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Immunostimulatory activity of brown seaweed-derived fucoidans at different molecular weights and purity levels towards white spot syndrome virus (WSSV) in shrimp *Litopenaeus vannamei*

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ABSTRACT

Fucoidan used as immunostimulant is commonly in the crude form. In this study, we investigated the effect of fucoidan in both crude and purified forms in their immunostimulatory activity. In addition, we studied the effect of low- and high-molecular weight fucoidan as hydrolysis products toward immunostimulatory activity. Four kinds of fucoidan were assayed for immunostimulant activity on the shrimp *Litopenaeus vannamei.* The parameters observed in the assay includes the mortality number, haemocyte, gene-related immunity (phenoloxidase, superoxide dismutase and transglutaminase) in the shrimps infected with viral WSSV. The assay results showed that pure fucoidan exhibited higher activity compared with that of crude fucoidan. Sulfate and carbohydrate content of HMW fucoidan are 7.8 % and 82.54 % with an estimated molecular weight of 8.28 $x10^4$ Dalton, and low molecular weight (LMW) fucoidan has 1.2% and 65.23% with an estimated molecular weight of 7.53 $x10^4$ Dalton. The transcriptional level of the immunity-related genes was found higher after feeding the infected shrimps with purified and HMW fucoidan. In particular, all of fucoidan forms increased the phenoloxidase gene transcription, suggesting that fucoidan have significant role in the production of phenoloxidase.

INTRODUCTION

White spot syndrome virus (WSSV) is one of the virulent pathogens and causes big economic losses to shrimp aquaculture (Flegel, 2011). Efforts to prevent outbreaks of WSSV in the cultivation method, include the bio-secured operating system, controlled environmental conditions and vaccination, the use of antimicrobial peptides, immunostimulants and RNA interference (RNAi) technology (Valerie *et al*., 2003). In general, most crustaceans have no immune system memory and only rely on innate immunity to protect themself against pathogen infection and other external factors that constantly threatens their lives (Sarathi *et al*., 2007). Research in the immune response of shrimp has flourished because of its importance in aquaculture and the increasing number of pathogens that arise are found, which may affect an organism's defense system (Bachere *et al*., 2000). To fight these pathogens, the shrimp using a variety of specific immune mechanisms, including haemocytes mobilization (Johansson and Soderhall, 1989); prophenoloxidase cascade formation (Sritunyalucksana and Soderhall, 2000). The immune system of shrimp is intimately related to its blood (hemolymph), because it contains haemocytes involved in cell's defense mechanisms such as phagocytosis, lysis of foreign units and encapsulation (Soderhall and Smith, 1983; Soderhall and Cerenius, 1992; Fuhua and Jianhai, 2013; (Dechamag *et al.,* 2006). Prophenoloxidase (proPO) system is an important defense mechanism in crustaceans. This system involves a complex molecular cascade triggered by components of the surface of immunostimulants. The zymogen proPO is converted to the active form, phenoloxidase (PO).

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After activation, PO subsequently initiates the molecular cascade and finally the formation of dark and insoluble melanin pigment. The parameters observed after fucoidan treatment on shrimp include: haemocyte (morphology, activation, cell size) and superoxide generation through proPO activation by haemocytes (Chen *et al*., 2014). Superoxide anions are the first products released during the respiratory burst (RB), and their derivatives have a bactericidal activity (Munoz *et al*., 2000). The superoxide anion is scavenged by superoxide dismutase (SOD) to form oxygen and hydrogen peroxide that is subsequently scavenged by peroxidase and catalase in the presence of a reducing agent (Fridovich *et al*., 1995; Holmblad and Soderhall, 1999).

The use of immunostimulants is considered as a more environmentally friendly approach and has been proven to enhance the innate immunity of shrimps and their resistance against pathogens (Sirirustanaun *et al*., 2011; Takahashi *et al*., 1998; Chotigeat *et al*., 2004; Pholdaeng and Sunanta, 2010). A number of immunostimulants that have been used in aquaculture are from the class of polysaccharides derived from bacteria and fungi such as -glucan, lipopolysaccharide (LPS), peptidoglycan, *Cynodon dactylon* and herbal immunostimulant. In addition to that, there are also immunostimulants derived from seaweeds such as alginate, carrageenan, laminarin and fucoidan (Grassian *et al*., 2012; Balasubramanian *et al.,* 2008; Suwaree *et al*., 2013; Doner and Whistler, 1973). Generally immunostimulatory substances that have been reported so far were less pure or in the form of crude extracts (Chin *et al*., 2013).

Fucoidan has no direct effect on virion, but it plays a role in the defense system through cellular and the humolar mechanisms to fight the virus. Mechanism of antiviral activity of fucoidan is to inhibit the absorption of viruses and the formation of virus-induced syncytium (Mandal *et al*., 2007). Fucoidan oral feeding may has protective effects through direct inhibition of viral replication and stimulation of the innate immune defense functions (Hayashi *et al*., 2008). The biologic activities of fucoidans are closely related to their molecular structures, which include fucose linkage, the sugar type, sulfate content, and molecular weight. Among these, molecular weight is one of the most important factors determining the biological activities of polysaccharides. The fucoidan with lower molecular weight showed higher resistance to cell transformation activity than high molecular weight (Jong and Hyun, 2013). Fucoidan purity and molecular weight fucoidan influence bioactivity in its function as an immunostimulant. This study was aimed at determining whether the molecular weight and purity of fucoidan are able to affect its immunostimulatory bioactivity in shrimps after the challenge test with WSSV infection.

