한국어병학회지 제 22 권 제 2 호 (2009)
J. Fish Pathol., 22(2) : 147 ~ 153 (2009)

Production and bioactivity of recombinant tilapia IL-1β

Suhee Hong†

Faculty of Marine Bioscience and Technology, Gangneung-Wonju National University, Gangneung 210-702, KOREA

To study the biological activity of interleukin-1β (IL-1β), a proinflammatory cytokine, in nile tilapia, *Oreochromis niliticus*, the recombinant tilapia IL-1β was produced in *E. coli* cells based on pQE vector. Ni-NTA (nitriloacetic acid) metal affinity chromatography was used to purify recombinant protein. The eluted fractions exhibited a single band of protein with a molecular weight of about 25kDa, which is in close agreement with 25.4 kDa predicted by the cDNA sequence. The biological activity of the purified recombinant tilapia IL-1β was tested through its effects on IL-1β gene expression, which are known as IL-1β inducible genes in mammals and fishes. IL-1β gene expression induced by poly I:C, a synthetic double stranded RNA, was also assessed in tilapia head kidney cells. IL-1β gene expression was analysed using Q-PCR (quantitative polymerase chain reaction). The ratio of the indicated gene expression was expressed as the relative mRNA level to β-actin mRNA level, which is constitutively expressed in macrophages. Consequently, head kidney cells incubated for three hours with rIL-1β (10, 2, 1 μg/ml) showed a dose dependent increase in IL-1β mRNA levels and 1 μg/ml of poly I:C was also able to induce IL-1β gene expression in head kidney in tilapia.

Key words: Nile tilapia, *Oreochromis niloticus*, IL-1β, Bioactivity, Recombinant protein, Q-PCR

Introduction

Interleukin-1β (IL-1β) is a cytokine playing the central role in vertebrate immune system, and a member of interleukin 1 family known as multifucntional cytokines, such as IL-1α, IL-1 receptor antagonist, and IL-18. The diverse biological effect of IL-1β on most cells and organ systems in mammals are also well known. In addition to systemic effects such as fever induction, its major function is an early initiator of inflammatory and immune responses in a cascade of the expression of a number of cytokines in many cells (Dinarello, 1984). One of its main immunological activities is the stimulation of T helper cells inducing secretion of IL-2 and expression of IL-2 receptors (Smith *et al*., 1980).

Comparing to a remarkable number of researches concerning IL-1β in mammals, only few studies have been done in non-mammalian species including fish. Clem *et al*.(1985), revealed that supernatant from LPS-stimulated monocytes of channel catfish had IL-1-like bioactivity allowing mitogen-induced proliferation and antigen-induced antibody production in macrophage-depleted lymphocytes. The secretion of an IL-1-like factor was also reported in carp macrophages and neutrophils (Verburg Van Kemenade *et al*., 1995), carp epithelial cell lines (Sigel *et al*., 1986) and macrophage cell lines (Weyts *et al*., 1997). In addition, the IL-1β gene was cloned and sequenced in several fish species including rainbow trout, Atlantic salmon, plaice and carp (Secombes *et al*., 1998; Bird *et al*., 1998; Zou *et al*., 1999; Fujiki *et al*, 1998).

Recently, tilapia IL-1β gene has been cloned and

†Corresponding Author : Suhee Hong, Tel : 033-640-2852
E-mail : s.hong@nukw.ac.kr

analysed its expression by Lee *et al*. (2006). However, its biological effect has not been studied yet. Due to the absence of isolated native IL-1β from fish, in this study, recombinant tilapia IL-1β (rIL-1β) was produced and its biological activity was investigated by assessing IL-1β gene expression induced by rIL-1β in tilapia head kidney cells.

