The protection capacity of the crude and whole protein spores of *Myxobolus koi* as an immunostimulant material development in goldfish (*Cyprinus carpio*) for preventing *Myxobolusis*

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Abstract. The aim of this research was to 1) isolate the crude and whole protein spores of *Myxobolus koi* using SDS-PAGE and 2) to analyze the profile of the *Myxobolus koi* protein spore that will be developed for use as an immunostimulant materials that can give protection to goldfish infected by *Myxobolus sp*. The method of this research was experimental, conducted in a laboratory. The research consisted of two stages; the culturing of the *Myxobolus* using spore scattering involving the characterization of the crude and whole protein spores using SDS-PAGE and the second stage was analyzing the protection capacity of the protein spore of Myxobolus koi in goldfish. This research is descriptive laboratory experiment. The results showed that there were bands of proteins with a molecule weight (MW) between 41.1 kDa – 230.1 kDa. Based on the protein MW identification of *Myxobolus koi* spores, there were 5 whole protein bands; MW 41.1 kDa, 51 kDa, 89.8 kDa, 121.7 kDa and a protein with MW 230.1 kDa. The molecule weight (MW) of the crude protein spores of the *Myxobolus koi* was 150 kDa and 72 kDa. The survival rate of pacific white shrimp that were exposed to the whole protein of *Myxobolus koi* was 87% and 82% when exposed to crude protein.

1. Introduction

The development of freshwater ornamental fish in Indonesia through aquaculture has bright prospects. This is due to the various ornamental fish having a high economic value. One of the fish species is the koi fish (*Cyprinus carpio* koi). Based on the data from the Ministry of Fisheries and Maritime Affairs in 2010, Indonesia still controls only 7.5% of the world's ornamental fish trade [1]. This has caused the development of koi fish to increase. However, there are many obstacles that can cause failure in the context of aquaculture activity, where the main obstacle that needs to be considered is the emergence of disease.

One of the parasitic diseases that can cause an outbreak is Myxobolusis, caused by *Myxobollus koi*. This disease, as of 2009, was included on the pest and quarantine class I list in Indonesia. It can cause serious problems in the koi fish population and death up to 60 - 90% [2]. Furthermore, it was said that in 1974 and 1978, there was a case of an outbreak of *Myxobolus sp* in Indonesia that caused the death of fry koi fish by up to 100%.
Fish that are attacked by myxobolusis show as having breathing difficulties because of the presence of nodules or cysts on the gill filaments. The spread of these parasites occurs because of the transfer of parasites from one fish to another, both directly and through the host between certain phases in the life cycle of the parasite [3].

The control of myxobolusis has been done using disinfectants and other chemicals. However, it has not been able to meet the target level of occurrence and it can still cause negative effects in the fish's body. For this reason, it is necessary to seek alternative prevention measures that do not cause negative impacts. One effort that has been developed is immunization [4]. We have succeeded in finding antigenic carbohydrates isolated from glycoprotein [5].

According to another study conducted by [4], the use of vaccines in the aquaculture industry is very important when it comes to controlling the disease. This type of vaccine has been developed for use in fish such as through whole cells (WC), outer membrane proteins (OMP), extracellular products (ECPs) and lipopolysaccharide (LPS). The previous study succeeded at obtaining [3-4]. It successfully identified the protein gene (MyxCP-1) Myxobolus cerebralis, which was then developed into a vaccine to prevent Myxobolus cerebralis infection in Rainbow Trout (Oncorhynchus mykiss). Another previous study [8] succeeded in isolating, identifying and characterizing the surface glycoprotein of Myxobolus koi as an immunogenic antigen for antibody production. Based on the background of the above problems, the research was carried out in search of material from the sub-unit of both the whole and crude protein from Myxobolus sp. in order to develop immunostimulants that can control myxobolusis in koi fish.

