The stability of Immunoglobulin Y (IgY) against streptococcal infection in tilapia aquaculture environment for the development of prophylactic and therapeutic agents

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Received: April 2021 Accepted: June 2021

Abstract

Immunoglobulin Y (IgY) can be used as a prophylactic and therapeutic agent for streptococcal infection in tilapia. This study explores the stability of IgY against water salinity and pH, temperature, and the presence of protease, which all stimulate the conditions of the digestive tract of tilapia. It was found that water salinity and pH levels of 0, 15, and 30 ppt as well as 6.5, 7.5, and 8.5 respectively, with an incubation period of six days, and temperatures of 25, 45, and 60°C for 60 minutes did not affect the molecular weight of IgY and resulted in positive ELISA test results. Extreme conditions at 80°C and incubation with the pepsin showed negative results in the ELISA test starting at minute 40. However, from its incubation at 80°C, IgY did not show any change in its molecular weight, as shown in the SDS PAGE gel. Trypsin also did not affect the molecular weight characteristics of IgY and showed a positive result in the ELISA test. However, IgY could not be detected after its treatment with pepsin. Based on this study, it can be concluded that IgY could lose its biological function due to heating to 80°C and exposure to pepsin. However, IgY is resistant to trypsin, water salinity, and pH levels in tilapia aquaculture.

Keywords: Aquaculture, Biological stability, Immunoglobulin Y (IgY), streptococcal infection, Tilapia

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Introduction

Streptococcal bacterial infection is one of the many bacterial diseases that infect tilapia and result in economic losses (Iregui *et al*., 2014). Several species of streptococcal bacteria that have been isolated from tilapia are *Streptococcus agalactiae*, *S. iniae* (Taukhid and Purwaningsih, 2011), *Lactococcus garvieae* (Anshary *et al*., 2014), and *Enterococcus faecalis* (Rahman *et al*., 2017; Rizkiantino *et al*., 2020a). The infection caused by the *Enterococcus faecalis* can lead to various clinical signs such as black spots at the eyes, operculum, and ventral mouth; opacity on the eyes; hemorrhage on the eyes and under the pelvic, dorsal, and pectoral fins; tail erosion; and exophthalmia (Rahman *et al*., 2017; Rizkiantino *et al*., 2021).

In several areas of Indonesia, the main efforts taken to treat and control this disease are through the use of antibiotics. However, the usage of inadequately controlled antibiotics can trigger resistance and make efforts to treat this disease more difficult. Immunoglobulin Y (IgY) from chicken eggs has recently become a more environmental-friendly treatment to be used as a prophylactic or therapeutic agent for several types of fish diseases. However, further studies need to be conducted on these protein molecules in the aquatic environment, especially in tilapia aquaculture, to determine how stable this molecule is to be used as a prophylactic and/or therapeutic agent.

Immunoglobulin Y is a water-soluble immunoglobulin isotype in the aves group (Inoue *et al*., 2015). Wibawan *et al*. (2008) reported that IgY from chicken egg yolk could sustain its activity for 30 minutes at a temperature of 65 °C, but that it will decrease in activity and break down through heating at 75°C for 30 minutes. Moreover, the presence of protease enzymes such as trypsin and pepsin and a pH level of 2 can cause IgY degradation, but the IgY molecule is stable at pH 4. Li *et al*., (2007) reported that alginate and chitosan microencapsulation technology could be used to protect IgY and stabilize its relative activity. Their study encapsulated IgY molecules with alginate-chitosan and incubated them in gastric simulative fluid (GSF) which contained pepsin and is at a pH level of 1.2. Their results found that the encapsulated IgY molecules had better stability than those without encapsulation. IgY molecules without encapsulation would rapidly hydrolyze, and the IgY activity disappears after one hour of incubation in the GSF.

