The Involvement of Pigment-Protein Fraction from Microalga Nannochloropsis oculata in Expression of Heat Shock Protein 70 with Nervous Necrosis Viral Infection on Grouper

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Abstract
The aim of this study is to investigate the expression of heat shock protein 70 (HSP70) on grouper infected by Nervous Necrosis Viral (NNV) with the involvement of pigment-protein fraction (PPF) from Nannochloropsis oculata (N. oculata). Fish treatments were divided into four groups, which are control, mixture with PPF from N. oculata, induction with NNV, and combination with PPF in NNV. After treatments with sonde method for 23 days for dosing up to 376 µL, fish were sacrificed, and their brain was harvested for protein analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fish blood was collected to examine the expression of HSP70 by haemagglutination and western blot analysis. The results showed that the expression of HSP70 was observed after treatment with PPF in NNV infected brain tissue and blood cell proved by SDS-PAGE and Western blot analysis. Thus, PPF is responsible for the expression of HSP70 in infected tissues, indicating that PPF appears to have a function as an inhibitor of proliferation of NNV.

INTRODUCTION
Heat shock proteins (HSP) represent as dominant microbial antigens during infection. For example, HSP70 plays a prominent role in the protection and defense of interference or factors of triggers disturbance. These proteins can be produced during a wide range of cellular stress, such as nutritional deficiencies, oxidative stress, increased temperature, ultraviolet irradiation, and exposure to chemicals, viral infection, bacterial infection, and necrosis [1,2].

HSP have found in common carp (Cyprinus carpio L.) [3], and also tilapia (Oreochromis niloticus) [4]. In normal cells, HSP is produced to regulate metabolism and to maintain cellular homeostasis. Under stress conditions, the function of HSP70 is to assist folding of newly synthesized polypeptide chains in its role as molecular chaperones, besides act as a mediator for repairing and degrading the proteins that undergo changes, damage or denaturation [5].

HSP is synthesized in all of the cells disturbed by the presence of environmental stress (hyperthermia, ultraviolet radiation, nutritional deficiencies, chemical treatments, a viral infection, ischemia, etc.), causing disturbance of structure and function of proteins in metabolic processes. The cells that influenced by environmental stress and viral infection will decrease in the transport process, the synthesis of DNA, RNA, and protein. Many HSP have the function to refold damaged proteins, unfold aggregated proteins, and to protect thermally damaged proteins, or target them for efficient degradation. The alteration in physiological such as cell wall restructuring, the synthesis of compatible solutes, and also contribute to cellular survival [6].

HSP is molecular chaperones resulted from the Heat Shock Response (HSR) that prevent the formation of aggregates of nonspecific protein and assist proteins in the acquisition of their native structures [7]. HSR is a response based on genetic function to induce genes encoding molecular chaperones, proteases and other proteins that are important in the mechanism of defense and recovery of cells to the induction of antigen or external stressor. This relates to cellular functions which is associated with the occurrence of misfolding of proteins cells. HSR serves to provide cell responses against a wide variety of physiological disorders or genetic basis. In the event of severe stress because virus infection will cause damage and the cell will

Keywords: PPF, grouper, HSP70, Protein, N. oculata, Inflammatory
die. While the advent of stress that is sublethal organism will trigger a cellular response in the form of HSR. The increased expression of HSP in a stressed cell is mediated primarily by heat shock transcription factors. HSF1 has a key role in the transcriptional regulation of HSP expression [6].

*Nannocloropsis oculata* (N.oculata) is a type of single-celled algae (unicellular) which has a very simple morphology and flat round shape, a small-sized spherical cell with a diameter of 4-5 μm. This microalgae contains rich proteins, pigments, and polysaturated fatty acids, and it is commonly used in aquaculture as feed for marine habitats and shrimp rotifer [8]. These algae have a unique composition in the form of fatty acids which are then consumed by rotifers, and then rotifers consumed by the larvae of humpback grouper (*C.altivelis*). In N.oculata, there is a macromolecule protein called pyrenoid [9] and pigment-protein fraction (PPF). This PPF has function as an anti-inflammatory for virus infection [10]. However, there is no report on the utilization of PPF from such microalgae as an inhibitor in tissues infected by NNV. In this study, the protection of grouper against NNV infection mediated by PPF from *N.oculata* was observed by investigating the expression of HSP70 on grouper infected by NNV with the involvement of PPF from *N.oculata*.

### EXPERIMENTAL

#### General

The experiment was designed using *C.altivelis* with the length of 10-15 cm obtained from Balai Perikanan Budidaya Air Payau (BBPAP) Situbondo, East Java, Indonesia. The fish are divided into control and treatment groups. Each group is consisting of 6 animal groupers. The samples group were splitted into four groups which are (A) Control group, (B) fish induced by PPF from *N.oculata*, (C) fish induced by NNV, and (D) fish induced by PPF from *N.oculata* and NNV.

