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## The Profile of Gill Protein Expression of Humpback Grouper (*Cromileptes Altivelis*) Injected with Per-Cp of *Halimeda Opuntia* and Viral Nervous Necrosis

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### ABSTRACT

The objectives of the research are to obtain the protein Per-CP Halimeda opuntia and to understand the cellular expression of the gill of the Humpback grouper (*Cromileptes altivelis*). The isolate of protein Per-CP H. opuntia is stored at -800C. Humpback grouper is assigned into three groups, which are normal grouper, grouper injected with protein Per-CP H. opuntia, and grouper injected with Per-CP H. opuntia and challenge-tested with VNN virus. The gill is taken out, charged with liquid Nitrogen and stored at -800C. The Result of research show that injection of Per-CP of H.opuntia into humpback grouper was able to increase protein expression of gill of Humpback grouper at molecule weight of 56 kDa and 5 kDa. By using western blot methods, it was clear that protein Per-CP of H.opuntia act as antiviral VNN expressed through protein 5 kDa of the gill organ

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## INTRODUCTION

Humpback grouper fish is a marine cultivated fish commodity that has developed widely in Indonesia. Last decades are witnessing the infection of marine cultivated fishes by virus. Therefore, early prevention against the possibility of a VNN epidemic at each cultivation location or at the float net cage is highly needed to prevent the virus from causing mass death. However, until now, effective medicine is not yet found and the fish with VNN symptom only waits for further extermination to prevent the epidemic. Comprehensive learning is, therefore, required.

*Viral Nervous Necrosis* (VNN) is a cause of deteriorated marine fish production in several fish cultivation locations in the worldwide, especially those in larva phase and juvenile. It also leads to death mass (Muroga, 1995, Nakai *et al.*, 1995). This pathogen has spread fast in some countries or even in almost all worldwide continents because this disease is quite harmful and able to spread quickly (Chi, 2005). The spreading of this pathogen includes Japan, Korea, China, Southeast Asia countries, Northern Australia, Austria, Iran, Israel, Greece, French, Norway, Canada and America. VNN disease can lead to death mass of the cultivated fish in relatively short term (Chi, 2006). It is also reported that fish species mostly attacked by VNN disease are humpback grouper, *Epinephelus spp.* in the Southeast and Japan regions; Barramundi (Asian seabass), *Lates calcarifer* (Bloch), in the Southeast, India and Australia regions; *Sea Bass, Dicentrarchus labrax* (L.), in the Northern Australia; *Striped jack, Pseudocaranx dentex*, in Japan; "Red drum", *Sciaenops ocellatus*, in Korea; and "Golden Grey Mullet", *Liza auratus*, in Iran. The infection of VNN virus against the Humpback grouper is induced by the deterioration of the immune system due to the deprivation of antibody production and phagocytosis activity.

In the cultivation of the Humpback grouper requires the information of marine natural resource which can be applied to the cultivation industry of Humpback grouper. The objectives of this research are to obtain the protein Per-CP *Halimeda opuntia* and to understand the cellular expression of the gill of the Humpback grouper in inhibit to proliferation of VNN virus.

## MATERIAL AND METHODS

The main objects of research are Humpback grouper fish and macroalga *Halimeda opuntia*. Additional materials include Tris HCL, SAS, aquades, PBS, NaCl, EDTA, clove oil, glycine and others. Humpback

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grouper fish is obtained from the Research Centre for Brackist Water Aquaculture, BBAP Situbondo, Indonesia. The method used in this research is an experiment.

#### **The Collection of Macroalga *Halimeda Opuntia*:**

Macroalga *Halimeda opuntia* is collected from the waters of the Kupang Bay, Nusa Tenggara Timur. The collected this macroalga is then stored at  $-80^{\circ}\text{C}$  as a sample. The collection of *Halimeda opuntia* is used on demand to produce the isolation of protein Per-CP.

#### **The Isolation of Protein Per-CP *Halimeda Opuntia*:**

*Halimeda opuntia*, after collecting from the waters of the Kupang Bay, is stored on  $-80^{\circ}\text{C}$ , and waited for preparation of other materials. The isolation method combines the method of both Weis *et al* (2000) and Yanuhar *et al.*, (2012). It is about 80 kg *Halimeda opuntia* charged with liquid Nitrogen and grinded by mortar to refine. Glycine  $\pm$  30 ml is then added and the solution is remixed. It is then moved into ependorf and centrifuged at  $4^{\circ}\text{C}$  in 17,000 rpm for 60 minutes. Supernatant is given with SAS and re-centrifuged at  $4^{\circ}\text{C}$  in 15,000 rpm for 30 minutes. Supernatant is dialyzed into Tris HCl solution at  $4^{\circ}\text{C}$  for 2x24 hours. It is then filtered with microphore and dialyzed into NaCl solution at  $4^{\circ}\text{C}$  overnight. The resultant is dialyzed (with protein Per-CP *Halimeda opuntia*) and stored at  $-80^{\circ}\text{C}$  as the stock.