Nevertheless, there is still a lack of studies on the stability of IgY molecules in the optimal environment of the digestive tract of tilapia (which contains the presence of protease enzymes, such as trypsin and pepsin). Further information on the stability of IgY is also useful for the production of fish pellets that require high temperatures above 60°C as the stability of IgY is feared to be damaged by high temperatures. Therefore, it is necessary to study the stability of IgY in the aquaculture environment and

physiological conditions of the digestive tract of tilapia to predict how stable this molecule will be when applied as a prophylactic and therapeutic against streptococcal infection in fish.

To complete this research gap, this study aims to explore the stability of the IgY against streptococcal infection against the impact of the aquatic environment, such as pH and salinity levels, extreme temperatures, and the presence of protease enzymes. This information can also help to improve proper packaging methods for the IgY against streptococcal infection pellets and optimize its function as a prophylactic and therapy.

Materials and methods

Microencapsulation of Immunoglobulin Y (IgY) against Streptococcal Infection

The IgY of anti-streptococcal infection was extracted from egg yolks and was purified by using the NaCl biocompatibility method (Wibawan *et al*., 2018), as obtained from a previous study (Rizkiantino *et al*., 2020b). In the subsequent examination, part of the IgY suspension was then microencapsulated as the IgY positive control. The microencapsulation was made by following Li *et al*. (2007) method. A 2% (w/v) sodium alginate solution in distilled water was then prepared. Pure IgY suspension was mixed into the 2% (w/v) sodium alginate solution with the ratio between IgY suspension and alginate solution being 40:60, then the mixture was homogenized.

The encapsulation medium solution was prepared by the following method. A total of 1 g of chitosan was dissolved in 100 mL of 1% (v/v) acetic acid solution to form a 1% (w/v) chitosan solution. The solution was then homogenized at room temperature and filtered by using filter paper to remove undissolved particles. A total of 0.2 mL of 1% (w/v) chitosan solution was mixed with 99.8 mL of 1.5% (w/v) calcium chloride ($CaCl_2$) solution to form a 0.2% encapsulation medium solution. The IgY microcapsules formed were then left in a solution of encapsulation medium for 30 minutes and then stirred by using a magnetic stirrer. Next, the IgY microcapsules were filtered with filter paper and then rinsed using distilled water. The rinse results were stored at −20°C until the next testing stage.

Molecular Characterization of the IgY against Streptococcal Infection from Chicken Egg Yolk

The characterization of IgY protein formed in non-microencapsulated and microencapsulated suspensions was characterized by using the Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS PAGE) method (modified He, 2011). The concentration of the stacking gel was 4%, and the separating gel used was 12%.

Preparation of a 12% separating gel was made by mixing 5.6 mL of ddH_2O, 5.2 mL of 1.4 M Tris-HCl solution pH 8.8 (separating gel buffer), 4.8 mL of 40% acrylamide solution, 400 µL of 20% SDS solution, 40 µL of 30%

ammonium persulfate (APS) solution, and 16 µL of tetramethylethylenediamine (TEMED) solution were inserted into the tube then the tube was closed and shaken slowly. The solution was immediately poured into a gel-forming glass plate using a 1 mL micropipette (taking care not to form air bubbles) to ±1 cm below the well-making comb. Aquadest-BuOH added slowly above the gel solution in a glass plate so that the surface of the gel was not wavy. The gel was allowed to solidify for approximately 30 minutes (marked by the formation of a transparent line between the Aquadest-BuOH boundary and the gel formed) then the distilled water-BuOH covering the separating gel was removed.

The 4% stacking gel was prepared in the same way in the separation gel preparation procedure with a volume of 1.95 mL of ddH_2O, 250 µL of 1 M Tris-HCl solution pH 6.8 (stacking gel buffer), 250 µL of 40% acrylamide solution, 50 µL of 20% SDS solution, 8 µL of 30% APS solution, and 4 µL of TEMED solution. After the separating gel solidified, the comb where the samples were injected was installed as a sample well mold then 4% stacking gel was poured into the plate through the glass slit and the comb was left for some time until the gel solidified. After solidifying then the comb was released.