Centrifugation was performed on Heraeus Pico 17 from Thermo Scientific. Protein contents were measured using a Nanodrop spectrophotometer (Nanodrop Technology, Wilmington, US) at a wavelength of 280 nm where one absorbance is equivalent to 1 μg/mL of protein. Molecular weight marker of the protein was performed using low range PRO-STAIN Intron Biotechnology, Sangdaewon-Dong. Protein analysis was carried out in a similar manner to the previously described by Laemmli as reported in 1970 using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% concentration in the buffer sample with 1:1 ratio [11]. Electrophoresis was performed by passing an electric voltage to 100V, and 400 mA for 100 min. Moreover, the visualization of brain tissue was performed using a scanning electron microscope (SEM) on FEI with a model of Inpect-S50 at 10 kV. The specimen was coated with a gold layer using sputter coater on SCT-620 Emitech. The optical microscope was also used for visualization of brain tissue using Olympus CX 330 with the magnification of 400x.

#### Isolation of PPF from *N. oculata*.

Isolation of PPF from *N. oculata* was done according to our previous report in 2015 [10]. Harvested *N. oculata* with wet weight of 50 g was homogenized for 1 h using a sterile mortar. After adding liquid nitrogen, the cell continues homogenized for 30 to 60 mins. Eight mL of 20 mM of KCl (pH 7.5) from Sigma-Aldrich and 50 mM of glycine (Merck) were added to the homogenated mixture. Furthermore, it was centrifuged at 17000 xg, 4 °C for 60 mins. The supernatant was collected and taken in the sterile eppendorf. Saturated ammonium sulfate solution (100% sat., Sigma-Aldrich) was appended incrementally until a final concentration to 30% sat. This solution was centrifuged by 15000 xg for 30 mins at 4 °C and recovered the supernatant. Before used, the dialysis tube was sterilized for 10 mins by boiling it in tris-ethylenediaminetetraacetate (TRIS-EDTA, Sigma-Aldrich) solution with a concentration of 0.1 mM and pH 7.3. The sample was dialyzed against 2 L of Tris–HCl solution (Sigma-Aldrich) with a concentration of 20 mM (pH 8.0) at 4 °C for 24 hrs while it was stirred. After the dialysis process, the sample solution was filtered using a disposable filter (0.22 μm, Sartorius). After the second dialysis, protein contents of the solution was determined using a spectrophotometer.

#### Induction of PPF from *N. oculata* in Groupers

Before treatment, fish was acclimated in the batch (aquarium) for 7 days with controlled environment parameter. Induction of PPF was applied by the sonde method (oral methods) through a hose feeding tube for six times. The sonde process was conducted on the first (1st), fifth (5th), ninth (9th), fourteenth (14th), nineteenth (19th) and twenty-three (23rd) days with a dose of 33.3, 307, 321, 331, 336, 346 and 376 μL PPF, respectively. The sonde process was applied for 6 times as a booster to increase the immune system in fish with different days. The measurements were conducted on the last day to obtain the highest protein titer response.

#### Protein monitoring and haemagglutination test

Monitoring protein was conducted using method as reported by Laemmli (1970) [11]. Protein separated using Electrophoresis SDS-PAGE with protein molecular weight marker was used. Electrophoresis was performed using 12.5% and 4% polyacrylamide gel (Merck) by passing an electric voltage to 100 V, and 400 mA for 100 mins using a coomassie brilliant blue staining (Merck).

Haemagglutination test was conducted based on Hanne and Finkelstein (1982) [12]. The blood was taken from the control fish using syringes 1 ml (GX26½", Terumo). The isolated blood was washed twice using PBS solution, homogenized and centrifuged by 3500 xg for 10 mins. Obtained Erythrocytes was diluted with PBS (1:200) for applying in the Haemagglutination test. The Haemagglutination test was conducted using microplate V (96 well). Negative control wells was filled by PBS only. Positive control wells was diluted using PBS (1:200) for applying in the Haemagglutination test. The Haemagglutination reaction was observed after 20 mins. The positive reaction was identified by the presence of dot in the bottom of the wells (erythrocyte sedimentation).