Materials and Methods

Production of recombinant tilapia IL-1β

The plasmid vector was constructed to express mature IL-1β with 6-histidine tag at N-terminus, which makes it easy to purify recombinant protein by binding to charged Ni metal, and the plasmid was transformed into *E. coli* cells. The *E. coli* was subcultured in 20ml of terrific broth (TB) medium containing ampicillin (50μg/ml) overnight at 37 ℃ in a shaker (200rpm), then cultured in 500ml TB medium for 4 hours, and incubated with IPTG (0.05mM) for 2 hours at 30 ℃. The *E. coli* was harvested by centrifugation at 4 ℃, resuspended in 6ml of lysis buffer (50mM Tris-Cl, pH 8, 500mM NaCl, 5mM $MgCl_2$, 10% Glycerol, 0.1% NP-40), and sonicated on ice to release accumulated recombinant protein in the cytoplasm. Induction of recombinant protein was confirmed by SDS-PAGE analysis of induced and uninduced bacterial cell suspension. Once the induced recombinant protein appeared on SDS-PAGE gel was observed, the cell lysate was applied to NTA column equillibrated with lysis buffer. Proteins lacking histidine tag were washed out by buffers containing 20 to 30 mM of imidazole, and the recombinant protein bound to the affinity column resin was eluted by 50 to 250 mM of imidazole. The protein concentrations of eluted fractions were determined by BCA method, and the size and purity of the eluted protein were confirmed by SDS-PAGE.

Western blot analysis

Induction of his tagged recombinant protein was also confirmed by western blot analysis using a histidine specific Ni-NTA-HRP conjugate. Cell lysate was separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Amersham). The blot was blocked with 5 % skimmed milk overnight, washed three times for 10 min with PBS (pH 7.4) containing 0.1% tween 20 (Sigma), and incubated with Ni-NTA-HRP (horseradish peroxidase) conjugate (Qiagen) at 1/200 dilution for 1 h. The reaction was visualised by the tetramethylbenzidine (TMB) substrate (Sigma) and photographed using a digital camera (Olympus).

Tilapia head kidney cell cultures

Head kidney was aseptically taken out from anaesthetised fish, and washed several times with L-15 medium (Leibovitz, Gibco) to remove blood. Leukocytes enriched total head kidney cells were prepared by pushing head kidney through a 100 μm-sized nylon mesh, then washed by centrifugation at 1600 rpm for 10min at 4℃, and suspended in L-15 medium containing antibiotics.

Stimulation of cells

For the gene induction experiment, freshly prepared head kidney cells were adjusted to 10^5 cells/ml, and stimulated with poly I:C (1μg/ml, Sigma), different dose (10, 2, 1 μg/ml) of rIL-1β or without any stimulant in cell culture dish in 5ml total volume at 22 ℃ for three hours. Head kidney cells were also incubated with elution buffer used for the elution of rIL-1β to test whether the elution buffer can affect the biological activity of rIL-1β. Three dishes of cell culture were used for each experimental condition. Poly I:C (polyinosinic:polycytidylic acid) is a synthetic double stranded RNA and used as a positive control since it is known to

induce IL-1β gene expression in fish head kidney cells.

Preparation of total RNA

Total RNA was extracted by using Trizol (Invitrogen) according to the manual. Briefly, the cell were homogenized in Trizol by repeated pipetting, extracted with 0.2 volume of chloroform, then precipitated with equal volume of chilled isopropanol, and washed by 75% chilled ethanol. The RNA pellet was resuspended in DEPC (diethyl pyrocarbonate)-treated water and stored at -80°C. The concentration was determined by UV spectrophotometry.

Reverse transcription

Reverse transcription to cDNA was carried out as described by Laing *et al*. (1996). Briefly, 3 μg RNA in 13 μl DEPC-water was incubated with 1 μl of oligo $(dT)_{12\text{-}18}$ (500 μg/ml, Promega) for 10 min at 70 °C. Then, 1 μl of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega), 4 μl 5X first strand buffer (Promega), 1 μl of 10 mM dinucleoside triphosphate (dNTP) mix (Promega) were added and the mixture incubated at 42 °C for 1.5 h. The reaction was terminated by heating to 94 °C for 15 min and after cooling, 30 μl of DEPC-water was added to make up the volume to 50 μl. The resulting cDNA was stored at -20 °C.

