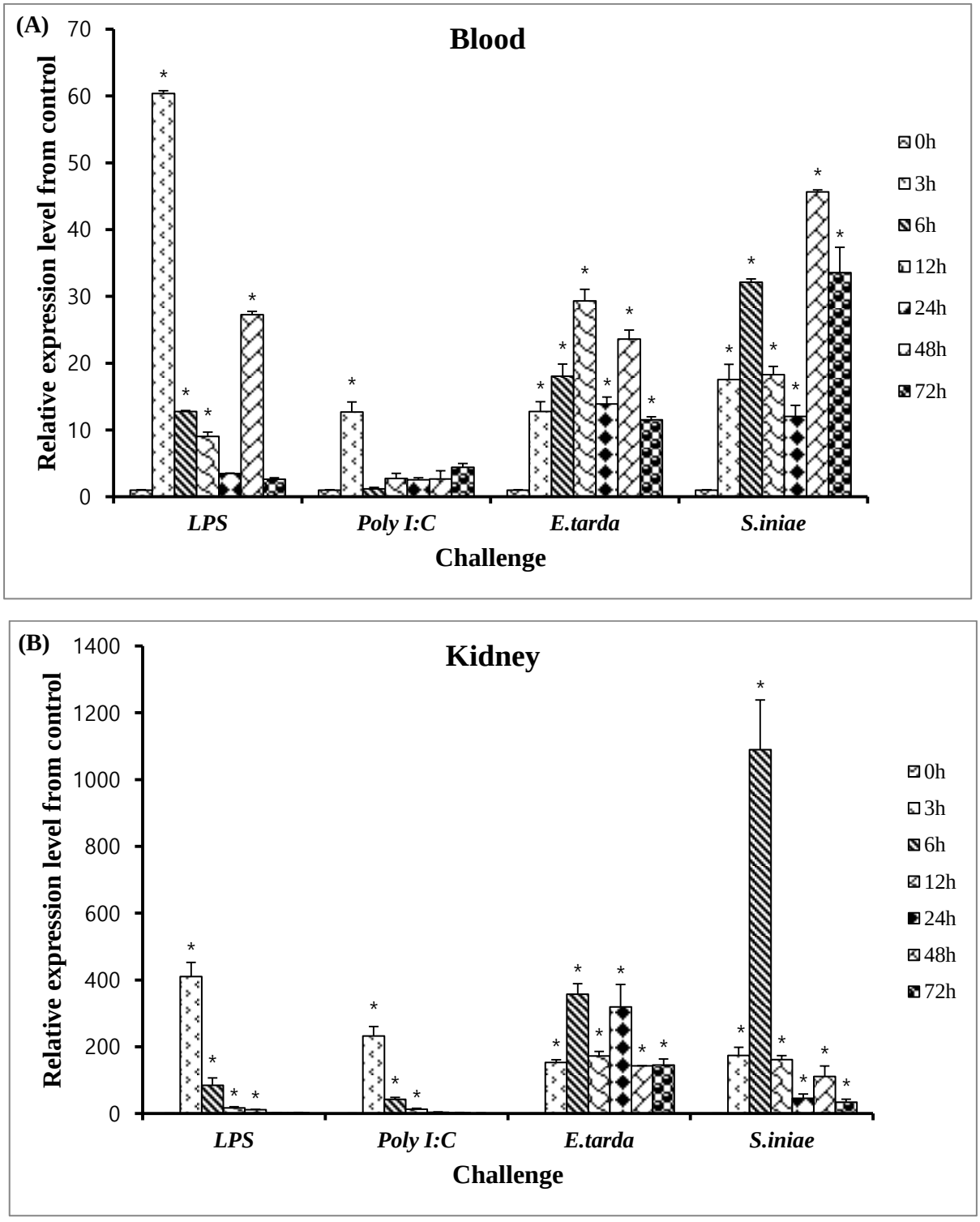


**Fig 15. The relative mRNA expression of ShTNF*α* in blood (A) and kidney (B) tissue** from big-belly seahorse challenged with LPS, Poly I:C, E.tarda, S.iniae and PBS(control). The mRNA levels were normalized to ShRPS7 mRNA levels. Gene expression in each control group (0 h) was set at 1, and expression in each challenge group was expressed relative to the expression in the respective PBS control group. Vertical bars represent the mean ± S.D. (n=3) and significant differences are indicated with * letters at $P < 0.05$.