#### Immunohistochemistry

Immunohistochemistry method was referred to avidin-biotin-complex (ABC) kit VECTASTAIN® (ABC-Elite, Vector Lab.) Protocols [13]. ABC kit was used to detect biotin using diaminobenzidine tetrachloride (chromogen). Tissue samples were dehydrated using alcohol and cleaned using xylene. The enzyme of endogenous peroxidase was incubated with 3% H₂O₂ in absolute methanol at ambient temperature for 5 mins. The microwave antigen was retrieved with 0.01 mol/L sodium citrate buffer (pH 6.0) and then was incubated in antibody monoclonal HSP70 (Santa Cruz) for 30 mins with dilution of 1:2,000. It was continued by washing for 5 mins in buffer. The secondary antibody biotin conjugated anti-IgG was added for 30 mins at...
ambient temperature and it was then washed for 5 mins in buffer. Slides were incubated with ABC reagent for 30 mins and then washed 5 mins in buffer. It was incubated in peroxidase substrate solution until desired stain intensity was achieved. It was rinsed in tap water.

**Western blot analysis**

Detection of HSP70 was conducted by western blotting technique, according to Towbin et al. (1979) [14]. Protein gel was placed on nitrocellulose (NC) paper in the semi-dry blotter (bidan) with 300 mA for 30 mins. The samples were stained using a ponceau 2% containing 3% trichloracetic acid (Merck). After that, it was rinsed with dH2O and blocked by TBE (Tris/Borate/EDTA, contains 3% albumin (Merck) at pH 7.4 added with 1% BSA (bovine serum albumin) (Merck). It was shaken for 2 hs for removing the dye. The samples were rinsed twice with an interval at 5 mins using TBE pH 7.4 solvent containing 0.05% Tween 20. Secondary antibody anti-HSP IgG mice was added with 1/1.000 concentration to TBE pH 7.4 and BSA 1%. The samples were shaken for 2 hrs and rinsed with TBE pH 7.4 solvent containing 0.05% Tween 20. Tablet Cip diluted in 10 mL H2O was used as staining material. The solution then was poured into NC paper and observed for the appearance of red color.

**RESULTS AND DISCUSSION**

Protein profiles of the brain of the grouper (*C. altivelis*) can be seen in Figures 1 and 2. The observation refers to the target of HSP70 kDa. Since the other bands are another protein response, it is not the target of this study, therefore it is not characterized. Through observations on the protein of grouper, the HSP molecule was expressed in all treatments. It is known as the HSP70 protein having 70 kDa in molecular weight.

![Figure 1. Protein profile of the brain of grouper (*C. altivelis*) for (A) marker, (B) fish control, (C) fish induced by PPF, and (D) fish induced by PPF and NNV, and (E) fish induced by NNV. Arrows indicate HSP70 (70 kDa).](image)

HSP is classified into groups based on phylogeny and structure or molecular mass in kilodalton (kDa) e.g. HSP70 family with a molecular weight of 70 kDa (classification useful for clinical laboratory analysis). HSP70 family is the major protein that is expressed as a response to the environmental stress [5]. Through observation using SDS-PAGE on tissue protein of Humpback grouper that had been treated with PPF, HSP70 molecules were expressed in all of the treatments. Expression of HSP70 seems obvious or less, depending on the response in each treatment as shown in Figure 1. From Figure 1, it is shown that the injection of PPF from *N. oculata* into the grouper produced an adhesion protein HSP70. Moreover, the results of the immunochemical test through the Western Blot showed the same ribbon band with molecular weight of 70 kDa. HSP70 adhesion protein identification results in the brain receptor showed that the molecule weight of brain of infected fish has protein of 70.2 kDa (Figure 1E and Figure 2A) whereas HSP70 adhesion protein is 70 kDa (Figure 2B).

Reaction test to the adhesion protein of PPF from *N. oculata* with brain receptors of *C. altivelis* infected with NNV was performed using Western blot methods as shown in Figure 2. The serology test through hemagglutination methods was carried out to determine the highest titer of erythrocytes of *C. altivelis* on PPF from *N. oculata* in the brain organ of groupers infected by NNV. Haemagglutination test is aimed to get the haemagglutinin protein because, in general, haemagglutinin protein is also an adhesion protein. Results of haemagglutination test of PPF from *N. oculata* against NNV of the erythrocytes of *C. altivelis* are shown in Table 1.

![Figure 2. Adhesion protein HSP70 of (A) Protein of brain fish induced by PPF and NNV; and (B) Western blot of HSP70.](image)

**Table 1. Results of haemagglutination test for adhesion protein molecule from *N. oculata***

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<tr>
<th>Sample group</th>
<th>Control</th>
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The visualization of brain tissue in the treatment of Table 1 with SEM and optic microscope images are shown in Figures 3 and 4, respectively. The treatment of PPF provided a visualization of the tissue surface that is better than those infected with NNV. This relates to the HSP70 functions as a protein that maintains the stressors to an antigen NNV.