Q-PCR analysis of Gene expression

PCR reactions were performed in 50 μl reactions containing 2 μl of cDNA, 2.5 μl (25pmol) of each primer, 5 μl of 10X reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1 % Tween-20, pH8.8, Promega), 1 μl dNTP mixture (2.5 mM for each base, Promega), 2.5 μl of $MgCl_2$ (50 mM, Promega) and 0.25 μl (1.25 U) of Taq polymerase (Promega), using a thermocycler (Px2, Thermo).

For each amplification procedure, a negative control was run using sterile water instead of cDNA template, to ensure that any amplified products generated from cDNA samples were not a result of any contamination i.e. DNA from pipettes or air. The PCR products were resolved on a 2% (W/V) agarose gel containing 0.1μg/ml ethidium bromide, using an UVP system to catch the images. To confirm successful isolation and reverse transcription of RNA from the cells, β-actin was used as a positive control for PCR, since the gene encoding β-actin is highly conserved and expressed constitutively in most cells.

To determine expression of tilapia IL-1β mRNA in isolated head kidney cells, quantitative real-time PCR (Q-PCR) reactions were carried out using the ABI 7900 HT real-time thermocycler (Applied Biosystems), using the SYBR Green detection system (Applied Biosystems). Gene-specific primers were chosen using Primer Express software (Applied Biosystems) to span exons in order to avoid co-amplification of genomic DNA: tilapia IL-1β, sense primer 5′-AGGTCAAACTGGAGCGCAAA -3′, anti-sense primer 5′-GCCAGGTGGT GGAAG TGTGT-3′ and tilapia β-actin; sense primer 5′-CAGTGAAAAGATGACCCAGAT CAT-3′, anti-sense primer: 5′-ACCATCACCG-GAGT CCATGT-3′. Q-PCR was performed in duplicate in 20 μl reaction mixtures using the following protocol: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min, followed by a final stage of 95 °C for 15 s, 60 °C for 15 s; and 95 °C for 15 s. The linearity of the dissociation curve was analyzed using the ABI 7900 HT software and the mean cycle time of the linear part of the curve was designated cycle time (Ct). Each sample was analyzed in duplicate and normalized to β-actin using the following equation: $\Delta Ct_{GENE} = Ct_{GENE} - Ct_{\beta\text{-actin}}$. The fold change of IL-1β gene expression relative to β-actin was calculated using

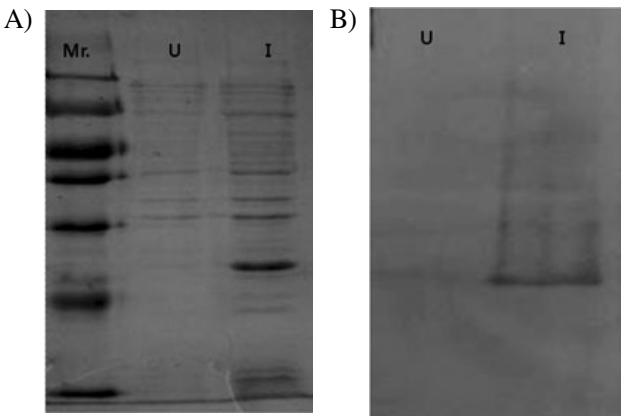

Fig. 1. Induction of recombinant protein was assessed by SDS-PAGE (A) and western blot (B) analysis. Transformed bacterial cells were induced (I) or uninduced (U) by 1mM IPTG and the cell lysates were subjected to SDS-PAGE and western blot analysis. Markers (Mr.) represent the proteins sized at 100, 70, 50, 40, 30, 20 and 15 kDa from the top. The predicted size of the recombinant IL-1β is 25kDa.