MATERIALS AND METHODS

Materials

The raw material used in this work was obtained from a brown seaweed *Sargassum binderi Sonder* collected from Binuangeun-Banten at the position S 06°50 727 E 105°53.337. Chemical reagents used include $CaCl_2$ (Merck), ethanol, $CHCl_2$, methanol, HCl, and H_2SO_4. Fucoidan standard from species *Fucus vesiculosus* was obtained from Sigma Aldrich. Healthy shrimp (6-8 g) were obtained from Situbondo Hatchery, East Java, Indonesia.

Fucoidan extraction

The fresh brown seaweed was selected and cleaned from other undesired substances. Briefly, fresh seaweeds were macerated in $MeOH:CHCl_3:H_2O$ (4:2:1) for 12 hours to remove fat and pigments washed with aceton and air dried (Duarte *et al*, 2001). The fucoidan from defatted algal powder was extracted by soaking 0.01M HCl of pH 4 at the ratio of 1: 10 (w/v), stirred for 6 hours and siefted through a 350 mesh nylon sieve, and added with 4 M $CaCl_2$, incubated for 30 minutes, and re-filtered using 500 mesh. Filtrate was diluted using distillated water until the final $CaCl_2$ concentration of 2 M, centrifuged at 10.000 rpm for 15 minutes, and then added with 3 M $CaCl_2$ and then centrifuged at a speed of 10.000 rpm for 15 minutes. All polysaccharides were dialyzed (cut off 10.000 Da) during 48 h (24 hours using 0.5 M NaCl followed by deionized water for another 24 hours). The sample was precipitated by addition of ethanol (1:2) then left overnight. The yield was calculated from the weights of crude fucoidan divided by defatted algal dry weight. The extraction was run in triplicate, and the extraction yield was expressed as mean ± standard deviation (SD). Each extract was combined and then analyzed.

Purification of fucoidan

Purification of fucoidan was conducted using DEAE Sephadex A-25. and eluted by gradient NaCl step wise elution (Duarte *et al*., 2001). Briefly, crude polysaccharides dissolved in 0.1 M buffer Tris-Cl pH 7.0 were loaded to a column of DEAE Sephadex A-25 (Sigma Aldrich) fast flow (4 cm × 25 cm), followed by step-wise elution using 50 mL of sodium chloride solutions (0.5- 2.5 M) at the flow rate of 1 mL/minute. Eluents (5 mL/tube) were separately collected and ethanol (HPLC grade) was added with ratio 1:2. All fractions were filtered, freeze-dried and stored at 4 °C until used.

The carbohydrate content in each eluent was determined using a phenol–sulfuric acid method (Dubois et al., 1956) using fucose (Sigma Aldrich) as the standard. Finally, all the fractions containing polysaccharides were dialyzed with deionized water and lyophilized for further study.

Depolymerization of fucoidan by mild acid hydrolysis (Stanislav *et al*., 2012)

Mild acid hydrolysis of crude fucoidan (CF) 1 g carried out using trifluoroacetic acid (1 N; 60 min; autoclave 121 °C, 5 mg/mL). The mixtures were neutralized with 5% NH_4OH solution in water and lyophilized. LMW fraction, CF supernatant, yield (62.4 ± 0.6) %, was obtained by fractionation in H_2O/EtOH 1:10, w/w; and precipitated as high molecule weight fucoidan (HMW) yield (37,3± 0,3) %.

Determination of chemical composition

Total sugar content of fucoidan was determined according to Dubois methods using phenol-H_2SO_4 reagent using L-fucose (Sigma) as the standard (Dubois *et al,* 1956). The sulfate content was quantified based on the $BaCl_2$-gelatin method using K_2SO_4 (Merck) as the standard and dehydrolyzed fucoidan as the positive control. Fucoidan sample was hydrolyzed (15 mg) in 3 M HCl for 17 h at 100 oC (Dodgson and Price, 1962).

Estimated molecular mass determination

Estimated molecular mass of fucoidan was determined by Gel Permeation Chromatography (GPC) using a Perkin Elmer Series 200 equipped with refractive index detector Series 200a on a Tosoh column (TSK-Gel G 5000 PWXL stainless steel column, 7.8 mm x 30.0 cm) using water as eluent at 65°C with a flow rate of 0.6 mL min-1. All samples were filtered through 0.22-mm filters before injection, in order to retain large aggregates (Rioux *et al.,* 2007). Average molecular weight of fucoidan was calculated by comparison of their retention times with the calibration curve using commercially available pullulans (Shodex P-82; Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers different standards (0.62, 1.0, 2.17, 4.88, 11.3, 20.0, 36.6, and 73.6 kDa).

Nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectroscopy analysis

FT-IR analysis

The qualitative analysis of the active principles of the fucoidan was done by Fourier Transmission Infra-Red (FT-IR; Shimadzu, Japan) method described by Kemp (1991). The frequency of the spectra set to analysis was between 4000 and 400 cm^{-1} wave number and the vibration spectrum was recorded as graphical.

NMR analysis

Fucoidan (40 mg) was dissolved in 0.7 ml deuterium oxide (D_2O), and ^1H and ^{13}C nuclear magnetic resonance spectra were acquired on a JEOL 400 MHz. resonance NMR spectrometer at 65 ^oC. ^1H and ^{13}C NMR chemical shifts were measured in ppm relative to internal reference D_2O at 4.7 ppm (Li *et al*., 2006).

Preparation of pellet feed supplemented with fucoidan

Four diets containing different concentrations of fucoidan were prepared by following the procedure described by Haryanti *et al*., 2014 (The basal diet contained 0.2% chitosan (without fucoidan) served as the control diet. The extracted fucoidan was added individually to the test diet at concentrations such as 0.3% with a corresponding decrease in the amount of cellulose. The crumble feed was dried by using hot air oven at 40 ^oC. After drying, the finished pellet feeds were stored in plastic containers at 4 ^oC until use. analysis of the basal diet was 40% crude protein, and 6.5% crude lipid, dietary fiber 2.2%, ash 13.0%.