The result of observations on the control treatment (group A) showed that red blood cell (RBC) precipitate formed RBC dot on the basis of well until the 1/4 dilution. This indicates a positive reaction caused by the bonding between the receptor of RBC and adhesin molecules contained in the fish brain organ. The sensitivity and magnitude of antibody titers to the control treatment is equal to a quarter due to the RBC agglutination reaction by adhesin molecule.
crude adhesin protein molecule that is identical to the haemagglutinin protein had cross-react with erythrocytes of *C. altivelis*. This suggests that the epitopes of the adhesin protein PPF bind the surface receptors on the *C. altivelis*.

The potency of PPF as an antiviral substance in fish showed after third treatment in which healthy fishes were induced by both crude PPF from *N. oculata* and NNV. This treatment resulted in the immune response HSP70 on brain tissue of fish. The analysis of the fish behavior in this treatment is not different from the fish that are subjected to crude treatment PPF from *N. oculata*. In the process of induction NNV, the fish began to show stress response. Fish swim passive and tend to be clustered. Fish colors look darker in the long term.

Observations using SDS-PAGE on the target organs of fish treated NNV infection showed that brain tissue expressed HSP70 molecules with molecul weight 70 kDa with a thicker band profile in greater numbers than other treatments. In this mechanism, NNV has a role as a foreign antigen causing the infection so that it will trigger the cell activation and immune cell differentiation. Effect of PPF induction was expected to induce the appearance of a primary immune response so that the fish is more health compared with other treatments. It has to effect on the quality of appetite. It can be confirmed by histological brain visualization as shown in Figures 3 and 4.

The advent of molecule HSP70 expression was due to the treatment PPF from *N. oculata* in the target tissues of *C. altivelis*. This function as a response to inflammatory effect which functions physiologically to inhibit the NNV attack. In the intracellular conditions, HSP can act as an anti-inflammatory which able to reduce proinflammatory cytokines through the immune effector cells, increases cytokine tolerance to cells and tissues, and debilitate the changes in the permeability of epithelium as a barrier. The release of HSP on the extra-cellular environment will evoke an immune response and was a “danger signal” to the immune system because HSP is costimulation of extracellular immune recognition [15]. The release of HSP to the extracellular environment is an interesting study because there is no evidence that the expression can be stimulated actively and passively. Various kinds of cells such as monocytes [16], tumor [17], glia [18], B cells and T cells [19] will actively release HSP70 into the environment of extracellular. However, the extra-cellular HSP70 can also be detected in the pathological and non-pathological state associated with the condition of the immune system to alter cellular functions.

These results provide an explanation that PPF from *N. oculata* has potential as an antiviral from biological material as an effort to control viral disease in the fisheries resources. Considering the diversity of pathogens in fish culture media, immunostimulant is an alternative an infectious disease control effort that should be done. Utilization of water resources in management activities can optimize fish production by increasing the body's resistance to infectious diseases, especially NNV.

**CONCLUSION**

Based on the results, treatment of PPF from *N. oculata* into the infected fish brain organ is able to agglutinate RBC of *C. altivelis* with the dilution 1/8. PPF was able to express the HSP70 molecule in the brain organ *C. altivelis* with NNV infection. The cells molecule of HSP70 capable to be expressed by the influence of PPF from *N. oculata* treatment so that the molecule is able to function as a proliferation inhibitor of NNV that be seen in the response to HSP70 and haemagglutinin...
results. HSP70 has anti-inflammatory activity, indicating that PPF of *N. oculata* appears to be able to use as an antiviral to control NNV diseases in *C.alivellis* culture.

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**REFERENCES**


**Abstrak**

Tujuan dari penelitian ini adalah untuk mengetahui ekspresi *heat shock protein* 70 (HSP70) pada ikan kerapu yang terinfeksi oleh Nervous Necrosis Virus (NNV) dengan keterlibatan pigment-protein fraction (PPF) dari Nannochloropsis oculata (*N. oculata*). Perlakuan ikan dibagi menjadi empat kelompok, yaitu kontrol, campuran dengan PPF dari *N. oculata*, induksi dengan NNV, dan kombinasi PFP dalam NNV. Setelah perlakuan selama 23 hari dengan metode sonde untuk dosis sampai dengan 376 µL, ikan dimakan dan otaknya diambil untuk analisis protein menggunakan sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Darah ikan diambil untuk diperiksa ekspresi HSP70 dengan analisis haemagglutinasi dan *western blot*. Hasil penelitian menunjukkan bahwa ekspresi HSP70 dioberservasi setelah perlakuan dengan PPF pada jaringan otak yang terinfeksi NNV dan sel darah yang terbukti dengan analisis SDS-PAGE dan *western blot*. Jadi, PPF bertanggungjawab untuk ekspresi HSP70 di jaringan yang terinfeksi, menunjukkan bahwa PPF memiliki fungsi sebagai penghambat proliferasi NNV.

**Kata kunci:** PFP, ikan kerapu, HSP70, Protein, *N. oculata*, Inflammatory