Sample injection and visualization began with preparation of 40 µL IgY protein samples mixed with 10 µL sample buffer and heated with a water bath at 95°C for 10 minutes. The sample was cooled at room temperature. The 7 µL of the sample was carefully inserted into the well using a 10 µL micropipette. The electrophoresis was performed on SDS PAGE gel with 100 V for 100 minutes. A 10–250 kDa protein ladder (Thermo Scientific™, Thermo Fisher Scientific Inc., UK) was used as a standard protein marker. The gel from the electrophoresis was then stained by immersing it in Coomassie blue staining solution for 30 minutes while shaking. The gel was then immersed twice in the bleach solution for each soaking duration of 3 hours until protein bands appeared.

Molecular Stability of the IgY against Streptococcal Infection against Water Salinity and pH

The extracts of the IgY against streptococcal infection in the phosphate buffer saline (PBS) pH 7.4 were obtained from previous studies (Rizkiantino *et al*., 2020b). The water salinity treatments were 0 ppt, 15 ppt, and 30 ppt, and the extracts were incubated at room temperature (25–28°C). The solutions with various salinities were prepared by using commercial sea salt (ASW Salt Mix+, Monsterlaut, Indonesia) according to the manufacturer's instructions. Salinity was measured by using a digital salinometer (Smart Sensor AR8012, Intell Instrument Pro, China). The salinity was measured during each addition of salt until the desired salinity level is reached. Water was treated to form water of pH 6.5, 7.5, and 8.5. The acidity level was added with 98% acetic

acid (CH_3COOH) and 1 N NaOH. The IgY was incubated at room temperature (25–28°C) with various water salinity and pH levels for 2, 4, and 6 days. The incubation results were evaluated in triplicate by measuring the IgY's relative activity by using an Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA).

Molecular Stability of the IgY Against Streptococcal Infection against Extreme Temperatures and the Presence of Protease Enzymes

The temperature treatments were conducted at 25°C, 45°C, 60°C, and 80°C. Immunoglobulin Y was incubated at room temperature for 25°C and in the water bath for 45°C, 60°C, and 80°C for 60 minutes and evaluated every 20 minutes. The protease enzyme treatment was conducted by adding trypsin, pepsin, and a non-enzyme was used for control. The trypsin solution was prepared by mixing 12.16 mg of trypsin powder (Herbsens Biotechnology, Xi'an, China) in 100 mL of 0.03 M NaCl pH 8 to simulate the optimal trypsin levels in the digestive tract of tilapia (Zhou *et al*., 2013). The pepsin solution was created by mixing 1,200 mg of pepsin powder (Herbsens Biotechnology, Xi'an, China) in 100 mL of 0.03 M NaCl pH 2.5. This condition was adapted to simulate the optimal pepsin levels in a tilapia's digestive tract (El-Beltagy *et al*., 2004; Zhao *et al*., 2011). The Immunoglobulin Y samples were incubated in these enzymes for 20, 40, and 60 minutes at 37°C. The incubation results from the extreme temperature and the presence of protease enzyme treatment were evaluated in triplicate by measuring the IgY relative activity by using an Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA).

Indirect Enzyme-Linked Immunosorbent Assay (Indirect ELISA)

The indirect ELISA method was followed by modifications stated by Shimizu *et al*. (1988) and Li *et al*. (2007) with the three-fold reiterative. A total of 100 µL of antigen coating was in the form of a suspension, whole cells of the *Enterococcus faecalis* strain 7INB (Rizkiantino *et al*., 2020a) were inactive in 0.5% formalin with a bacteria concentration of 10^9 CFU mL^{-1} in a 0.05 M carbonate-bicarbonate coating buffer solution (pH 9.6), which was then was pipetted into 96 microplate wells (MICROLON®, Greiner Bio-One Indonesia). The positive control used a suspension of microencapsulated IgY against anti-*Enterococcus faecalis* and was confirmed by the agar gel precipitation test and stored at an ideal temperature of −20°C. In contrast, the negative control consisted of a suspension of egg yolk extract that did not contain the IgY against anti-*Enterococcus faecalis* and was confirmed by the agar gel precipitation test. The microplate was then covered with aluminum foil and incubated overnight at 4°C. Next, the microplates were washed four times by using a phosphate buffer saline Tween 20 (PBST) 0.05% solution of pH 7.4. Blocking on microplates was done by