Feeding experiment

Healthy shrimp (6-8 g) were obtained from Situbondo Hatchery, Indonesia kept in bio-filter laboratory tanks containing artificial seawater at 26 ^oC. After measuring the length and weight, uniform size of *L. vannamei* post larvae at the PL30 stage were selected from the acclimatized stock and transferred in to individual experimental tanks (0% control and 0.3% fucoidan supplemented pellet feeds, each containing 95 L of filtered seawater in 100 L capacity FRP tanks at ambient temperature (28 ^oC) and salinity (32 ppt). The shrimp post larvae were maintained at the stocking density of 1/5 L. Mild aeration was given continuously in order to maintain the optimal oxygen level. Feeding regime was applied to all tanks throughout the experiment, and the food amount was adjusted 3 times a day (7:00, 14:30 and 20:00 h) @ 30, 30, and 40% of pellet feed, respectively. Healthy shrimp (5-8 g) were divided into five groups (20 shrimp/ group) and each group assayed in duplicate (2 shrimp/assay). Group1 was composed of shrimp fed with untreated fucoidan followed by saline injection (normal control (CN)). For group 2-5 delivered varian type of fucoidan with concentration 300 mg/ kg weight shrimp respectively. Group 2 was composed of shrimp fed with LMW fucoidan. Group 3 was composed of shrimp fed with HMW fucoidan. Group 4 was composed of shrimp fed with extract fucoidan (CF). Group 5 was composed of shrimp fed with purified fucoidan (PF). Shrimp were fed triplicate daily with threated. The control group was fed with fucoidan un supplemented pellet feed. The unfed was collected after the respective hours of feeding and 50% water was exchanged daily during the experimental period. The feeding experiment was prolonged for 15 days. Simultaneously, duplicated were maintained in each group.

WSSV challenge experiment

After 15 days of feeding experiment, the immune response was done by a challenge test with WSSV. The WSSV infected *L. vannamei* shrimp (7.96.0 g ± 1.5 g) were used tested shrimp. Aquarium tank with size of 60x40x39.5 cm, approximately capacity of 95 L seawater were used in the presented trials. Each aquarium was stocked with 20 shrimps and in duplicated. Infected WSSV fresh shrimp were use as feed and fed to the shrimp once were use as feed and fed to the shrimp once time a day (10 g/ aquarium). The challenge test was done for 72 hours. The hemolymph was taken every 24 hours for observation of haemocyte and gene immunity expressions was done using RTq-PCR. The WSSV infection level was analyzed through PCR using WSSV (93 bp) primer.

The immunological parameters such as total mortality, Relative Percent Survival (RPS), total haemocyte count (THC), prophenoloxidase activity (PO), superoxide dismutase activity (SOD) and transglutaminase activity were analyzed in the haemolymph samples of shrimps before injection of WSSV (0 hours) and 24, 48, 72 hours of challenge experiment. During the challenge experiment 50% water was exchanged daily in order to remove uneaten food materials and shrimp waste materials in the experimental tanks.

Analysis of immunological parameters

Total haemocyte count

Hemolymph was collected from shrimp from ventral-sinus cavacity using 25-gauge needle and 1 mL syrenge filled with anticoagulant solution. Anticoagulant solution, modified with K-199 solution (Itami et al., 1994) became KC-199 with the addition of 2.38 g/L HEPES and 5% L-cystem. Total haemocyte cell was calculated based on cell number from hemolymph (0.1 mL) of challenged shrimp in anticoagulant KC-199 solution with amount of 0.4 mL, by using a hemocytometer under light microscope with 400x magnification.

Isolation of total RNA and cDNA synthesis

Hemolymph was taken using a 25-G needle with 1mL syringe containing 0.3 mL of cold anticoagulant solution (2% NaCl; 0.1M glucose; 30 mM Na-citrate; 26 mM citric acid; 10 Mm EDTA) then mixed and centrifuged at 12.000 rpm, 4 ^oC for 15 minutes. Hemocite pellets obtained were washed with cold anticoagulant solution. Total RNA extraction was done using lysis solution with modified methods.

Synthesis of complementary DNA (cDNA) was performend using Ready-To-Go-You-Prime First Strand Beads (GE Healthcare, USA). The concentration of total RNA was as much as 3 mg in 30 mL DEPC, homogenized using a low speed vortex. Total RNA in microtube were incubated at 65 ^oC for 10 minutes and subsequently transferred into new microtube and incubated on ice for 2 minutes. RNA solution was transferred into the first strand reaction mix beads tube. Primer race vect dT3 oligo was used to synthesize cDNA (concentration of 100 pico mole) and added as much as of 3 mL into reaction and left to stand for 1 minute. Microtube was incubated at 37 ^oC for 1 hour. cDNA solution was then added of 50 µL ddH_2O and stored at -20 ^oC for further analysis.