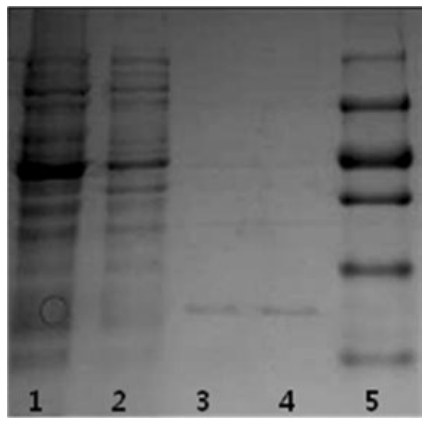

Fig. 2. SDS-PAGE analysis of purified recombinant tilapia IL-1β using Ni-NTA affinity chromatography. Lane 1: flow-through, lane 2: wash, lane 3-4: eluted rIL-1β, lane 5: markers (100, 70, 50, 40, 30 and 20 kDa).

the following equation: $\Delta\Delta Ct_{GENE} = \Delta Ct_{GENE}$ of the control - ΔCt_{GENE} of each sample. Values are mean fold change $\pm$ SD.

Results

Production of rIL-1β in *E. coli* cells

The recombinant tilapia IL-1β was produced in *E. coli* cells based on pQE vectors, SDS-PAGE analysis of the whole cell lysate from induced or uninduced cells showed the expression of recombinant protein only in induced cells (Fig.1A). Western blot analysis was also performed using HRP (horseradish peroxidase) conjugated Ni-NTA specifically binding to histidin and confirmed that the induced protein is tagged by histidin (Fig 1B). Once the expression of his-tagged recombinant protein was verified, Ni-NTA metal affinity chromatography was used to purify recombinant protein, SDS-PAGE analysis of purified recombinant protein exhibiting a single band of about 25kDa protein (Fig. 2). This is in close agreement with 25.4 kDa predicted by the cDNA sequence.

Bioactivity of recombinant IL-1β (rIL-1β)

It was shown that the positive control, poly I:C, significantly increased IL-1β gene expression in tilapia head kidney cells after 3 hours incubation. Cells incubated with rIL-1β at the various doses of 10, 2, 1 μg/ml showed a dose dependent increase in mRNA levels (Fig. 3). The cells treated with 10 μg/ml of rIL-1β showed a significantly higher IL-1β gene expression than 2 and 1 μg/ml treated cells while similar to the positive control. There was no significant difference between 2 and 1 μg/ml treated cells in the IL-1β gene expression level (Fig. 3). Indeed, the elution buffer showed no effect on IL-1β gene expression in tilapia head kidney cells since the expression level was similar to the negative control.

Discussions

IL-1β is a β-trefoil tertiary structured protein lacking a signal peptide. Biologically inactive precursor (31kDa) is cleaved to active mature peptide (17kDa) by ICE (IL-1 converting enzyme), a major protease released at inflammatory sites, that cleaves

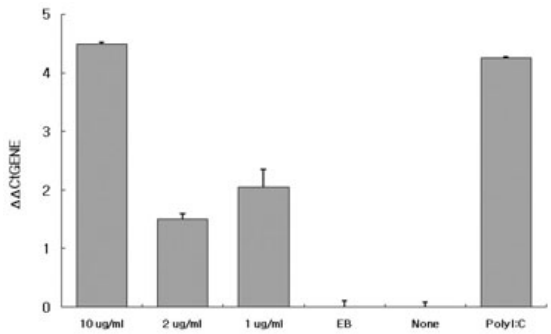

Fig. 3. Bioactivity of recombinant tilapia IL-1β on IL-1β gene expression in tilapia head kidney cells analysed by Q-PCR. Freshly prepared head kidney cells (10^5 cells/ml) were incubated with poly I:C (1μg/ml), different doses (10, 2, 1 μg/ml) of rIL-1β, elution buffer(EB) or without any stimulant (None) in cell culture dish in 5ml total volume at 22 °C for three hours. Three dishes of cell culture were used for each experimental condition.

after an aspartic acid (i.e. a caspase) (Jobling *et al*. 1988). In mammals, it has been known the basic biologic properties of IL-1β is the effects on the expression of various genes, especially associated with inflammation and immune response including IL-1β itself, by inducing the transcription or increasing the steady-state mRNA levels.