adding 100 µL of PBST-Bovine Serum Albumin (BSA) (PBST-BSA) 1% solution in each well and then covering them again with aluminum foil and incubating them for 2 hours at 37 °C. The microplates were then washed four times by using PBST 0.05% pH 7.4.

A total of 100 µL IgY samples were incubated at various variables then put into the microplate wells. The microplate was covered with aluminum foil and incubated for 2 hours at 37°C. The microplate was then washed four times by using PBST 0.05% pH 7.4. A total of 100 µL of the goat IgG secondary antibody anti-IgY chicken conjugated by horseradish peroxidase (HRP) (Invitrogen, Thermo Fisher Scientific, US) 1:3.000 dilution in each PBS solution was pipetted into microplate wells. The microplate was then covered with aluminum foil and incubated for 2 hours at 37°C, then washed four times with PBST 0.05% pH 7.4. A total of 100 µL of substrates 3.3',5.5'-tetramethylbenzidine (TMB) (1-Step™ Ultra TMB-ELISA, Thermo Scientific, US) were put into microplate wells, which was then incubated for 15 minutes at room temperature and dark conditions. The evaluation indicator was successful when there was a change in the color blue after incubation. The color intensity was measured by using optical density (OD) at a wavelength of 630 nm by using a microplate reader (BioTek 800TS, BioSPX, The Netherlands).

The validation of the test was determined by looking at the comparison of the OD value of the positive control (P) with the OD value of the negative control (N) (P/N) 5.0 and the OD value of the negative control <0.2. The average OD value was then converted into the S/P ratio with the following formula:

$$\frac{S}{P}\text{ ratio} = \frac{(\text{Sample OD average value} - \text{Negative control OD average value})}{(\text{Positif control OD average value} - \text{Negative control OD average value})}$$

The percentage of S/P (S/P %) was also determined by multiplying the value of the S/P ratio by 100. The cut-off value for the test results that were declared positive was calculated by adding up the average OD value of the sample with three-fold the standard deviation (SD) value. The value of the S/P ratio was then compared with the cut-off value. The test results would be declared positive if the value of the S/P ratio is the same or more than the cut-off value (Indriani and Indi Dharmayanti, 2013; Adji *et al*., 2015).

Data Analysis

Data analysis was conducted qualitatively, both on the results of characterization of the IgY protein by using the SDS PAGE method and the results of confirmatory testing by using indirect ELISA. The results of the SDS PAGE were analyzed for changes in the molecular weight of the IgY at the end of the incubation period, while the results of the ELISA test were analyzed for the value of the S/P ratio, which is an indicator that could be used to determine the sample's positive or negative results.

Results

The molecular characterization of the anti-streptococcal infection IgY protein that has undergone various treatments were conducted by using SDS PAGE gel and the results are presented in Figures 1 and 2.