#### **Treatment of Humpback Grouper (*Cromileptes altivelis*) using Protein Per-CP of *Halimeda Opuntia*:**

Humpback grouper is acclimated in the streaming water system batch for more or less 7 days at BBAP Situbondo. Three treatment groups are made. Treatment I is normal Humpback grouper (Control group). Treatment II is the Humpback grouper injected with protein Per-CP *Halimeda opuntia*. Treatment III is the Humpback grouper injected with protein Per-CP *Halimeda opuntia* and challenge-tested with VNN virus and During the rearing, Humpback grouper is fed by fresh puffy fish.

In treatment I (Normal Group), the fish are acclimated into the streaming water system batch. The Group is fed only with fresh puffy fish and observed for the stability (bright color, agile, seawater quality) for 3 or 4 days in rows. Each individual fish in the Treatment II, after acclimation stage, fish is injected with protein Per-CP. The intensity of injection is five times at five different periods to obtain the Humpback grouper that has an immune-ready. In the final period of injection of protein Per-CP, the fish is ready to harvest. In treatment III, the fish must pass an acclimation stage. Every individual fish is injected with protein Per-CP *Halimeda opuntia*. The intensity of injection is five times at five different periods to increase the immune system of the fish before the fish are challenged-tested with VNN virus. After the injection of protein Per-CP in final day, the fish is challenged-tested through oral system. Fresh food is then given. Next days, the virus (encapsulation in feed) is provided through oral system in alternate ways between virus and fresh food.

#### **Isolation of Fish Gill:**

After challenge-test is finished, the fish is made slept with the mixture of clove oil and sea water. The fish is dissected to take out gill organ. Gill organ is then charged with liquid Nitrogen and stored at  $-80^{\circ}\text{C}$  for the preparation and the observation of SDS-PAGE.

#### **Protein Characterization of Fish Gill Using Gel Electrophoresis SDS-Page:**

Electrophoresis SDS Page using Laemmli methods (Laemmli, 1970). The gills of all three groups are removed from storage at  $-80^{\circ}\text{C}$ , and then all are prepared for each treatment. The running of SDS-PAGE to obtain an electrophoresis protein gel is used Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis at a concentration of 12.5%. Electrophoresis protein gel is scanned to obtain an electrophoresis gel slide picture of each gill. RF is counted based on the comparison between the ribbon movement from early site and the distance of color movement from color site.

Molecular weight of protein isolate from Humpback grouper's kidney organ is determined using SDS-Page based on Laemmli's Method (1970). Protein sample is warmed at  $100^{\circ}\text{C}$  for 5 minutes in the supporter solution which contains of 5 mM Tris CHL at pH 6.8, 5% 2-mercapto ethanol, 2.5 % w/v sodium dodecyl sulfate, and 10 % v/v glycerol. Tracker dye of bromophenol blue is used with 12.5 % mini-slab gel and 4 % tracking gel. Color material is *coomassie blue* and standard molecule of *sigma low range marker*.

#### **Western blot Methods:**

When the response of Per-CP *H. opuntia* as antiviral is confirmed, it is then SDS-Page gel moved into nitrocellulose paper using Biorad's semi dry blotter device. The applied electrical current is 300 mA for 30 minutes and followed by dyeing with 2 % poncho which contains of 3 % TCA. The dyeing is aimed to ensure whether the sample protein has moved into nitrocellulose paper, and if it is confirmed, a specific sign is given to determine the molecular weight. Nitrocellulose paper is cut based on the well path. Rinsing with  $\text{dH}_2\text{O}$  is made to eliminate poncho dye. Nitrocellulose paper is blocked with TBE at pH 7.4 with the supplement of 3 %