Real-time quantity polymerase chain reaction (RT-qPCR) analysis of the immune gene of *L. vannamei*

The analysis of transcriptional expression profiles of immune-related gene after challenge with WSSV were used RT-qPCR with specific primers following Wang *et al*., (2010) consisted of (prophenoloxidase, superoxide dismutase, and transglutaminase). RT-qPCR was performed using the ABI PRISM 7500 detection system sequences with 5x Hot firepol Evagreen qPCR mix (ROX). cDNA amplication reaction volume was 20 µL as the final concentration of 1x hot master mix (ROX); primary F/R, 10 picomole of each 250 mM; ddH_2O was added up to volume of 20 µL and cDNA (0.01 ng/uL). Conditions of thermal cycling for RT-qPCR were consisted of initial denaturation temperature of 95 ^oC (15 minutes, 15 seconds) followed by annealing temperature of 60 ^oC (30 seconds) and a final extension at 72 ^oC for two minutes by 40 cycles. Calculation of “Ct” from PCR cycle threshold (Ct) gene were tested and then normalized relative to Ct 18 sRNA on the sample. ΔCt value calculated from (sample group tested challenged)-Ct (basic expressions, example exposure to 0 hour). Relative representation of different multiples of basic expression can be calculated by $2^{-\Delta Ct}$.

RESULTS AND DISCUSSION

Chemical analysis, molecular mass and structure of fucoidan

The crude extract yield obtained was 4.22 ±0.20% of the seaweed dry weight. The results of measuring carbohydrate and sulfate contents as well as molecular weight for all of four kinds fucoidan are presented in Table 1. The higher sulfate content was found in HMW fucoidan, because depolymerization of crude fucoidan with the acid causes the sulfate removal. This made the significant different of sulfate content between LMW fucoidan and HMW fucoidan, suggesting that the acid hydrolysis not only results in oligomers but also causes the release of sulfate groups.

Table 1: Results of Analysis Chemical All Kinds Fucoidan.

Kinds of Fucoidan	Total carbohydrate (%)	Total sulfate (%)	Molecular Weight x10^4 Dalton
LMW	65.23	1.2	7.53
CF	74.25	8.44	10,68
HMW	82.54	7.8	8.28
PF	70.23	3.23	8,00
Fucoidan commercial (FK) *Fucus vesiculosus*	84.23	7.82	8.18

FT-IR spectroscopy is used to identify where the sulfates are positioned in the structure of fucoidan. The main information for the position of sulfate groups is contained in the ranges of wavenumber 1500-700 cm^{-1}. It has been reported that the broad signal at 1259 cm^{-1} (-S-O antisymmetric stretching vibration of the sulfate group) is representative of the total sulfate esters in polysaccharides. The signal at 823 cm^{-1} characterize the sulfation the equatorial position where the sulfate ester binds to the of fucose to form sulfate fucose. This result is in agreement with IR spectrum indicating that fucoidan being studied have close structural similarity to commercial fucoidan (Figure 1) (Zvyagintseva *et al*., 1999; Usov and Bilan., 2009). The NMR spectroscopy is used to identify the composition and the structure of fucoidan. The NMR spectroscopy is used to identify the composition and the structure of fucoidan. The NMR profiles described below are only for LMW and PF (Figure 2). The NMR profiles for HMW and CF are not shown here due to the overlapped peaks. Based on our ^1H NMR data, the presence of the chemical shift for the methyl group was observed at 1.0 ppm. Monosaccharides generally do not have alkyl group, and therefore their chemical shifts were in the range of 3-4 ppm instead of 1 ppm. The chemical shift of 1 ppm is not common in carbohydrate. This chemical shift showed the specificity of fucose which has the methyl group (CH_3) at C-6 position. The chemical shift at 96 ppm ^{13}C NMR is charaterisctic of carbons of C-1 of carbohydrate. The peak appears at the most downfield shift (5 ppm) indicating a proton bound to the carbon containing a sulfate group. This is supported by the appearance of the peak at 75 ppm ^{13}C NMR spectrum (Figure 2b).

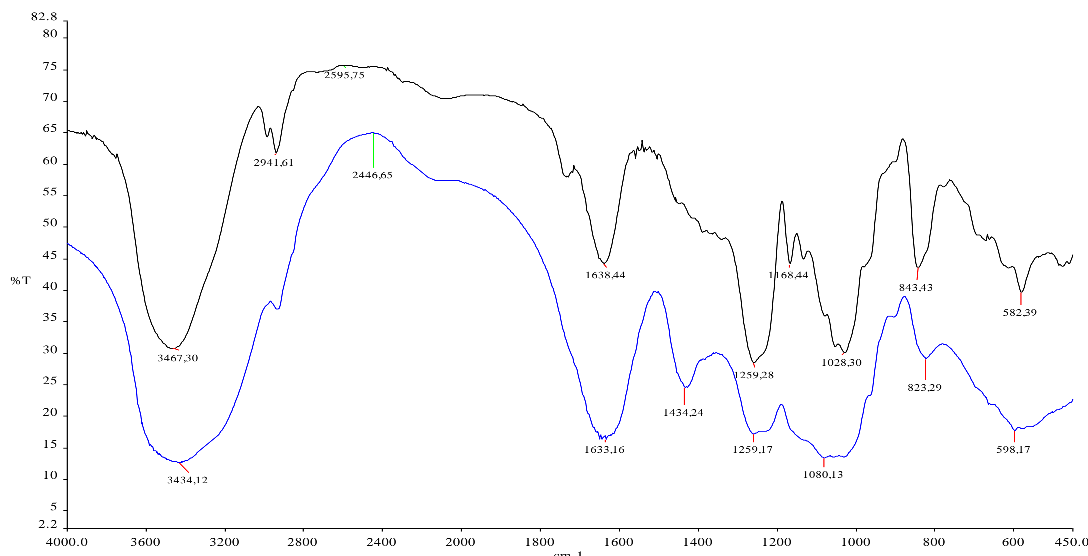

Fig. 1: FT-IR spectra of crude fucoidan *Sargassum binderi* Sonder and a commercial fucoidan from *Fucus vesiculosus*.