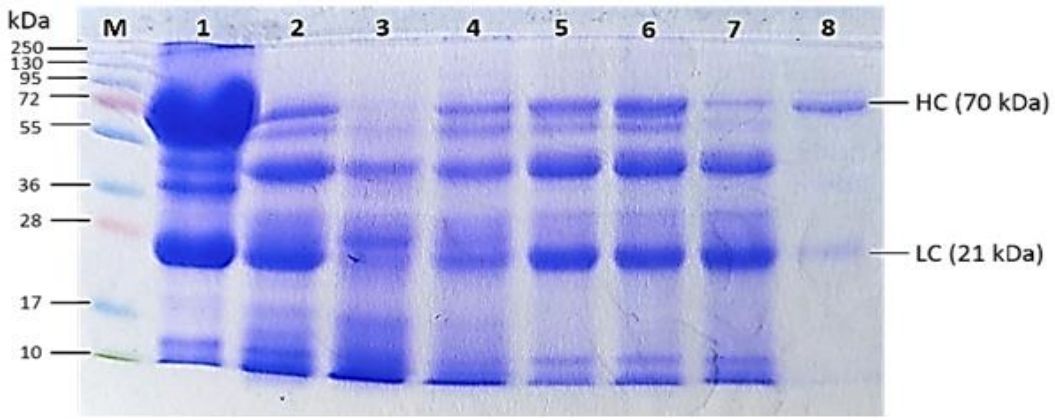

Figure 1: Characterization results in the SDS PAGE gel of The IgY against streptococcal infection protein molecule incubated for six days at various salinities and water pH levels. (M) Standard 250 kDa protein marker. (1) IgY suspension stored at ideal conditions of −20°C. (2–4) IgY suspensions were incubated at 0 ppt (2), 15 ppt (3), and 30 ppt (4) water salinities. (5–7) IgY suspensions were incubated at water pH 6.5 (5), 7.5 (6), and 8.5 (7). (8) Microencapsulated IgY suspension with the alginate-chitosan solution as a positive control. (HC) IgY heavy chain. (LC) IgY light chain.

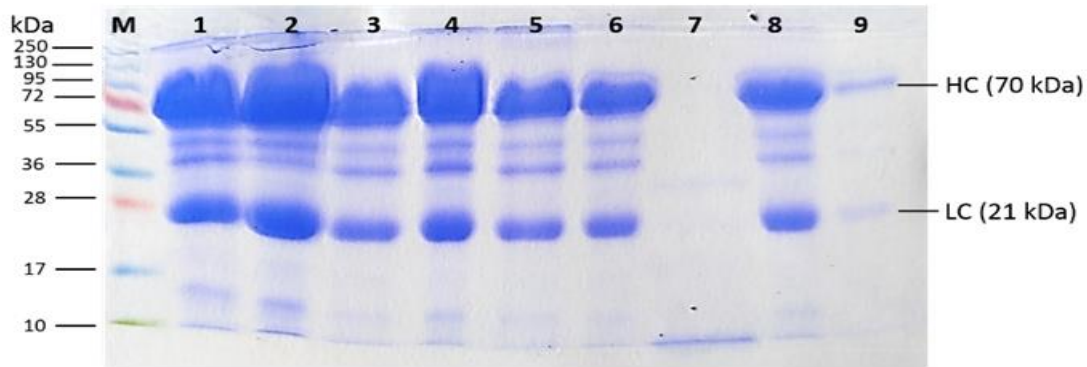

Figure 2: Characterization results in the SDS PAGE gel of The IgY against streptococcal infection protein molecule incubated for 60 minutes at various temperatures in the presence of protease enzymes. (M) Standard 250 kDa protein marker. (1) IgY suspension stored at ideal conditions of −20°C. (2–5) IgY suspensions were incubated at 25°C (2), 45 °C (3), 60°C (4), and 80°C (5). (6–8) IgY suspension resulted from incubation in non-enzyme solution (6), the presence of pepsin (7), and the presence of trypsin (8). (9) Microencapsulated IgY suspension with the alginate-chitosan solution as a positive control. (HC) IgY heavy chain. (LC) IgY light chain.

Based on six days of observation, the incubation of IgY at various salinities and water pH did not result in any qualitative changes in the molecular weight of IgY. The results showed that the heavy and light chains of IgY were still detected compared to the control IgY stored under ideal conditions and the microencapsulated IgY. The same results were also found in IgY that were incubated at various temperatures and in the presence of trypsin for 60 minutes. The two IgY chains were still clearly detectable in the characterization stage with SDS PAGE gel. However, the opposite was found in IgY that was incubated in the presence of pepsin for 60 minutes. The SDS PAGE gel with the IgY treated with pepsin did not show the presence of protein bands at the size of 21 kDa and 70 kDa where the light chains and heavy chains of IgY should appear, respectively. This result indicates that the IgY molecule was lost and could not be detected on SDS PAGE gel.

