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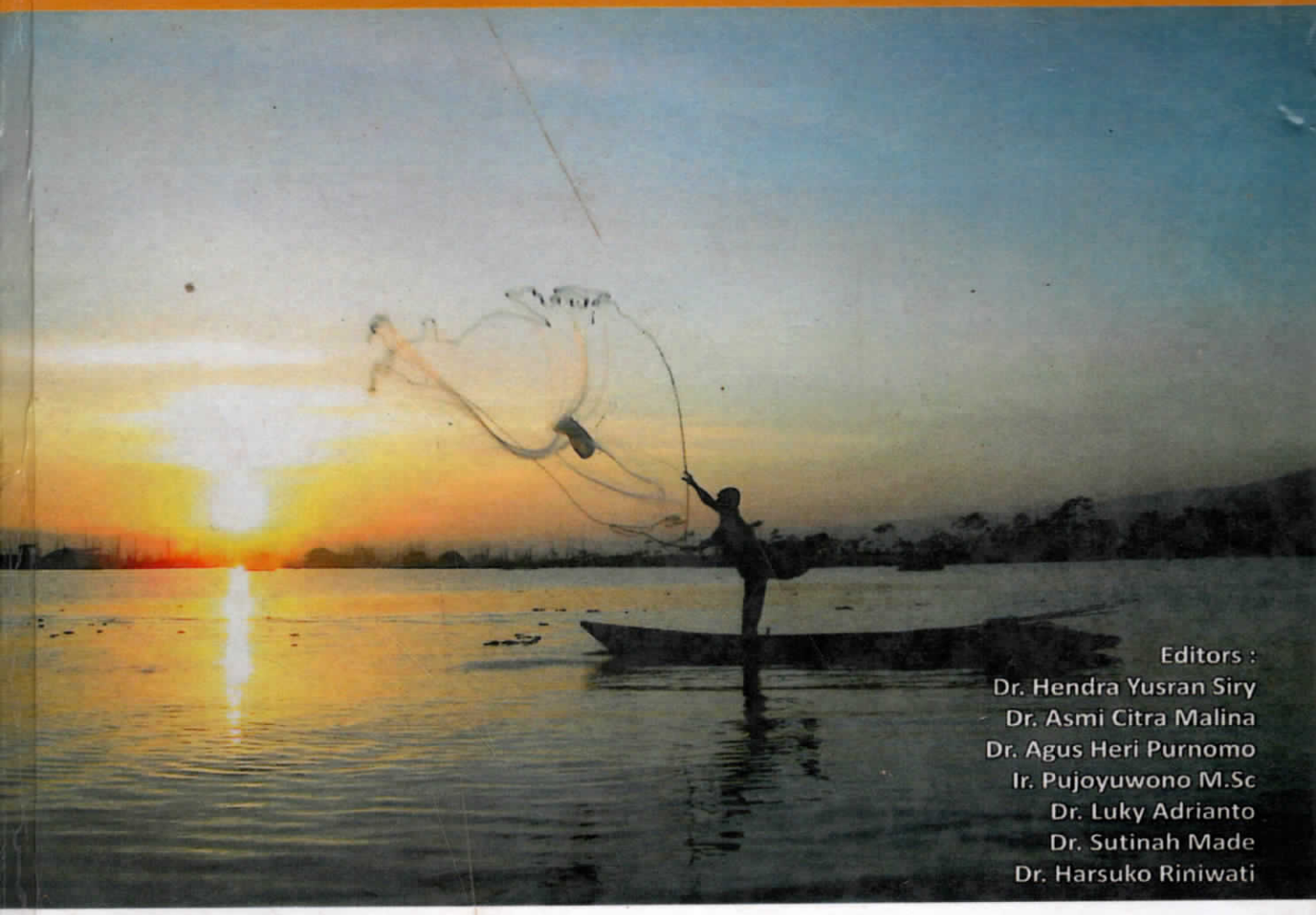

Editors :
Dr. Hendra Yusran Siry
Dr. Asmi Citra Malina
Dr. Agus Heri Purnomo
Ir. Pujoyuwono M.Sc
Dr. Luky Adrianto
Dr. Sutinah Made
Dr. Harsuko Riniwati

ENHANCING INDONESIAN FISH PRODUCTION AND COMPETITIVENESS IN INTERNATIONAL MARKET

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Dr. Harsuko Riniwati

Copy-editor :

Nurhendra

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Research Center for Marine and Fisheries Socio Economics
Jl. K.S Tubun - Petmburan VI Jakarta Pusat
Phone : (021) 536 50162 - 53650475 - Fax : (021) 536 50159
E-mail : pt.sosek@gmail.com
web : www.bbrse.kkp.go.id