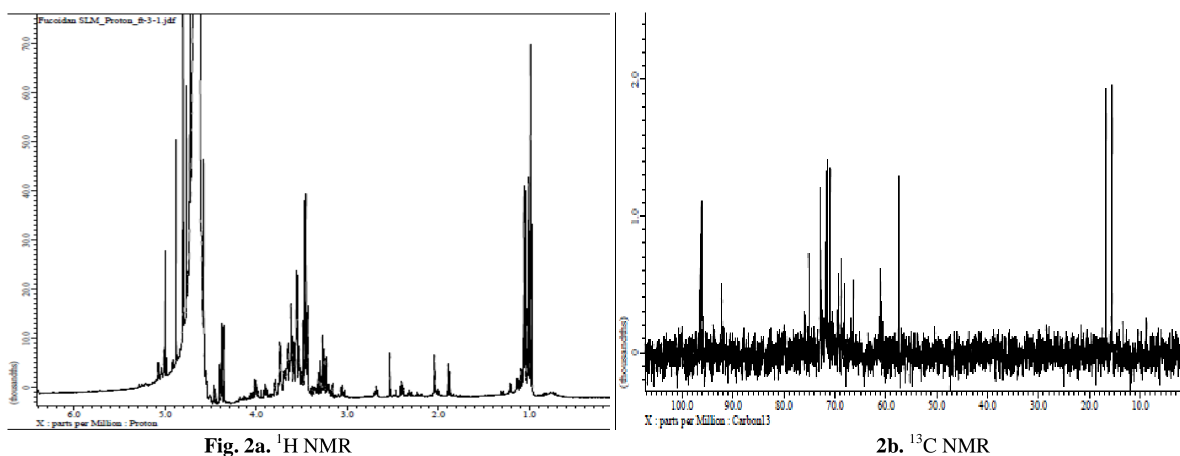

Fig. 2a. ^{1}H NMR **2b.** ^{13}C NMR

Fig. 2: NMR Spectra of fucoidan extracted from brown seaweed *Sargassum binderi* Sonder.

Immunostimulant and resistance against WSSV infection of fucoidan

Nutrition composition of the given food for assay (in the form of crumble, PV feed code 1 type) contains protein (40%), fat (6.5%), hoarse (2.2%), ash (13.0%), and water content (11.0%). The statistical calculation revealed that HMW fucoidan enhanced the growth of the shrimp *L. vannamei* significantly, as indicated with the increased body length (8.35 ± 0.59) cm and weight (10 ± 0.29) g if compared with the controls (without fucoidan) that have growth length of 7.45 ± 1.27 and weight of 9.95 ± 0.60. Similar result was reported by Traifalgar *et al.,* (2009) on PL 15 shrimps *P. monodon* for 30 days increased the weight (13.5 ± 0.59 mg) with the specific growth rate (SGR) of 8-7.9%/day. Our feeding observation was done on shrimps cultivated in the tank containing sea water with salinity of 32 ppt, pH of 8, temperature of 28 ± 1 °C and the oxygen levels of 6-7 ppm.

The lowest mortality of the infected shrimps were observed for treatment with HMW fucoidan about 10%, followed with LMW fucoidan (40 %), CF (45 %), PF (55 %), and CN (55 %). We assumed that the low mortality for HMW fucoidan was related to sulfate content of HMW fucoidan. Sulfate content HMW fucoidan (7.8%) is higher than LMW fucoidan (1.2%). The sulfate content may contribute to the effect of stimulant to shrimp. However the effect stimulant is not only cause by sulfate content, but also is influenced by molecule size. To digestion higher moleculer weight require longer time.

After 24 hours of infection, the shrimp individuals fed with CF showed a 10 % mortality (Fig. 4). The shrimps fed without fucoidan supplement (negative control) showed 80 % mortality at 48 hours and at 72 hours 90 % mortality after infection. After 72 hours of infection, 80 % mortality was observed for the shrimps fed with HMW fucoidan; whereas 70 % mortality was found for those treated with CF and LMW fucoidan. The survival rate of the shrimps fed with all four kinds of fucoidan was in the range of 20-30 %, which was twice than the negative control (20 % survival rate). Similar result was also observed after 72 hours of infection, in which the survival rate of the fucoidan-fed shrimps was twice than the negative control.