The results of the ELISA tests for the IgY against streptococcal infection based on treatments with different levels of water salinity, pH level, temperature, and the presence of protease enzymes are presented in Tables 1, 2, 3, and 4. The P/N value showed a result of 7.880 with an average negative control OD value of 0.078. These values validate the results from the ELISA tests. The cut-off value of the positive sample on the water salinity parameter is 0.641; water pH is 0.642; temperature was 0.710; and the presence of a protease enzyme was 0.759.

Table 1: The indirect ELISA results for the IgY against streptococcal infection at various water salinities.

Incubation duration	S/P ratio	S/P %	Interpretation*
	Water salinity 0 ppt		
Day 2	0.895	89.5	Positive
Day 4	0.885	88.5	Positive
Day 6	0.878	87.8	Positive
	Water salinity 15 ppt		
Day 2	0.918	91.8	Positive
Day 4	0.897	89.7	Positive
Day 6	0.880	88.0	Positive
	Water salinity 30 ppt		
Day 2	0.897	89.7	Positive
Day 4	0.890	89.0	Positive
Day 6	0.888	88.8	Positive

* The cut-off value of the positive sample is ≥ 0,641

Table 2: The indirect ELISA results for the IgY against streptococcal infection at various water pH.

Incubation duration	S/P ratio	S/P %	Interpretation*
	Water pH 6.5		
Day 2	0.921	92.1	Positive
Day 4	0.897	89.7	Positive
Day 6	0.890	89.0	Positive
	Water pH 7.5		
Day 2	0.905	90.5	Positive
Day 4	0.893	89.3	Positive
Day 6	0.884	88.4	Positive
	Water pH 8.5		
Day 2	0.912	91.2	Positive
Day 4	0.910	91.0	Positive
Day 6	0.867	86.7	Positive

* The cut-off value of the positive sample is ≥ 0.642

Table 3: The indirect ELISA results for the IgY against streptococcal infection at various temperatures.

Incubation duration	S/P ratio	S/P %	Interpretation*
	Temperature 25°C		
Minute 20	0.920	92.0	Positive
Minute 40	0.915	91.5	Positive
Minute 60	0.893	89.3	Positive
	Temperature 45°C		
Minute 20	0.880	88.0	Positive
Minute 40	0.876	87.6	Positive
Minute 60	0.875	87.5	Positive
	Temperature 60°C		
Minute 20	0.878	87.8	Positive
Minute 40	0.848	84.8	Positive
Minute 60	0.853	85.3	Positive
	Temperature 80°C		
Minute 20	0.870	87.0	Positive
Minute 40	0.636	63.6	Negative
Minute 60	0.631	63.1	Negative

* The cut-off value of the positive sample is ≥ 0.710

Table 4: The indirect ELISA results for the IgY against streptococcal infection against the presence of protease enzymes.

Incubation duration	S/P ratio	S/P %	Interpretation*
	Non-enzyme		
Minute 20	0.898	89.8	Positive
Minute 40	0.893	89.3	Positive
Minute 60	0.884	88.4	Positive
	Pepsin		
Minute 20	0.788	78.8	Positive
Minute 40	0.563	56.3	Negative
Minute 60	0.465	46.5	Negative
	Trypsin		
Minute 20	0.954	95.4	Positive
Minute 40	0.850	85.0	Positive
Minute 60	0.760	76.0	Positive

* The cut-off value of the positive sample is ≥ 0.759

The results from the incubation of IgY at water salinity levels of 0, 15, and 30 ppt for six days showed positive results as IgY could still be detected by the ELISA test. The decrease in S/P % obtained from incubation in water salinity levels of 0, 15, and 30 ppt on the sixth day of observation were 12.2%, 12%, and 11.8%, respectively.