Indonesian Marine and Fisheries Socio-Economcs Research Network
c/o Jl. K.S Tubun - Petmburan VI Jakarta Pusat
Phone : (021) 536 50162 - 53650475 - Fax : (021) 536 50159
E-mail : pt.sosek@gmail.com
web : http://imfisern.wordpress.com/

Faculty of Fisheries and Marine Science, Hasanuddin University
JL. Perintis Kemerdekaan KM. 10, Kampus Tamalanrea, Makasar 90245. Indonesia
Web : www.fikp.unhas.ac.id

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ACTIVATION OF CYTOKINE GENES IN COMMON CARP (*Cyprianus carpio* L.) MACROPHAGES STIMULATED BY CpG DNA

Asmi Citra Malina[1] and Masahiro Sakai [2]

[1]Faculty of Marine Science and Fisheries, Hasanuddin University, Makasar

[2]Faculty of Agriculture, University of Miyazaki, Japan

ABSTRACT

Synthetic oligodeoxynucleotides with CpG motifs (CpG-ODNs) have strong innate immune responses. CpG-ODNs promote the production of T-helper 1(TH1) and pro-inflammatory cytokines. However, analysis of the effects of CpG-ODNs on the expression of cytokine genes in fish is inadequate for fish. This study presents analysis of in-vitro addition of CpG-ODNs induced the expression of pro-inflammatory cytokines in common carp head kidney leucocytes. Pro-inflammatory cytokines interleukin (IL)-1b, CXC and CC-chemokines and tumor necrosis factor (TNF)-α were expressed at an early time point. This result suggests a robust immune stimulation can be achieved by CpGs and has a potential as an immunostimulant in aquaculture.

Keywords : CpG, DNA, Fish, cytokine genes, common carp kidney, tumor necrosis factor

INTRODUCTION

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides within the context of certain flanking bases (CpG motifs) have been shown to induce potent innate immune responses. Direct and indirect CpG ODN-induced gene expression and cytokine secretion occurs in a variety of cell types. In fish, CpG-ODNs were shown to induce the production of interferon-like cytokines (ILC) in Atlantic salmon (Jørgensen et al., 2001a; 2003) and rainbow trout (Jørgensen et al., 2001b). To date, there have been very few reports about the in vitro effects of CpG-ODNs on the expression of cytokine genes in fish. The aim of the present study was to determine if CpG ODNs could affect in vitro on the expression of cytokine genes such as IL-1β, CXC and CC-chemokines and TNF-α in the head kidney of common carp (*Cyprinus carpio* L.).

MATERIALS AND METHODS

A total of 200 common carp (mean weight = 100g) was obtained from Sunaso Fisheries farm in Miyazaki, Japan. The oligodeoxynucleotides were purchased from SAWADY (Japan), with the following sequences: B = GCTAGACGTTAACGTT, C = ATCGACTCTCGAACGTTC TC. The oligodeoxynucleotides (ODNs) were solubilized in sterile water at a concentration of 1 μg/μl, and stored at –20 °C. The head kidney phagocytic cells of the carps were isolated according to the modified method described by Braun-Nesje et al. (1982). HK cells of 24 fish were stimulated by treatment with 10 ng/ml ODNs for 0.5, 1, 2, 4, 12, and 24 h in RPMI 1640 (Nissui, Japan) medium supplemented with 5 % carp serum and 1 % Streptomycin/Penicillin (S/P, Gibco, USA). The HK cells stimulated for different time points were pooled together and stored in ISOGEN (Nippon Gene, Japan) at – 80 °C for further RNA isolation. Total RNA was isolated from head kidney of carp using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. cDNA synthesis was performed using ReverTra Dash (Toyobo, Japan). The cDNA was then used for PCR. PCR products were electrophoresed on a 1.5 % agarose gel to detect the specific bands.

Semi-quantitative analysis was carried out according to the method described by Kono et al. (2004). In order to obtain an optimum semi-quantitative approach to analyse carp cytokine genes expression, both carp cytokine genes and β-actin genes were amplified using a series of cycle numbers (20-33). By reducing the cycle number from 33 to 20, it was possible to select a cycle number that just gave a clear product, which in the case of cDNA of carp were 26 cycles for β-actin and 33 cycles of

carp cytokine genes. After determining the optimal cycle number, specific PCR was conducted three times. The relative levels of RNA were quantified for each gene by densitometry, which was performed by measuring the photostimulated luminescence values using Science Lab99 Image Gauge software (Fujifilm, Japan). Ratios of cytokine genes/β-actin product were subsequently calculated for each gene of interest and used to assess the differences in expression levels between control and CpG-ODNs treated group.