2. Material and methods

2.1 Material

Koi fishes ranging between 7 - 10 cm from Kemloko Village, Nglegok, Blitar were used. The used materials included NaCl, ethanol, percoll gradient solvents, pepsin, HCL, EDTA, KCL, KH2PO4, Na2HPO4, trypsin, sodium citrate, NaHPO4, H2), NaHCO3, glucose, phenol red 0.5%, NaOH, 0.22 um filter, bovineserum albumin, dextrose, ETOH, proteinase, primary pair ForwardERIB1 5’-ACCTGGTTGATCCTGGCAG-3’ (2-20) and Reverse ERIB10 5’- CCTCCGCAAGGTCACTACCG-3’ (2079-2059), 400 um DNTP, 3 um MgCl2, yellow and blue dye, agarose, TAE buffer, sybrsafe, DNAladder 100 bp and 1 bp, loading dey, tris-HCL, 2-mercaptoethanol, sodium deodecyl sulfate (SDS), bromphenol blue, glycerol, SDS loading buffer, polyacrylamide, stacking gel, ammonium peroxidaisulphate (APS), TEMED and glycine.

The equipment used consisted of a haemocytometer, rubber pollen, a microscope micrometer, autoclave, centrifugal tube, swinging rotor, water bath sonicator and 1 set of SDS-PAGE electrophoresis equipment [9].

2.2 Method

2.2.1 Whole and crude protein preparation

This research study was an experiment with the aim of determining and analyzing the Myxobolus protein as a sub-unit vaccine that could provide protection for koi fish against myxobolusis attacks (Steel and Torrie, 1992). This study consisted of a Myxobolus culture and the characterization of protein spores with SDS-PAGE. The test was focused on the protective ability of both whole and crude spore proteins that were laboratory tested [10].

The research method used in this study was a descriptive laboratory experiment method that was conducted to determine the value of an independent variable, either one variable or more (independent) without making comparisons or linking it with other variables [11]. It was expected that this research could answer the question of what the protein character is of the nodules containing spores of Myxobolus sp.

Testing the whole and crude protection ability of Myxobolus sp. in koi fish used 4 aquariums with a capacity of 15 lt of water each. The fish stocking density was 5 fishes / aquarium with a fish size of 7 -
10 cm. The fish were taken from Bilatar and taken to the FPK-unair Laboratory using a pastry bag supplied with oxygen. The dose of whole protein *Myxobolus koi* was 1 µl protein / fish given through an intramuscular injection referring to the research conducted by other researchers [12]. The koi fish were kept in water from the pond where the fish had been kept for 28 days. During maintenance, the water quality was checked as supporting data.

2.2.2 *Myxobolus* sp. spores
The *Myxobolus* sp. spores were obtained from goldfish infected with myxobolusis. The infected fish were first cleaned using aquadest. The nodules were picked off manually using tweezers.

2.2.3 Isolation of whole protein *Myxobolus* sp. spore
The spores were transferred to a test tube and 5 ml of PBS PMSF 5 ml was added; the combination was put in a vortex for 10 minutes. The sample was authenticated using an old zinc sonicator for 10 minutes and then kept at room temperature. Next, the sample was centrifuged at a speed of 6000 rpm for 15 minutes. The precipitate part and the supernatant had absolute ethanol added at a ratio of 1:1, which was then stored in the refrigerator overnight until the precipitate formed. This was then centrifuged at a speed of 10,000 rpm for 15 minutes to form a pellet at the bottom of the tube. The resulting pellets were dried to eliminate the smell of ethanol. The pellets were stored at 4°C in a freezer with 10 ml of Tris-Cl Buffer solution as much as 10 ml. This was then directly analyzed using the SDS-PAGE method [13]. The samples obtained were taken in part to measure the protein concentration using UV-Visible.

2.2.4 Isolation of crude protein from the *Myxobolus* sp. spores
The spores were given PBS and then centrifuged at a speed of 5000 rpm for 10 minutes [11]. The pellets plus the 500 µl lysis buffer were then authenticated in ice (1 minute sonication, ½ minute break), repeated 10 times. The sonication results were then distorted (1/2 minute vortex 1 minute break) in ice, repeated 15 times. The vortex results were centrifuged at a speed of 12000 rpm for 5 minutes; the supernatant that was formed was collected and SDS-PAGE analysis was performed [13].