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### 6.5. Expression and purification of rShTNFα

The recombinant ShTNFα was overexpressed in *E. coli* BL21 (ED3) cells by IPTG induction and purified as an MBP fusion protein. Aliquots of different fractions during the purification steps were analyzed by SDS-PAGE (Fig 16). Induction of ShTNFα upon IPTG, could be clearly identified (lane 2) compared to un-induced cells (lane 1). The molecular mass of the purified recombinant ShTNFα fusion protein (rShTNFα) was approximately 68.5 kDa, as determined by SDS-PAGE (lane 5). Since the molecular mass of MBP is 42.5 kDa, predicted and determined molecular mass values of ShTNFα appeared to be the same (26 kDa).

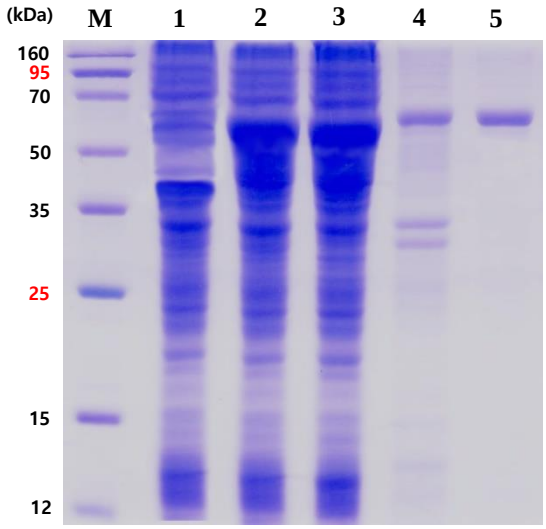


**Fig 16. SDS-PAGE analysis of recombinant ShTNFα protein.** M: protein marker, lane 1: un induced, lane 2: induced protein, lane 3: supernatant after pelleted, lane 4: pellet and lane 5: purified protein elution.

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### 6.6. Functional characterization of recombinant SHTNFα

#### 6.6.1. NO induction assay

ShTNFα was examined to check whether it has a decisive effect on NO production. NO production induced by ShTNFα was assessed using macrophage RAW 264.7 cells. The positive control cells, which had been treated with 1 ng/mL LPS, promoted NOs induction. The rShTNFα treatment at 0.0625, 0.125, and 0.25 μg/mL encouraged the NO production from RAW 264.7 cells in a dose-dependent manner as similar as positive control. (Fig 17).