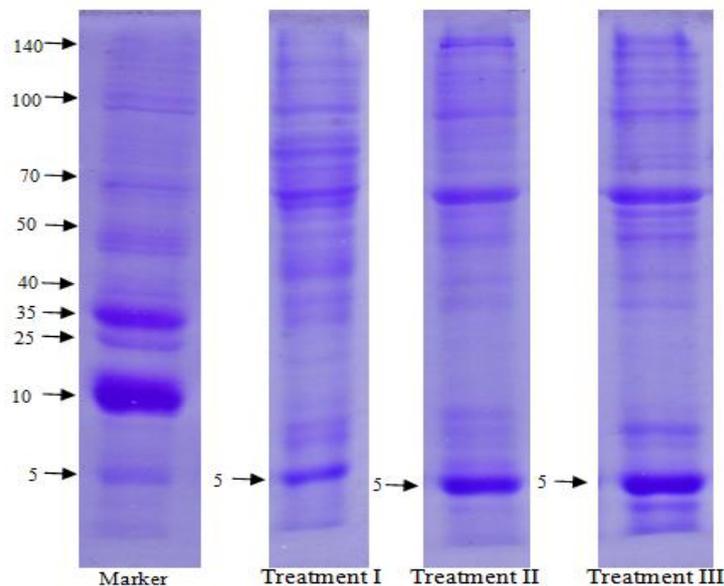
albumin. The addition of 1 % BSA is given before paper is shaken for 2 hours. Twice washing is then conducted with interval of 5<sup>1</sup> using TBE solution at pH 7.4 with the supplement of 0.05 % Tween 20. Secondary antibody (IgG-anti mouse) labeled as AP is added at concentration 1/1000 into the solution comprising to TBE at pH 7.4 and 1 % BSA. Direct light is avoided. Nitrocellulose paper is shaken for 2 hours and washed for twice for 5 minutes using the solution containing of TBE at pH 7.4 and Tween 20 ± 0.05 %. Color material involves tablet β Cip mixed into 10 ml H<sub>2</sub>O. It is then poured onto nitrocellulose paper and observed for red color. Nitrocellulose paper is then rinsed with H<sub>2</sub>O and aerated (Towbin, 1979).

#### The Analysis of Data:

Data analysis is conducted through description which is by comparing between normal gill and gill after treatment II and treatment III.

### RESULT AND DISCUSSION

Result of SDS-PAGE protein profile of the gill of the Humpback grouper (*Cromileptes altivelis*) in normal condition (normal gill/treatment I), that of injected with protein Per-CP *Halimeda opuntia* (treatment II), and that of injected with protein Per-CP *Halimeda opuntia* and challenge-tested with the VNN Virus (treatment III), is shown in Figure 1.



**Fig. 1:** The Profile of Gill Protein of Humpback Grouper (*C.altivelis*).

As shown in Figure 1, protein ribbon bands produced by normal gill tissue cells of Humpback grouper (Treatment I) are counted to 27 and these proteins are ordered from the biggest molecular weight, such as: 111.4 kDa, 103.2 kDa, 92.6 kDa, 86.7 kDa, 81.7 kDa, 74.1 kDa, 66.5 kDa, 61.6 kDa, 59.4 kDa, 54.1 kDa, 50.9 kDa, 47.3 kDa, 44.5 kDa, 41.9 kDa, 38.2 kDa, 35.1 kDa, 31.5 kDa, 26.0 kDa, 23.5 kDa, 19.4 kDa, 17.5 kDa, 15.0 kDa, 13.6 kDa, 12.5 kDa, 10.5 kDa, 5.3 kDa, 4.2 kDa and 4.0 kDa.

Treatment II produce the protein ribbon bands up to 27 band protein. In ordered manner, these bands are arranged from the biggest molecular weight, which are: 93.5 kDa, 87.4 kDa, 80.4 kDa, 73.7 kDa, 71.9 kDa, 68.9 kDa, 64.3 kDa, 57.6 kDa, 51.5 kDa, 44.2 kDa, 39.6 kDa, 34.0 kDa, 26.1 kDa, 22.3 kDa, 20.9 kDa, 17.9 kDa, 12.8 kDa, 10.9 kDa, 8.2 kDa, 5.2 kDa, 4.2 kDa, 4.0 kDa, 2.7 kDa, 2.2 kDa, 1.9 kDa, 1.7 kDa and 1.4 kDa.