IL-1β was the first cytokine gene found in fish. Secombes *et al*. (1998) sequenced the cDNA complete coding region of rainbow trout IL-1β for the first time out with mammals. The first fish cytokine was produced as a recombinant protein in *E. coli* cells and its biological activities were assessed by analysing the induction of gene expression after stimulating rainbow trout cells with rIL-1β *in vitro* and *in vivo* (Hong *et al*., 2001, 2003).

In this study, the recently cloned tilapia IL-1β was produced as a recombinant protein to test its biological activity in immune system of tilapia. For this, tilapia rIL-1β was expressed in *E. coli*, purified using affinity column, and studied its bioactivity in tilapia head kidney cells *in vitro*. IL-1β gene induction was observed as indicator gene of a bioassay to test the bioactivities of rIL-1β since the autocrine effect of IL-1β is known in mammals and previous studies reported that IL-1β gene expression was induced by rIL-1β in rainbow trout (Dinarello, 1984, Hong *et al*., 2001 and 2003). Consequently, IL-1β gene induction were observed by increased mRNA level in primary head kidney cells after stimulating with polyI:C and rIL-1β. It was demonstrated that rIL-1β increased IL-1β gene expression in dose dependent manner. This is in agreement with other study demonstrated by Waner *et al*. (1987) that recombinant human IL-1β (1-100ng/ml) induced concentration dependent increases in IL-1β mRNA levels in human saphenous vein endothelial cells.

In conclusion, the present study demonstrated that the produced rIL-1β induced IL-1β gene expression as well as mammalian IL-1β known as an important cytokine in mammalian immune system and as a potential immune adjuvant.

Acknowledgement

This work was supported by academic research development grant of Gangneung-Wonju National University.

References

Alvi, S.A., Rajasingam, D., Brown, N.L., Elder, M.G., Bennett, P.R. and Sullivan, M.H.: The production of IL-1beta from human fetal membranes is not obligatory for increased prostaglandin output. Immunology, 97: 249-256, 1999.

Bird, S., Zou, J. and Secombes, C.J.: Evolution of IL-1β gene in vertebrates. EMBL accession No. AJ010640, 1998.

Blecha, F., Reddy, D.N., Chitko-Mckown, C.G., Mcvey, D.S., Chengappa, M.M., Goodband,

R.D. and Nelssen, J.L.: Influence of recombinant bovine interleukin-1β and interleukin-2 in pigs vaccinated and challenged with *Streptococcus suis*. Vet. Immunol. & Immunopathol., 44: 329-346, 1995.

Cannon, J.G., Clark, B.D., Wingfield. P., Schmeissner, U., Losberger, C., Dinarello, C.A. and Shaw, A.R.: Rabbit IL-1: Cloning, expression, biological properties, and transcription during endotoxemia. The journal of immunology, 142(7): 2299-2306, 1989.

Clem, L.W., Sizemore, R.C., Ellsaesser, C.F. and Miller, N.W.: Monocytes as accessory cells in fish immune responses. Developmental and comparative Immunology, 9: 803-809, 1985.

Diaz, A., Chepenik, K.P., Korn, J.H., Reginato, A.M. and Jimenez, S.A.: Differential regulation of cycloxygenases 1 and 2 by interluekin-1 beta, Tumor necrosis factor-alpha, and transforming growth factor-beta 1 in human lung fibroblasts. Experimental cell research, 241: 222-229, 1998.

Dinarello, C. .: Interleukin 1. Rev. Infect. Dis., 6:51-95, 1984.

Dinarello, C.A., Ikejima, T., Warner, S.J.C., Orencole, S.F., Lonneman, G., Cannon, J.G. and Libby, P.: Interleukin 1 induces interleukin1. I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro. J. immunology, 139: 1902, 1987.

Flodstrom, M., Chen, M.- C., Smismans, A., Schuit, F., Pipeleers, D.G. and Eizirik, D.L.: IL-1beta increases arginin accumulation and activates the citrulline-NO cycle in rat pancreatic β-cells. Cytokine, 11(6):400-407, 1999.