2.2.5 Analysis of the protein concentration
The analysis of the protein concentration in the study was assessed using the Biuret Method and this was read by the UV-Visible spectrophotometer [13]. The protein content was calculated by converting the Bovin Serum Albumin (BSA) curve, which has a known concentration.

The steps involved in determining the protein content using the Biuret method were carried out in several stages, such as the preparation of the BSA stock solution and the determination of the BSA wavelength, creating a BSA standard curve and calculating the sample protein content [13].

2.2.6 Total Hemocyte Count (THC)
Hemocytes were taken from the ventral part of the second abdominal segment using a 25 G needle and a one ml syringe that inserted 0.2 milliliters of cold Alsever modified solution (US 19.3 mM; Na citrate 239.8 mM, NaCl 182.5 glucose and 6, 2 mM EDTA, pH 7.2) as an anticoagulant [14]. The calculation of the hemocyte count was carried out using the May Grunwald-Giemza method [14] which used a bright microscope (LM) at 1000x magnification. This was then calculated using the Coulter counter model ZM (Counter Electronic Ltd). The hemocyte particle sizes ranged from 0.4-800 µm; the supporting data could be observed with an electron microscope (EM) by first centrifuging 700x of gravity at 40 C for five minutes.

2.2.7 Differential Hemocyte Count
The blood was dripped onto the object glass and stained with giemsa [15] for cell type identification. The hemocyte differential aimed to determine the number, type and percentage of hemocyte cells. The hemocytes that were analyzed were classified according to [16]’s instructions. The number of
hemocytes was counted to 100 cells and the percentage of each type was sought. This was carried out close to the end of the study [16].

2.2.8 Research parameters and data analysis
The main parameters of this study were the *Myxobolus* protein profile, survival rate (SR) and the blood picture of the koi fish exposed to the *Myxobolus* protein. The data obtained consisted of both qualitative and quantitative data. The qualitative data was data obtained from the determination of gill pathology and the blood picture used in the analysis of the koi fish immune response exposed to both whole and crude *Myxobolus* spore protein as an ingredient for immunostimulatory development. The quantitative data consisted of the results of the determination of SR and the blood analyzed using the ANOVA statistical test (Analysis of Variance) with four treatments and four replications. If there were any differences, then the researcher continued with Duncan's Multiple Range Test at a 5% confidence level in order to know the difference between the treatments [17].

3. Result and discussion
3.1 Identification of *Myxobolus* in Koi fish
The *Myxobolus* identification showed that the morphology was in accordance with the identification keys of [18-19], namely that the *Myxobolus* spheres had an ellipse or oval shape. Inside the *Myxobolus* spore, there were two poliform polar capsules located anteriorly. The observation of the clinical symptoms in the pond indicated that the koi fish swam abnormally and had difficulty breathing due to the presence of reddish white nodules in the gill filaments that caused the operculum to not close.

3.2 Characterization of protein spores with SDS-PAGE
The characterization of the whole protein spores of *Myxobolus* sp. was done using the SDS-PAGE method. There were 5 protein bands with a molecular weight of (BM) 230.1 kDa, 121.7 kDa, 89.8 kDa, 51.0 kDa and 41.1 kDa. The results of the protein characterization determined using the SDS-PAGE method can be seen in Figure 1.

![Figure 1](image_url)

*Figure 1. Results of the Whole Protein Characterization conducted using the SDS Method - PAGE, M: Protein Markers and N1, N2: Protein Samples.*
Figure 1 showed the same protein band between the N1 and N2 protein samples. This showed the consistency of the protein nodules containing *Myxobolus* spores. It can be seen that the protein band of BM 89.8 kDa had a thicker band than the other protein bands.

3.3 Isolation of *Myxobolus koi* protein spores

The isolation results of the crude *Myxobolus* protein from the koi fish can be seen in Figure 2. The profile picture of the crude protein and soluble protein from the *Myxobolus* spores showed that the molecular weight of the crude *Myxobolus* protein in this study was 150 kDa and 72 kDa. For the soluble protein, the weight was 73 kDa.