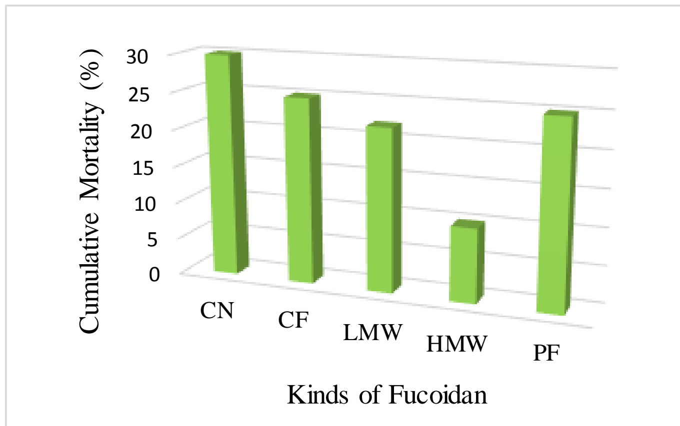

Fig. 3: Mortality Shrimp during Experiments

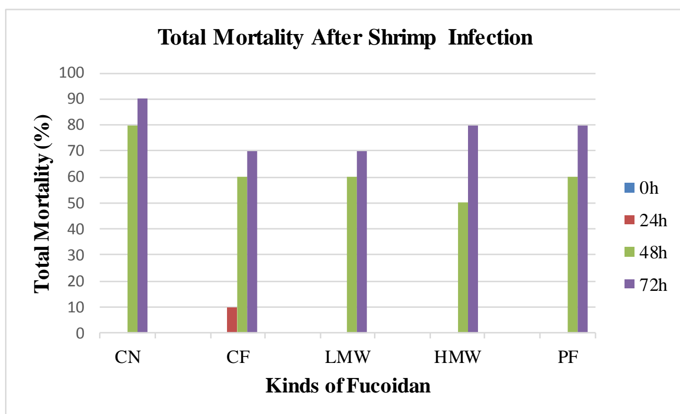

Fig. 4: Mortality Shrimp After Challenge Test with WSSV

The highest total haemocyte number (1,9x10^5 cell/mL) was observed on the non-infected shrimps fed with HMW fucoidan. The lowest haemocyte number (1.4 x 10^5 cell/mL) was found in the non-infected inividuals, administered with CF. This suggests that HMW fucoidan resulted from the depolymerization of CF was more easily absorbed by the shrimps compared with CF. Similar study was reported by Traifalgar *et al.,* (2013) showing that feeding with immunostimulant (*Vibrio harveyi* lipopolysaccharide (VLP) and β -glucan) increased the total haemocyte number.

The infection of the virus stimulates the activation of the cascade proPO system, in which PPAE (*prophenoloxidase activating enzyme*) as a terminal-specific protease converted prophenoloxidase (proPO) into the active form phenoloxidase (PO) through proteolytic activity. Futhermore, activated PO oxidizes phenol to form quinone. Futher polymerization of quinone leads to formation of melanin that is responsible of killing pathogen and healing wound caused by encapsulation and regeneration of ROS (Cerenius *et al.*, 2010); Nappi and Christensen, 2005; Pakkakul *et al.,* 2015).

The highest total haemocyte number (2.2 x 10^5 cells/mL) was found for the infected individuals fed with HMW fucoidan at hour 0. Whereas the lowest number (0.6x10^5 cells/mL) was observed on those administered with LMW fucoidan. In general after 24 hours of infection, the total haemocyte number decreased significantly. The 24-hour infected shrimps physically looked less moving, appetite and stressful. This is confirmed by a large number of the remaining food residues in the experimental tub. In the challenge test for 48 hours of infection, total haemocyte number for the CF-fed individuals decreased 82.3 %. Similar result was observed for the PF-fed individuals, in which the total haemocyte number showed the decrease of 35.3%. In contrast, the individuals fed with LMW fucoidan exhibited significant increase of total haemocyte number 40.0%. Total haemocyte number of the HMW fucoidan-fed shrimps begun to increase slightly if compared with the 24-hour observation.

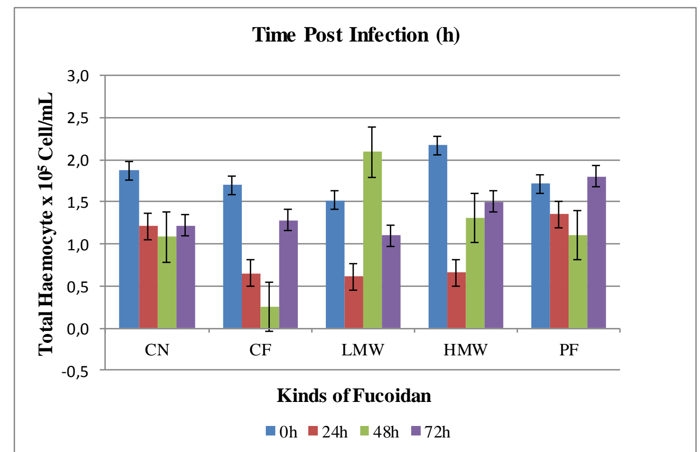

Fig. 3: Total Hemocyte Count after Challeng Test with WSSV

In the challenge test for 72 hours of infection, the LMW fucoidan-fed individuals exhibited the decrease in total haemocyte number 26.6%. Interestingly the total haemocyte number of the PF-fed individuals increased slightly by 5.8% compared with the hour 0. Another treatment with HMW fucoidan showed slight increase by 15.%. Surprisingly significant increase by 333.3% in total haemocyte number was found in the individuals fed with CF in comparation with the 48-hour observation.

Based on the results described above, it is likely the treatment of the infected shrimp with LMW fucoidan stimulates the fast increase of total haemocyte number in short period of time (24 hours). We assumed that LMW fucoidan is more easily transported across the cell membrane; and therefore it immediately interacts with the haemocyte receptors. This was also reported by Tawut *et al.*, (2015) that polysaccharide with lower molecular weight provides greater opportunities for material immunodulator in the haemocyte pathway. Three other treatments showed the slow increase after 48 hours of infection. Shortly after feeding with fucoidans, the total haemocyte number did not increase immediately, because it required time to metabolize them. In particular, HMW fucoidan consumed by the infected shrimps may undergo hydrolysis into smaller MW units that are subsequently transported into the cell via cell membrane. Inside the cell, the immun system is stimulated by interaction between the fucoidan sulfate groups and the haemocyte receptor, as has recently been by

Tawut *et al*., (2015) for HMW sulfated galactan from *Gracilaria fisheri* (100 kDa).

Expression of gene-related immunity

After feeding for 15 days, WSSV challenge tests were conducted at the level of gene expression for prophenoloxidase (proPO), superoxide dismutase (SOD) and transglutaminase (Tgase) using RT-qPCR. The test results were presented in Figures 6A, 6B and 6C.