Treatment III produce the protein ribbon bands up to 25 band protein. These are arranged by ordering them from the biggest molecular weight, which are 108.4 kDa, 97.3 kDa, 91.2 kDa, 84.5 kDa, 77.9 kDa, 71.1 kDa, 64.5 kDa, 60.4 kDa, 57.6 kDa, 52.8 kDa, 46.6 kDa, 42.5 kDa, 40.5 kDa, 38.2 kDa, 35.8 kDa, 30.6 kDa, 26.3 kDa, 20.9 kDa, 15.4 kDa, 13.5kDa, 12.5 kDa, 11.0 kDa, 5.2 kDa, 4.2 kDa and 4.0 kDa.

Data of cellular expression in Figure 1 indicate that cellular expression is different across three treatment groups if compared to marker protein. The dominant cellular expression in protein marker is found between 15 kDa and 35 kDa. Dominant cellular expression resulted by treatment I (controlled gill) are observed from 5 kDa to 10 kDa and also from 40 kDa to 80 kDa. Dominant cellular expression resulted by treatment II occurs from 5 kDa to 15 kDa and from 40 kDa to 80 kDa. Dominant cellular expression of treatment III are from 4 kDa to 15 kDa and 45 kDa to 140 kDa.

Cellular expression profile of controlled gill (treatment I) is similar to that of protein after treatment II. Cellular expression profiles obtained from treatment I and treatment II are different if compared to cellular expression of treatment III. Treatment III express two proteins which distinguish it from result of other treatment which are 4.2 kDa and 4 kDa.

All of treatment was result an expression protein at 56 kDa (P56), but in treatment III, the P56 expression was more stronger than treatment II and the P56 expression in treatment II was more stronger than treatment I. It indicate that Per-CP from *H. opuntia* in fish body was able to generate immune system in grouper. The contribution of this treatment is immediately perceived because it does not give harmful effect to Humpback grouper infected by VNN Virus and even it has 100 % survival rate. This result is supported by previous research which states that peridinine has an ability to mediate the path causing dead cell receptor (Sugawara *et al*, 2007). Peridinine has some functions such as anti-inflammatory activity, anti-tumor activity, immune response modulation and immunoregulatory (Park *et al*, 2010).

This different expression has reflected the readiness of the immune system of the Humpback grouper (*C. altivelis*) as shown in gill organ of normal and treatment Humpback groupers (Figure 1). Before treatment with protein Per-CP *H. opuntia* at molecular weight of 5 kDa as antiviral, Humpback grouper indicates weak cellular expression. A similar trend is also observed at molecular weight 50 kDa. Humpback grouper treated with protein Per-CP *H. opuntia* shows strong cellular expression at molecular weight 5 kDa or 50 kDa, meaning that Humpback grouper with Per-CP *H. opuntia* treatment experiences the establishment and improvement of the immune system. Humpback grouper with immune readiness, if treated with VNN Virus, shows that protein Per-CP *H. opuntia* in the fish cells will activate the proliferation of antiviral function to produce antiviral mechanism.

Data of cellular expression in Figure 1 show that the protein of gill organ of Humpback grouper has different protein expression character across each treatment. The gill of normal Humpback grouper has different expression from that of Humpback grouper injected with protein Per-CP *H. opuntia* and also differs in expression from Humpback grouper injected with Per-CP *H. opuntia* and challenge-test with VNN Virus. Gill organ of treatment III has the protein ribbon with small molecular weight. It has the strongest cellular expression at molecular weight of 5 kDa and followed by 51.5 kDa. Gill organ of treatment III has protein ribbon with small molecular weight and has the strongest cellular expression at molecular weight of 5 kDa followed by protein ribbon with molecular weight by 52.8kDa. Normal gill organ (Humpback grouper without injection of protein Per-CP *H. opuntia*) in protein ribbon with small molecular weight has the strongest cellular expression of 5.5kDa followed by protein ribbon with molecular weight by 50.9 kDa.

The strongest cellular expression of the gill organ obtained from treatment II is found in protein ribbon with small molecular weight. But, in general, the strong cellular expression in this treatment is dominated by protein ribbon with big molecular weight (Figure 1). The reason may be related to the relationship between the complex amino acid compounds in the big molecular weighted protein ribbon and the concentration of certain amino acid in the small molecular weighted protein ribbon.

The strongest cellular expression of VNN Halimeda gill organ (Humpback grouper injected with protein Per-CP *H. opuntia* and challenge-tested with VNN Virus) is observed in the ribbon with small molecular weight. Two final protein ribbons have smallest molecular weight, but they have strong cellular expression. Strong cellular expression in the VNN Halimeda gill treatment is dominated in average by a protein ribbon with big molecular weight (Figure 1). It is caused by the relationship between the complex amino acid compounds in the big molecular weighted protein ribbon and the concentration of certain amino acid in the small molecule weighted protein ribbon.