The data was expressed as mean ± S.D. The data was analysed using Student's *t*-test.

RESULTS AND DISCUSSION

The expression of IL-1β in the head kidney leucocytes of carp incubated with 10 ng of ODNs is shown in Fig. 1. In comparison with the control leucocytes, the cells incubated with CpG-ODN B showed a significantly enhanced IL-1β expression at 0.5 and 12 h post-stimulation ($*P < 0.05$). At 2 and 24 h post-stimulation, IL-1β expression in the head kidney of carp was significantly enhanced by CpG-ODN C ($*P < 0.05$). The expression of CXC-chemokine gene in head kidney of carp incubated with CpG-ODN C demonstrated a significantly higher level of expression ($*P < 0.05$) than those of controls at 2 h after stimulation (Fig. 2). The CXC-chemokine gene expression in the head kidney cells incubated with CpG-ODN B did not show a significantly higher level of expression than those of controls. The head kidney cells isolated from fish incubated with CpG-ODNs B & C were also found to express CC-chemokine. It was shown that CC-chemokine ($*P < 0.05$) [Fig. 3] demonstrated a similar pattern of expression as that seen for IL-1β. However, CC-chemokine expression was lower in the cells isolated from fish incubated with CpG-ODN B at 12 h post-treatment. The expression of TNF-□ genes were significantly increased in the head kidney of carp incubated with CpG-ODN B at 2 and 4 h and CpG-ODN C at 2 h post-stimulation ($*P < 0.05$) [Fig. 4].

In the present study the in vitro effects of CpG-ODNs on the expression of cytokine genes in the carp head kidney cells were investigated. The results showed that CpG-ODNs B and C significantly increased the inflammatory cytokines IL-1β, CXC and CC-chemokines and TNF-α. CpG-ODNs challenge at various time intervals indicated that those genes are induced at an early time point and the message is strong in the early period of induction.

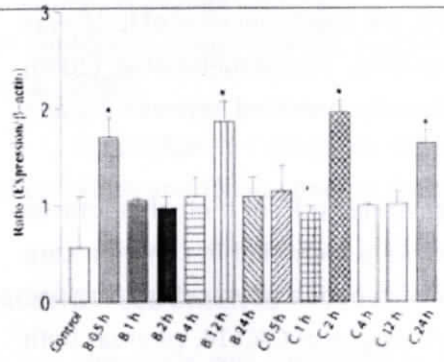

Fig. 1. Densitometric quantification of IL-1β expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12, and 24 hrs). Values are mean ± SD in 4 fish. *$P < 0.05$

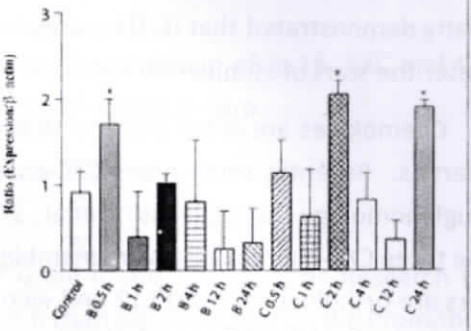

Fig. 3. Densitometric quantification of CC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish. *$P < 0.05$

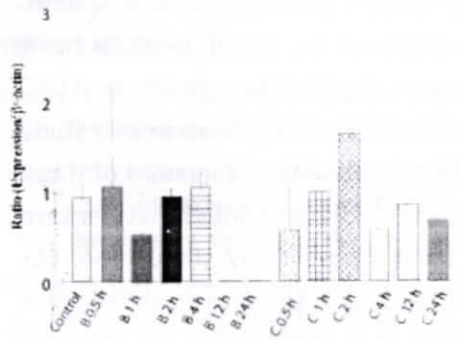

Fig. 2. Densitometric quantification of CXC-chemokine expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish. *$P < 0.05$

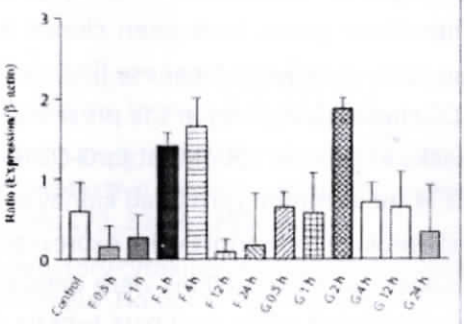

Fig. 4. Densitometric quantification of TNF-α expression relative to the β-actin transcript in head kidney leucocytes isolated from control fish incubated with PBS and CpG-ODNs treated fish (0.5, 1, 2, 4, 12 and 24 hrs). Values are mean ± SD in 4 fish. *$P < 0.05$