The results from the incubation of IgY at various water pH levels of 6.5, 7.5, and 8.5 for six days showed positive results in which IgY can still be detected by the ELISA test. The decrease in S/P % obtained in incubation at pH 6.5, 7.5, and 8.5 on the sixth day of observation were 11%, 11.6%, and 13.3%, respectively.

The results from the incubation of IgY at various temperature conditions at 25°C, 45°C, and 60°C for 60 minutes showed that IgY still obtained positive results from the ELISA test. However, the sample that was incubated at extreme conditions at 80°C for 40 minutes obtained negative ELISA test results. The decrease in S/P % obtained in incubation at 25°C, 45°C, 60°C, and 80 °C at the 60th minute were 10.7%, 12.5%, 14.7%, and 36%, respectively. These results indicate that the decrease in S/P % is directly proportional to the increase in temperature. Therefore, the higher the incubation temperature, the higher the decrease in S/P % of the IgY against streptococcal infection.

Furthermore, IgY was incubated in the presence of the protease enzyme trypsin to simulate the physiological conditions of the digestive tract of tilapia and in the absence of enzymes for 60 minutes. Both treatments resulted in positive results from the ELISA test. However, the incubation with the presence of the pepsin resulted in negative results starting at minute 40. The decrease in S/P % obtained from incubation under non-enzyme, pepsin, and trypsin conditions at the 60th minute of observation were 11.6%, 53.5%, and 24%, respectively.

Discussion

The results of the characterization of IgY protein in this study are in line with Wibawan *et al*. (2018) who found that by using biocompatibility with NaCl for their purification process, that the light chain of IgY can be detected in the molecular weight range of 21 kDa and that the heavy chain of IgY is in the range of 70 kDa. The microencapsulation results obtained in this study are also in line with the research conducted by Li *et al*. (2007). The results of IgY with microencapsulation on SDS PAGE gel only showed the presence of IgY bands in the light chain and heavy chain without any other protein bands. This is presumably because the microencapsulation can also act as ultrafiltration that is capable of perfectly purifying the IgY molecules so that only the encapsulated protein molecules are left behind.

Protein molecules are not static. They are dynamic and can change due to different conditions and purposes. The dynamics and conformational changes of these proteins allow them to act as nanoscale biological machines that are very important in the physiological system of life (Bu and Callaway, 2011). Therefore, IgY's conformation and its surrounding environment can certainly influence its structure as a macromolecule of the protein group.

Based on the test results on the effect of water salinity, it can be seen that the IgY against streptococcal infection can still be detected after six days of incubation in various salinity levels. The tested salinity levels represented various types of water, namely freshwater (0 ppt), brackish water (15 ppt), and saltwater (30 ppt) (NOAA, 2021). Moreover, the S/P ratio measured using the ELISA test showed a positive result, thereby indicating that IgY was stable at various water salinity levels for six days. Huang *et al*. (2012), through an in silico experimental approach with molecular dynamics simulation methods, reported that salt molecules at various concentrations did not significantly affect the conformational complex of bond interactions between human Immunoglobin G1 (hIgG1) and protein A from *Staphylococcus aureus* (SpA). Therefore, both proteins could still bind stably in the salt solution. This is indicated by the average root-mean-square deviation (RMSD) SpA in a stable NaCl solution ranging from 2.6–3.3 Å, which is only slightly different from the RMSD value of the SpA protein in water (2.7 Å). This is also thought to have occurred in the IgY against streptococcal infection produced from *Enterococcus faecalis* in this study.

The existence of non-polar interactions and electrostatic interactions when a protein is in a salt solution can increase the contribution of bond-free energy to several critical residues in the protein so that the binding affinity of the protein becomes stable. Shimizu *et al*. (1992) stated that in high salt conditions or the addition of a stabilizing reagent can increase IgY

resistance from high temperatures, extreme acid pH, and high pressure. Thus, although there may be a conformational change in the fragment of antigen-binding (Fab) and the fragment of complement (Fc) of the IgY against streptococcal infection due to its incubation in saline solutions of various concentrations, the effect is not significant enough to be detected in biological function testing by the ELISA test.