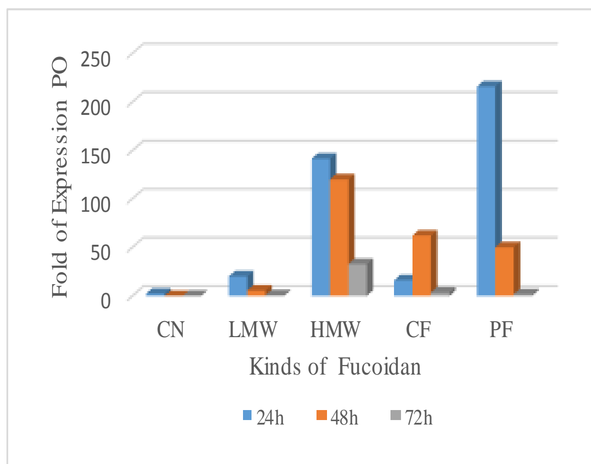

Fig. 6A. Fold of Expression PO

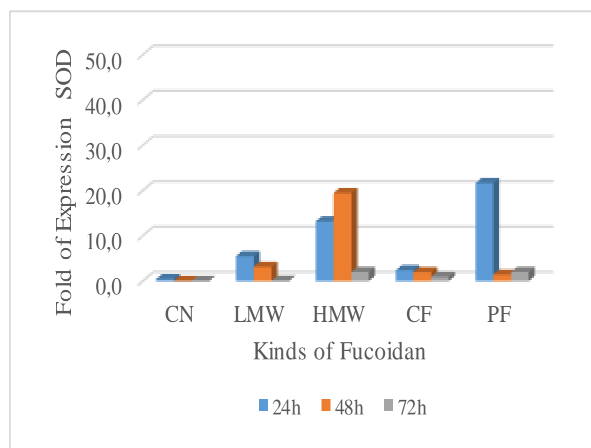

Fig. 6B. Fold of Expression SOD

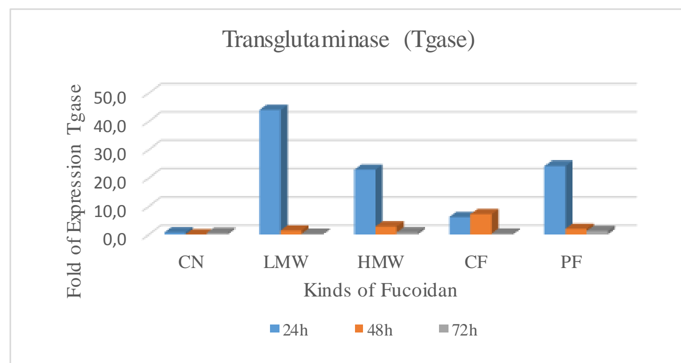

Fig. 6. Expression of gene-related immunity

Prophenoloxidase (proPO)

Detection using RT-qPCR is intended to know the transcription level of proPO gene during WSSV infection. Expression levels of proPO gene were individually normalized using 18s RNA as the endogenous reference to indicate the normal condition (without infection) of the challenge test at hour 0. ProPO gene activation system was significantly improved upon feeding with fucoidans. The highest trancriptional level of proPO was observed on PF treatment, which was 100 times compared with the control at the hours 24. Similar result was observed for other fucoidan treatment. However at hour-48, gene expression of proPO decrease, except for CF. The highest level of proPO gene transcription was found in the individuals fed with PF, followed with HMW fucoidan, LMW fucoidan and CF. The highest transcriptional level of proPO was observed at the 24-hour infection. However, at the 48-hour infection, the trancriptional level of proPO gene decreased. In general, feeding with fucoidan improved proPO gene transcriptional level in the range 8-100 times compared to control.

In shrimps, proPO is localized in the haemocytes, but it plays a major role in the humoral response that is triggered by immunostimulatory activity of fucoidan. The proPO cascade in shrimps commonly occur through recognition of the cell wall components of bacteria or fungi by pattern recognition proteins (Fagutao et al., 2011). In this present study, we used fucoidan to stimulate the proPO cascade through proteolitic activity. This proPO cascade process is initiated by serine protease cascade activation that converts the proPO-activating enzyme (PPAE) to an active proteinase. Then proteinase catalizes the conversion of the inactive enzyme precursor, proPO, into phenoloxidase (PO). PO, a copper containing oxidase, eventually catalyzes the oxidation of tyrosine to produce toxic quinone substances and other short-lived reaction intermediates that lead to the formation of melanin. It has been shown that melanin binds to the surface of bacteria and increase the adhesion of haemocytes to bacteria, thus accelerating their removal by nodule formation (Cerenius et al., 2008). Melanization, which is a major innate defense system in invertebrates, is controlled by the enzyme phenoloxidase. However, PO activity does exist in the plasma of haemolymph without pathogenic infections (Masuda *et al.,* 2012). The type proPO in which Vannamei shrimps was conducted proPO1 refer to NCBI data base (AF 521948) (Amparyup *et al*., 2009). The expression level of proPO increases as a response towards WSSV infection, leading to the increased defense system (Wang *et al*., 2010).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is an antioxidant enzyme mediating oxidative damage. On the crustacean haemocyte, the antimicrobial activity is a derivative of reactive oxygen species (ROS) during and after phagocytosis. This ROS production could lead to tissue damage on the shrimp. ROS could be a source of disease due to the oxidative stress. To prevent the formation of ROS, it requires an anti-oxidative substance. SOD serves as the first eliminator of ROS and converts it into hydrogen peroxide and

oxygen. In the case that ROS is formed excessively, it can cause damage to the host cells. Under this condition, SOD acts to control excessive ROS (Fuhua and Jianhai, 2013). In this study, the level of SOD gene expression after WSSV challenge test increased during 24-48 hours of infection, as shown in the Figure 6B.