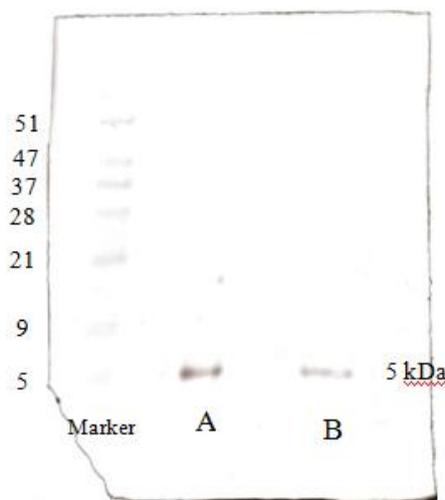
The strongest cellular expression of normal gill organ (treatment I) is shown in protein ribbon with small molecular weight. In general, strong cellular expression of normal Humpback grouper is dominated, in average, by a protein ribbon with big molecular weight (Figure 1). It may correspond to the relationship between the complex amino acid compounds in the big molecular weighted protein ribbon and the concentration of certain amino acid in the small molecule weighted protein ribbon.

The response of cellular protein from Humpback grouper gill with treatment can be understood further through Western blot analysis (Towbin, 1979). The result of western blot analysis was shown in Figure 2.

Data of cellular response of Humpback grouper (*C. altivelis*) in Figure 2 has shown that protein Per-CP *H. opuntia* at molecular weight of 5 kDa can produce antiviral. It is validated by the fact that controlled gill (Treatment I), gill in treatment II and treatment III can provide the strongest cellular expression at molecular weight of 5 kDa. It means that at molecular weight of 5 kDa, all gills can give expression/response to the activity of VNN Virus. Indeed, protein Per-CP *H. opuntia* activates the proliferation of antiviral function in the gill organ of Humpback grouper at molecular weight of 5 kDa. It is shown that Per-CP *Halimeda opuntia* will induce the activity of protein 5 kDa to work on the function of anti-virus. The result was strengthened by Yoshida *et al* (2007) who state that Halocynthiaxanthine and Peridinine regulate the gene of DR5 (death receptor 5). It has function for blocking the receptor so the virus could not attach on the cell. This mechanism

can prevent the fish from virus attack. It prove from this research result that no death fish occurred after treated by Per-CP of *H.opuntia* while the fish injected by VNN virus.

Data of VNN antiviral response in Figure 2 have described that Per-CP *H. opuntia* at weight 5 kDa is reacting as a very strong antiviral if the fish is exposed to RNA VNN Virus. Indeed, the presence of RNA VNN Virus delivers a signal to induce the activity of Per-CP to prevent the proliferation of virus. In this condition, Per-CP has functioned as the immune to cope with the virus such that the strength is weakened. Previous research has reported that peridinine works to prevent the strength of lipid oxidation reaction but cannot show number of antioxidants if there is ROS outside liposome which is anti-lipoperoxidation in nature (Barros *et al*, 2001).



**Fig. 2:** Cellular Responses of the Gill of Humpback Grouper (*C.altivelis*) as Antivirus Consequence Produced by Per-CP *H.opuntia* at 5 kDa.

Per-CP *H. opuntia* provides a very strong reaction against VNN Virus presence, the expressed form of antiviral response is similar to that of both Spot A and B (Figure 2). It is related with the molecular mechanism between the activity of immune cell chained by peridinine and VNN virus. The result, however, depends on virus infection rate and the ability of NFkB cell molecule to induce T-cell differentiation (especially, T-cell in low proliferation capacity to produce IFN-gamma, IL-10, and TGF- $\beta$ ). Therefore, there is a possibility that NFkB cell molecule plays an important role in the treatment of immune system reactivity (Gusman, 2010). Previous research has reported that peridinine inhibits the strength of lipid oxidation reaction and cannot show number of antioxidants if there is ROS outside liposome which is anti-lipoperoxidation in nature (Barros *et al*, 2001).

### Conclusion:

Based on the data of the gill protein profile of Humpback grouper, it is concluded that: injection of Per-CP of *H.opuntia* into humpback grouper was able to increase protein expression of gill of Humpback grouper at molecule weigh of 56 kDa and 5 kDa. By using western blot methods, it was clear that protein Per-CP of *H.opuntia* act as antiviral VNN expressed through protein 5 kDa of gill organ.

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