![Figure 2. Protein profile using the SDS-PAGE method. M = marker 20-180 kDa, S1 = crude protein sample, S2 = protein soluble sample.](image)

3.4 Calculation results of the erythrocytes in Koi fish given protein spores.

The results from determining the blood picture of the koi fish exposed to spore protein after 14 days of maintenance, as presented in Table 1.

| Treatment                  | Blood Picture of the Koi Fish after 14 days (%) |
|----------------------------|-----------------------------------------------|
|                            | Erythrocyte | Leukocyte   |
| Control, No Protein        | 87,35±5,18  | 8,78±2,98   |
| Whole Protein *Myxobolus*  | 35,24±4,25  | 71,45±2,63  |
| Crude Protein *Myxobolus*  | 32,56±3,46  | 65,38±1,76  |
| PBS                        | 65,58±1,18  | 27,37±3,21  |

Description: The use of the different superscripts in the same column showed there to be significant differences (p <0.05)
3.5 Calculation of the leukocytes in the koi fish given the spore protein

The results of the differential determination of the white blood cells in the koi fish exposed to the spore protein after 14 days, with a dose of 600 µl, have been presented in Table 2.

Table 2. Differential determination results of the leukocytes of the exposed koi fish to the spore protein after 14 days.

| Treatment                      | Differential Leukocytes in the Koi Fish after 14 days (%) |
|-------------------------------|----------------------------------------------------------|
|                               | Lymphocytes | Monocytes | Eosinophil | Neutrophil |
| Control, No Protein           | 74.42       | 3.11      | 2.65       | 11.35      |
| Whole Protein Myxobolus       | 81.43       | 4.22      | 6.11       | 17.45      |
| Crude Protein Myxobolus       | 87.98       | 6.13      | 7.89       | 24.33      |
| PBS                           | 71.67       | 3.21      | 4.98       | 10.13      |

3.6 SR of the koi fish

The SR of the Koi fish has been presented in Table 3. It showed that in the Myxobolus spore protein protection test, there were significant differences (p < 0.05) between the treatment against the average SR between those exposed and those not exposed to the Myxobolus spores after 14 days.

Table 3. SR of the koi fish exposed to the protein spores after 14 days.

| Treatment                                         | SR±mean SD             |
|---------------------------------------------------|------------------------|
| No infection, no protein (Control)                 | 96.00±2.24             |
| Myxobolus infection, exposed with protein spore of 600 µl/fish | 86.00±6.52             |
| Myxobolus infection, no protein                   | 10.00±3.54             |
| No infection, exposed with protein spore of 600 µl/fish | 98.00±2.94             |

Description: The use of different superscripts in the same column showed there to be significant differences (p < 0.05)

3.7 Water quality

The water quality measurement data in the aquaculture ponds in Kemloko Village, Nglegok, Blitar showed the average temperature of the pond as being 28°C. The average pH of the pond water was 6 while the ammonia level was 0 mg / l. The salinity was 1 ppm and the oxygen dissolved showed as 9 mg / l. This showed that the condition of the water quality of the koi fish ponds in Kemloko Village, Nglegok, Blitar was of a normal condition. However, some of the conditions of the water quality in the koi ponds were not suitable or not within the normal range for the survival of koi fish. The water quality data before and after the treatment media has been presented in Table 4.
Table 4. Water quality data before and after the treatment.

| No. | Treatment | Parameter       | Before  | After |
|-----|-----------|----------------|---------|-------|
|     |           | Temperature (°C) | pH (ppm) | DO (ppm) |
| 1.  | No infection, no protein (Control) | 29 | 8 | 5 | 29 | 6 | 4 |
| 2.  | Myxobolus infection, exposed with protein spore of 600 µl/fish | 29 | 8 | 5 | 29 | 8 | 5 |
| 3.  | Myxobolus infection, no protein | 29 | 8 | 5 | 29 | 7 | 4 |
| 4.  | No infection, exposed with protein spore of 600 µl/fish | 29 | 8 | 5 | 29 | 7 | 5 |

3.8 Discussion

Myxobolus is a parasitic disease in fish caused by Myxosporea from the genus Myxobolus (which can be identified by its spore morphology, and the number and location of polar filaments [20-22]). In the study of [23], it was stated that the protein from the Myxobolus cerebral spores had BM 7 kDa, 45 kDa, 60 kDa and 130 kDa. In our study, protein bands with BM 41.1 kDa, 51 kDa, 89.8 kDa and 121.7 kDa were probably proteins from Myxobolus sp. spores, whereas the protein band of 230.1 kDa was thought to be a Myxobolus nodule.