IL-1 is one of the pivotal early response pro-inflammatory cytokines that enables organisms to respond to infectious non-self challenges and induces a cascade of effects leading to inflammation. Many of these effects are mediated indirectly through up- or down regulation of other cytokine (Dinarello, 1997). A cytokine analogous to IL-1β was cloned in several teleost fish (Zou et al., 1999; Fujiki et al., 2000). Previous in vivo study (Tassakka and Sakai, 2004) has shown that CpG-ODNs B and C activate the expression of IL-1β gene in the head kidney of carp. In vitro study demonstrated that CpG-ODN 1668 activates trout macrophages to express IL-1β and IFN-like cytokines, while the non-CpGs tested were ineffective (Jørgensen et al., 2001b). Furthermore, IL-1β was induced with similar kinetics by LPS and CpG-ODN, but the CpG response was lower. Hirono and Aoki (2003) noted that the expression of IL-1β in Japanese flounder was apparent after 1 h stimulation with Con A and PMA, but no expression was found

after that. On the other hand, in the presence of LPS, the maximum level of IL-1β was expressed at 3 h as compared to 1 h, 6 h after stimulation. Pleguezuelos et al. (2000) similarly demonstrated that IL-1β expression significantly increased between 2.5 and 4 h after the start of stimulation with LPS.

Chemokines are small proteins that derive their name from their chemotactic properties. Recently, three novel CXC-chemokines were identified in common carp through homology cloning (Huising et al., 2004). Phylogenetic analyses show that one of the three CXC-chemokines is an unambiguous orthologue of CXCL14, whereas both others are orthologues of CXCL12 and were named CXCL12a and CXCL12b. All three novel carp CXC-chemokines are expressed during early development, in contrast to established immune CXC-chemokines. These chemokines must play key roles in the patterning and maintenance of the (developing) vertebrate central nervous system. CC-chemokine genes have been cloned in carp (Fujiki et al., 1999), rainbow trout (Dixon et al., 1998) and Japanese flounder (Kono et al., 2003). The activations of CXC and CC-chemokine genes in the present study confirm the results from in vivo study (Tassakka and Sakai, 2004) that CpG-ODNs B and C increased the expression of these genes in the common carp head kidney cells. Savan et al. (2003) noted that the carp CXC-chemokine transcripts were expressed after 1 h of LPS and Con A stimulation. CC-chemokine gene expressions were detected in blood and in the muscle of Japanese flounder injected by plasmid DNA (pCMV-CCC) [Kono et al., 2003].

Similar to IL-1β and chemokines, TNF-α is an important component of early inflammatory events. TNF-α is a 17-kDa protein that is synthesized by different cells types upon stimulation with endotoxin, inflammatory mediators, or cytokines such as interleukin-1 and, in autocrine manner, upon stimulation with TNF itself (Hirono and Aoki, 2003). Multiple isoforms of TNF-α have been cloned in common carp; TNF-1α, TNF-2α (Saeij et al., 2003) and TNF-3α (Savan and Sakai, 2004). Activity of tumor necrosis like factor has also been reported in fish. Recently, there is a report showing enhanced leucocytes migration and phagocytic activity of rainbow trout macrophages when incubated with recombinant trout TNF proteins (rTNF) [Zou et al., 2003]. Pro-inflammatory expression also increased significantly by the rTNF protein. In this study, the expression of TNF-α genes were significantly increased in the head kidney of carp incubated with CpG-ODN B at 2 and 4 h and CpG-ODN C at 2 h post-stimulation. The present study is the first to report the stimulation of CpG-ODNs on the expression of TNF-α genes in fish. In mice, pretreatment with CpG-ODN enhanced the production of TNF- α and type-1 cytokines, including IL-12, IFN-γ and the IFN-γ-dependent ELR- CXC chemokines IFN-γ-inducible protein-10 and monokine induced by IFN- γ in response to

Klebsiella challenge (Deng et al., 2004). It has been shown that TNF- α can be induced in rainbow trout and carp macrophages by stimulation with LPS (Zou et al., 2002; Saeij et al., 2003).

In conclusion, CpG-ODNs stimulate an early expression of IL-1β, CXC and CC-chemokines and TNF-α genes in the head kidney of common carp.