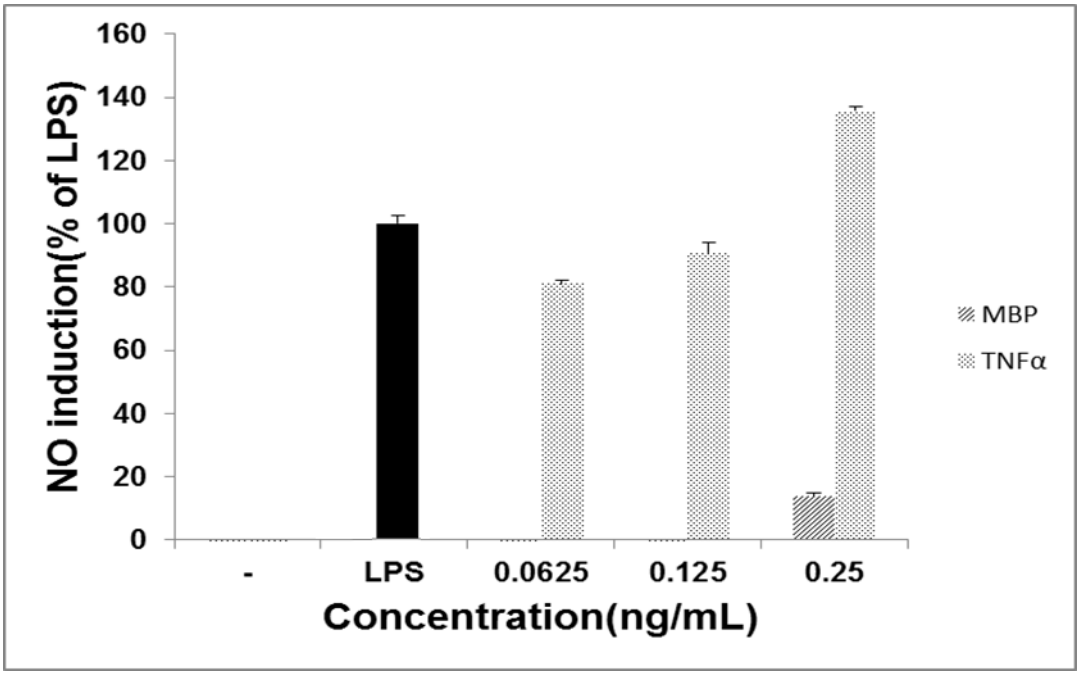


**Fig 17. Effect of rShTNFα on the NO production in stimulated RAW 264.7 cells.** Cell were incubated with the indicated concentrations (0, 0.0625, 0.125, 0.25 μg/mL) of each rShTNFαs for 24 h. The positive control cells had been treated with 1ng/mL LPS for 24h. The data expressed as means ± S.E. of four determinations.

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### 6.6.2. Western blot

To determine whether ShTNFα have an effect on the expression of pro-inflammatory factors, the expression levels of iNOS and COX-2 upon TNFα induction were examined in a dose-dependent manner using western blot technique. As shown in Fig 18, expression levels of pro-inflammatory factors were induced in LPS-treated cells. The up-regulation pattern of ShTNFα treatment also appeared to be similar in a dose-dependent manner and expression level were higher than positive control in both of the instances; iNOS and COX-2 proteins.

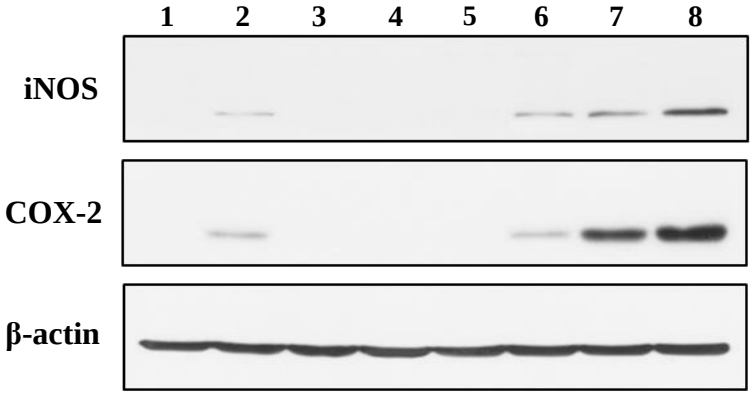


**Fig 18. Expression of iNOS and COX-2 in RAW 264.7 cells treated with MBP and rShTNFα.** Cells were treated with indicated concentration (0.0625, 0.125, 0.25 μg/mL) of each MBP and rShTNFα for 24 h and the negative control cells were stimulated with LPS (1ng/mL) for 24 h. The protein levels were determined by western blot analysis. Lane 1: negative control, Lane 2: positive control, Lane 3-5: MBP treated cells (0.0625, 0.125, 0.25 μg/mL), Lane 6-8: rShTNFα treated cells (0.0625, 0.125, 0.25 μg/mL).

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## 7. Discussion

In this study, the TNFα of big-belly seahorse (ShTNFα) has been characterized. Here, the transcriptional characterization upon immune challenges and their pro-inflammatory properties were studied.

Inflammation is an important part of immune system of fish to defend against extrastimulation such as pathogens, irritants etc. TNFα is an important cell signaling protein which is primarily generated as stable homotrimeric cytokine formed from its precursor form that involved in type II transmembrane region by activated macrophages, lymphocytes and many other cells. Thereafter, the hydrophobic transmembrane domain is cleaved by proteolytic enzyme; the TNFα converting enzyme (TACE) (Luettig et al., 1989). After being processed by TACE, the soluble TNFα (sTNFα) is secreted as two types of TNF receptors (Moss et al., 1997). Molecular characterization of ShTNFα revealed a CDS encoding a protein of 243 aa with a transmembrane region at the N-terminal and a conserved TNF domain as mature domain at C-terminal.