Fuhlbrigge, R.C., chaplin, D. D., Kielly, J.M. and Unanue, E.R.: Regulation of IL-1 gene expression by adherence and LPS. J. Immunol., 138:3799-3802, 1987.

Fujiki, K., Nakao, M., Shin, D. and Yano, T.: cDNA cloning of carp homologue of mammalian IL-1β. EMBL accession No. AB010701, 1998.

Haskill, S., Johnson, C., Eierman, D., Becker, S. and Warren, K.: Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. J. Immunol., 140:1690-1694, 1988.

Holland, J.: The generation of eicosanoids in rainbow trout with particular reference to gills and leukocytes. Ph. D thesis, University of Wales, 1997.

Jobling, S.A., Auron, P.E., Gurka, G., Webb, A.C., Mc donald, B., Rosenwasser, L.J. and Gehrke, L.: Biological activity and receptor binding of human prointerleukin-1beta and subpeptids. J. Biol. Chem., 31:16372-16378, 1988.

Komaki, G., Arimura, A. and Koves, K.: Effects of intravenous injection of IL-1β on PGE2 levels in several brain areas as determined by microdialysis. Am. J. Physiol., 262:E246-E251, 1992.

Laing, K.J., Grabowski, P.S., Belosevic, M., Secombes, C.J.: A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. Immunol. Cell Biol., 74(4):374-379, 1996.

Lee, D.-S., Hong, S., Lee, H.-J., Jun, L.-J., Chung, J.-K., Kim, K.-H. and Jeong, H.-D.: Molecular cDNA cloning and analysis of the organization and expression of the IL-1β gene in the nile tilapia, *Oreochromis niliticus*. Com. Bio. Phy. partA, 143:307-314, 2006.

Schindler, R., Clark, B.D. and Dinarello, C.A.: Dissociation between IL-1β mRNA and protein synthesis in human peripheral blood mononuclear cells. J. Biol. Chem.

265:10232-10234, 1990.

Secombes, C., Zou, J., Daniels, G., Cunningham, C., Koussounadis, A. and Kemp, G.: Rainbow trout cytokine and cytokine receptor genes. Immunological reviews, 166: 333-340, 1998.

Sigel, M.M., Hamby, B.A. and Huggins, E.M.Jr.: Phylogenetic studies on lymphokines. Fish lymphocytes respond to human IL-1 and epithelial cells produce an IL-1-like factor. Verterinary immunology and immuno pathology, 12:47-58, 1986.

Smith, K.A., Lachman, L.B., Oppenheim, J.J. and Favata, M.M.: The functional relationship of the interleukins. J. Exp. Med. 151:1551-1556, 1980.

Verburg Van Kemenade, B.M.L., Weyts, F.A.A., Debets, R. and Flik, G.: Carp macrophages and neutrophil granulocytes secrete an IL-1-like factor. Developmental and comparative Immunology, 19:59-70, 1995.

Warner, S.J.C., Auger, K.R. and Libby, P.: Interleukin 1 induces interleukin-1. II. Recombinant human interleukin 1 induces interleukin 1 production by adult human vascular endothelial cells. The journal of immunology, 139(6):1911-1917, 1987.

Weyts, F.A.A., Rombout, J.H.W.M., Flik, G. and Verburg Van Kemenade, B.M.L.: A common carp leucocyte cell line shares morphologic and functional characteristic with macrophages. Fish and shell fish immunology, 7:123-133, 1997.

Zou, J., Grabowski, P.S., Cunningham, C. and Secombes, C.J.: Molecular cloning of interleukin-1beta from Rainbow trout reveals no evidence of an ice cut site. Cytokine, 11(8): 552-560, 1999.

Zou, J., Cunningham, C. and Secombes, C.J.: The Rainbow trout *Oncorhynchus mykiss* interleukin-1β gene has a different organization to mammals and undergoes incomplete splicing. Eur. J. Biochem., 259(3):901-908, 1999.

Manuscript Received : May 27, 2009
Revised : July 24, 2009
Accepted : August 2, 2009