Fish have a bodily defense system to fight various diseases. The defenses consist of specific non-specific and specific defenses. Non-specific defenses consist of the skin, scales and lenders, which are the leading bodily defenses in the face of the fish being attacked by various incoming microorganisms. Specific defenses in fish consist of macrophages, leukocytes and Natural Killer cells. Specific defense systems need time to recognize their antigens before they can respond [24].

Changes in the total number and type of leukocytes could be used as indicators of the presence of certain infectious diseases that occur in fish. Leukocytes are one of the blood components that function as specific body defenses that can neutralize and destroy pathogens through the phagocytosis process. Lymphocytes are a form of leukocytes. The lymphocyte observation results showed the highest percentage of 63.3% in treatment D when compared with treatment A (control) of 55.8%. The increased lymphocyte count in fish infected with Myxobolus spores was the response of the fish's body defense system to the entry of the pathogens. This was in accordance with the opinion of [25], stating that the lymphocytes functioned as antibodies involved in immunity from diseases.

Lymphocyte cells consist of two populations, namely B cells and T cells. B cells have the ability to transform into plasma cells, which are cells that produce antibodies. T cells play a role in cell-mediated immunity (cytotoxic T cells) and controlled immune responses (suppressor T cells) [26]. After antigen binding with the lymphocyte cell antigen receptors, the lymphocyte cells divide and differentiate into effector cells and memory cells [27].

The concentration of the crude and whole protein Myxobolus sp. was measured using UV-Visible spectrophotometry, with a result of 5116.25 µg / ml. According to Scope, in a previous study [28], it was stated that the lowest protein concentration needed for protein analysis was 1.2 µg / ml. It can therefore be concluded that there was the presence of the crude Myxobolus sp. nodule protein. Protein analysis was performed using SDS-PAGE electrophoresis.

The results showed that the Myxobolus spore protein was able to protect the koi fish against myxobolus. This was evidenced by an increase in the survival of the koi fish, which shows that it could decrease fish mortality. The results showed that there were no differences between the whole and crude Myxobolus spore proteins in enhancing the immune response and providing protection to the koi fish against myxobolus. This was evidenced by an increase in the survival of koi fish during the 28 days. The use of whole and crude Myxobolus spore proteins could stimulate immune cell activity in fish and it can also be used as a material in developing immunostimulants. It could therefore increase
the activity of the fish body defense cells against myxobolusis. The fish respond to vaccination by synthesizing antibodies known as immunoglobulins. The previous study told us that the antigens enter the host cell or the body of the fish. As the antigen is presented by MHC, the antigen can be captured by the receptor on the T helper cells [29]. T helper cells secrete cytokines i.e. IL-2, IL-4 and IL-6, thus increasing the number of lymphocytes in the blood aimed at the differentiation and proliferation of B cells. B cell differentiation produces plasma cells and memory cells [30]. Furthermore, the plasma cells synthesize specific antibodies that bind the antigen so as to prevent the antigen movement and to facilitate the phagocytosis process. High protein concentrations can provide high protection. For the fish life level, the effectiveness of the immunostimulant material was considered good if the SR value was ≥ 50%.

4. Conclusion
There were 5 profiles for the whole protein spores of Myxobolus with a molecular weight of 41.1 kDa, 51 kDa, 89.8 kDa, 121.7 kDa and 230.1 kDa. There were 2 profiles for the crude spore proteins with 150.6 kDa Molecular Weight and 72.34 kDa. There was a difference between the whole and crude protection of Myxobolus spore protein in relation to increasing the immune response and providing protection to the koi fish.

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