Pairwise sequence analysis of ShTNFα was exhibited more than 50% aa sequence identity with teleost TNFα sequences where higher vertebrate TNFα showed less than 30% identity. ShTNFα consisted with a TNF domain ranged in the CDS and our multiple sequence analysis results revealed its high conservation residues within the teleost TNFα counterpart. Similar to mammal TNF, fish TNFα has been reported to have these same features, suggesting that the TNFα of big-belly seahorse is also a TNFα homologs. The phylogenetic analysis amply validated that ShTNFα belongs to the teleost TNFα.

The ShTNFα transcriptional expression was remarkably expressed in healthy skin and gill tissues. TNFα is engaged in first defense responses in inflammation against stimuli which synthesized in white blood cells such as macrophages and lymphocytes as well as in

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other cells (Pennica et al., 1983; Kriegler et al., 1988; Luettig et al., 1989). Skin and gill of fish perform a key role as the first physical barrier to extrastimulation (Uribe et al., 2011). In human skin, pre-formed TNFα exists as a predominant source, which can be released upon inflammatory stimulus (Walsh et al., 1991). Therefore, expression of ShTNFα tissue distribution mRNA profiles may suggest the importance of inflammation responses in innate immune system of big-belly seahorse.

Different from those found in mammals, kidney is considered as an important lymphomyeloid organ in fish. Macrophages and lymphocytes are produced in lymphomyeloid organ such as kidney and disembogued into blood. Subsequently, they secrete TNFα into tissues upon extrastimulation (Uribe et al., 2011). Accordingly, ShTNFα mRNA levels in kidney and blood tissues were determined post-immune challenges of this study. In present study, the early phase immune responses and susceptible expression were detected in both kidney and blood tissues upon pathogenic stimuli i.e. LPS, poly I:C, *E.tarda* and *S.iniae*.

TNFα regulates immune response by binding to two receptors, TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2). TNFR1 is expressed in almost all tissues and can be activated by both the membrane-bound and soluble trimeric forms of TNFα. Whereas TNFR2 is existed only in immune tissues and respond to the membrane-bound form of the TNFα homotrimers. Upon binding with TNFα causes a conformational change to occur in the receptor and it activates the NF-κB pathway in consecutive order (Wajant et al., 2003). Subsequently, NO and PGE2 were expressed and cause inflammatory responses.

The appropriate concentrations of ShTNFα for cellular treatment were determined by NO induction assay (data not shown). The highest concentration level of ShTNFα (0.25 $\mu g$ /mL) influenced the NO induction of mouse macrophage RAW 264.7 cells up to positive

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control, LPS (10 ng/mL) and increased in a dose-dependent manner. These results suggest that ShTNFα affects the inflammation in macrophages. Accordingly, to investigate the downstream events related to inflammatory responses, western blot assay was performed and determined the expression levels of pro-inflammatory factors; iNOS and COX-2. Both expression levels of iNOS and COX-2 were increased in a dose-dependent manner. This result may speculate that ShTNFα mediates inflammatory responses through NF-B pathway.

In conclusion, the results suggest that activation of ShTNFα induces the expression of pro-inflammatory factors and inflammatory reactions via NF-κB pathway. The expression of ShTNFα occurs constitutively in various tissues and is upregulated by pathogenic infections such as bacteria and virus. ShTNFα is an inflammatory mediator of the innate immune system in response to invading bacteria and virus that cause serious detrimental effects on the host apart from the beneficial effects.

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## 감사의 글

생물학과에서 4년의 학부생활과 해양생명과학과에서 2년의 석사과정을 마치며, 그동안 저를 아껴주시고, 여기까지 성장할 수 있게 이끌어주고 도와주신 모든 분들께 감사의 말씀을 전하고자 합니다.

우선, 대학원 석사과정동안 해양생명과학과라는 분야에서 아무것도 몰랐던 저를 받아주시고 챙겨주셨던 이제희 교수님께 깊은 감사의 말씀과 존경의 마음을 표현합니다. 그리고 저의 부족한 논문을 꼼꼼히 수정해 주시고 지도해 주신 완창 박사님과 한국해양과학기술원의 오철홍 박사님께도 깊은 감사를 드립니다. 석사과정동안 깊은 학문적 지식을 가르쳐 주신 허문수 교수님, 여인규 교수님, 정준범 교수님, 이승헌 교수님께도 감사의 말씀을 드립니다. 항상 저의 인사를 밝게 받아주시는 전유진 교수님, 그리고 아직 뵙지 못하였지만 김기영 교수님께도 감사의 말씀을 전합니다.

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