In this study, the manganase superoxide dismutase (MnSOD) type plays an important role in crustacean immune defense reaction by eliminating oxidative stress (Zhang *et al*., 2007; Zhao *et al*., 2014). At the infection of 48 h, the SOD transcriptional level of shrimps treated with all four kinds of fucoidan (in particular HMW and PF) were higher than the control, suggesting that during the phagocytosis on WSSV, the level of ROS increases and more SODs are needed to reduce the effects of ROS. The highest increase in SOD was found at the individuals fed with PF, followed with HMW fucoidan, LMW fucoidan and CF in the range 2-21 times compared to the control.

Transglutaminase (Tgase)

Transglutaminase clotting system plays a role in shrimp. Level of Tgase gene expression for all fucoidans treatment was significantly higher compared to the control. The treatment of fucoidans enhance the Tgase level. The highest level of Tgase was observed in the individuals fed with LMW fucoidan, then PF, HMW fucoidan and CF in the range 2-44 times compared to the control at the 24-hour infection. In the injured shrimp, Tgase acts quickly to prevent the loss of the hemolymph. Tgase with its cofactor Ca^{2+} plays a role in the coagulation system, which triggered polymerization with hemolymph protein clotting. Protein clotting acts to prevent the spread of pathogens through hemocoel. The requirement of the natural form of ε- (γ- glutamyl) Tgase catalyses cross binding between glutamine and lysine, and therefore it inhibits the blood coagulation in the gills, liver, intestinal, hepatopancreatic, haemocyte, and lymphoid (Sritunyalucksana and Kenneth, 2000).

In general, the enhancement of the shrimp immune system was indicated by proPO; SOD or Tgase during 24 to 48 hours of infection. After that, the gene expression levels associated with its immunity decreased downhill. This is a constraint on the effectiveness of immunostimulant, in which time its effectiveness tends to be short. Several of the research that has been done by Sung *et al*., (2001), they observed that turned out to be the effect of glucan on soaking *P. monodon* Vibrio bacteria or that benefit lasts for a maximum of 24 hours before finally descending to the level of control. Effect of carrageenan by way of soaking as much as 200, 400, 600 mg/L in *Litopenaeus vannamei*, after 3 hours of tested challenge virus WSSV improved immunity (PO activity, activity of the serine protease) and then decreased after 5 hours (Yu *et al.,* 2014).

Sargassum hemiphyllum var.chinensis administration increases the number of haemocytes, phenoloxidase (PO) activity, respiratory burst and lysozyme activity after 3 hours, after 5 hours return to forming of basal (Truong *et al*., 2011). This is the difference immunostimulant vaccine, which vaccine immune system at the same antigens tends resistant for a prolonged time (Valerie *et al.,* 2003). One way to increase the effectiveness immunostimulant time is by giving in a prolonged time and the amount added. Feeding *Sargassum wightii* on *P. monodon* over 90 days can increase the maximum survival of 96.66% (Huxley and Lipton, 2009). The crude fucoidan of *Sargassum horneri* on juvenile crayfish with a concentration of 0.05%, 0.1% and 0.2% per kg body weight for 7 weeks can increase SOD after the challenge test *Aeromonas hydrophila* (Qing *et al*., 2014).

Recently, a set of immune pattern recognition receptors (PRRs) that play important roles in innate resistance have been identified in penaeid shrimp including lipopolysaccharide and β-1, 3-glucan binding protein (LGBP), and toll receptors. Recognition of pathogens by PRRs triggers activation of a serine protease cascade which subsequently cleaves prophenoloxidase (proPO) to generate phenoloxidase (Yu *et al.,* 2016). It has been shown that the amino acid sequence of LGBP deduced from LGBP cDNA of Penaeus chinensis contains a potential recognition motif for β-1, 3-linkage of polysaccharides (Chizhov *et al.,* 1999). Sulfate galactan's structure of *G. fisheri* contains the β-1, 3-linkage which may interact with LGBP localized on the membrane of haemocytes with subsequent generation of active phenoloxidase enzyme. Another receptor activity that plays a key role in the innate immune system involves the Toll-like receptors (TLRs).

The results of the present study suggested that the brown seaweed *Sargassum wight ii* was major source of fucose and sulfate containing biologically active polysaccharide-fucoidan, it may be considered as good immunostimulant in shrimp diseases additionally or instead of commercial antibiotics. The revealed structures of (1-6)-β-D-galactose, α-L-fucose and β-D mannuronic acid and it was suggested that the sulfates of the fucoidan act against WSSV infection while fucose, galactan and mannuronic acid stimulate the immune system of shrimp (Grassian *et al*., 2012; Tawut *et al*., 2015). The efficiency of antiviral agent activity of SPs depends on the density and position of the sulfate groups on sugar residues, and it was reported that SPs from seaweed contain as many as 35-60 sulfate groups per one hundred sugar residues demonstrated a strong antiviral agent activity (Marais *et al.,* 2001) These protective antioxidants are increased at the level of transcription (Kanokpan *et al*., 2014) for the rapid elimination of extreme stress-related reactive oxygen species (ROS) induced by pathogens. It is possible. Therefore, to speculate that sulfate galactans bound to TLRs in *L. vannamei,* which up-regulates the antioxidant enzyme systems and eliminates extreme ROS thus preserving immune homeostasis. At the same time, considering the viruses host interaction, it could be postulated that SG binding with TLR interrupts the viral usage of the TLR-NF-kB pathway for viral replication in the host cell (Witvrouw *et al.,* 1997).

CONCLUSION

Immunostimulatory activity levels of fucoidan toward shrimps infected with WSSV virus are affected by the molecular weight, sulfate content, and purity of fucoidan. Among the three immunity-related genes tested, the transcriptional level of PO gene

have the most significant effect of improving the shrimp's defense system.

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