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REFERENCES

Braun-Nesje, R., Kaplan, G., Sejelid G., 1982. Rainbow trout macrophages *in vitro*: morphology and phagocytic activity. Dev. Comp. Immunol. 6, 281-291.

Deng, J.C., Moore, T.A., Newstead, M.W., Zeng, X., Krieg, A.M., Standiford, T.J., 2004. CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary Klebsiella infection. J. Immunol. 173, 5148-5155.

Dinarello, C.A., 1997. Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. J. Biol. Regul. Homeost. Agents. 11, 91-103.

Dixon, B., Shum, B., Adams, E.J., Magor, K.E., Hedrick, R.P., Muir, D.G., Parham, P., 1998. CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunol Revs. 166, 341-348.

Fujiki, K., Shin, D-H., Nakao, M., Yano, T., 1999. Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression substractive hybridization. Immunogenetics 49, 909-914.

Fujiki, K., Nakao, M., Shin, D., Yano, T., 2000. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A. Fish Shellfish Immunol. 10, 229-242

Hirono, I., Aoki, T., 2003. Immune-related genes of the Japanese flounder, *Paralichthys olivaceus*. Aquatic Genomics. Springer-Verlag Tokyo, 286-300.

Huising, M.O., van der Meulen, T., Flik, G., Verburg-van Kemenade, B.M., 2004. Three novel carp CXC chemokines are expressed early in ontogeny and at nonimmune sites. Eur. J. Biochem. 271, 4094-4106.

Jørgensen, J.B., Johansen, A., Stenersen, B., Sommer, A.I., 2001a. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. Dev. Comp. Immunol. 25, 313-321.

Jørgensen, J.B., Zou, J., Johansen, A., Secombes, C.J., 2001b. Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1β and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. 11, 673-682

Jørgensen, J.B., Johansen, L-H., Steiro, K., Johansen, A., 2003. CpG DNA induces protective antiviral immune responses in Atlantic salmon (*Salmo salar* L.). J. Virol. 77, 11471-11479.

Kono, T., Kusuda, R., Kawahara, E., Sakai, M., 2003. The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder *Paralichthys olivaceus*. Vaccine 21, 446-457.

Kono, T., Ponpornpisit, A., Sakai, M., 2004. The analysis of expressed genes in head kidney of common carp *Cyprinus carpio* L. stimulated with peptidoglycan. Aquaculture 235, 37-52.

Pleguezuelos, O., Zou, J., Cunningham, C., Secombes, C.J., 2000. Cloning, sequencing, and analysis of expression of a second IL-1β gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics. 51, 1002-1011.

Saeij, J.P., Stet, R.J., de Vries, B.J., van Muiswinkel, W.B., Wiegertjes, G.F., 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev. Comp. Immunol. 27, 29-41.

Savan, R., Kono, T., Aman, A., Sakai, M., 2003. Isolation and characterization of a novel CXC chemokine in common carp (*Cyprinus carpio* L.). Mol. Immunol. 39, 829-834.

Savan, R., Sakai, M., 2004. Presence of multiple isoforms of TNF alpha in carp (*Cyprinus carpio* L.): genomic and expression analysis. Fish Shellfish Immunol. 17, 87-94.

Tassakka, A.C.M.A.R., Sakai, M., 2004. Expression of immune-related genes in the common carp (*Cyprinus carpio* L.) after stimulation by CpG oligodeoxynucleotides. Aquaculture 242, 1-12.

Zou, J., Grabowski, P.S., Cunningham, C., Secombes, C. J., 1999. Molecular cloning of interleukin-1β from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ICE cut site. Cytokine 11, 552-560.

Zou, J., Wang, T., Hirono, I., Aoki, T., Inagawa, H., Honda, T, et al, 2002. Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 28, 229- 237.

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C., Ellis, A.E., Secombes, C.J., 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 27, 813-822.

Indonesia fisheries development contains challenges to accelerate the efforts to the trends and emerging requirements that concerns on quality and environmental sustainability as well as certifying fair trade standards and social and labor related standards. Apart of reuired several improvements, it needs several alternative and creative ways to improve competitiveness of Indonesian fisheries products.

This book is a complication of research paper presented at the International Seminar on Indonesian Fisheries Development: Enhancing Fish Production and Competitiveness in International Market. It addresses major themes related to fisheries production and competitiveness. It examines meticulously Indonesian fisheries development empirically in the context to enhance its fish production and competitiveness in international market. This book intends to serves as a reference for those who wish to research further on respective topics, besides acting as wake-up call on the importance of finding ways for Indonesian fisheries in competitive market. Contents of this book are relevant to the current initiative of the Ministry of Marine Affairs and Fisheries

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