The stability of the IgY against streptococcal infection at water pH levels of 6.5, 7.5, and 8.5 at room temperature (25–28°C) was tested and no negative result from the ELISA test was seen during six-day incubation. This finding is in line with the study reported by Punyokun *et al*. (2012), which stated that the IgY against anti-*Vibrio harveyi* can be stable at natural pH (6–8) levels with an incubation duration of 4 hours but will begin to decrease when incubated at a pH below 4 and ultimately lose its activity at pH 2. The IgY against anti-*Vibrio harveyi* activity will also decrease at pH 10, and the percentage of activity will be constant at pH 11 (63%) and 12 (42%) after incubation for 4 hours. Moreover, Huang *et al*. (2012) also reported that the affinity of SpA protein with new hIgG1 would decrease at pH 3.0 and strong electrostatic rejection begins at a low pH (3.0) and becomes dominant between the SpA protein and hIgG1. Then, the binding affinity that arises between the two proteins would become weak and unstable. Li *et al*. (2007) also stated that IgY could be significantly damaged when incubated at pH 1.2. Shimizu *et al*. (1988) further reported that the conformational changes of the protein surface and the destruction of the Fab region of the immunoglobulin responsible for the antigen attachment site could also occur due to the influence of an acidic pH environment. Additionally, Jaradat and Marquardt (2000) found that IgY was still stable when stored at 21°C and 37°C for six weeks and was stable enough without affecting its activity if the shelf life was extended to 14 weeks with or without protective materials. However, the IgY molecule will lose its activity if stored at a temperature of more than 50°C for more than 14 weeks. This finding is in line with the current study, which showed that the IgY against streptococcal infection that was incubated at various salinities and water pH levels in an aquaculture environment for six days at room temperature (25–28°C) could still have its heavy and light chains bands detected in the SDS PAGE gel and obtain positive results from ELISA tests. The results showed that IgY can be stable in the water pH levels for tilapia aquaculture environment.

Furthermore, the results showed that the optimal pH conditions of the water in tilapia culture (6.5–8.5) did not significantly affect the relative decrease in the IgY against streptococcal infection activity and that the IgY structure remained stable when it was incubated for six days in an aquatic environment. Likewise, the stability of IgY at various salinities indicates its

potential as a prophylactic or therapeutic agent that can be applied to various fishery commodities in various types of aquatic habitats, such as freshwater, brackish water, and saltwater. Therefore, the biological properties of the IgY against streptococcal infection, which is relatively stable at various water salinity and pH levels, can be used as a foundation for further research on its potential utilization to eradicate infectious diseases in aquaculture ecosystems.

Moreover, due to its stability, it can also be used in fish pellets as a passive immunity measure. This is not only intended for fish or aquaculture commodities but also for wild fish around the aquaculture who can eat the pellets debris, or the remaining pellets that were not consumed by the aquaculture commodity or fall to the bottom of the water. This is expected to cause a domino effect on the immune system of wild fish as they could be carriers of bacteria that cause streptococcal infection without clinical symptoms. By consuming the leftover pellets, the pathogens in these wild fish can be neutralized due to the action of IgY.

The incubation of the IgY against streptococcal infection at 80°C showed a decrease in S/P % of 36.4% at 60 minutes of incubation when compared to the results of incubations at 25°C, 40°C, and 60°C, which is only 10.7–14.7%. This decrease causes a negative interpretation of the results and suggests that IgY has lost its biological function and cannot be detected on the ELISA test. This is in line with the findings reported by Punyokun *et al*. (2013) who found that incubation at a temperature of 80°C for 2 minutes can cause the percentage of IgY activity to decrease. The decrease in the percentage of activity began to occur when the temperature reached 70°C. Additionally, Shimizu *et al*. (1988) stated that a temperature of 70°C with an incubation period of 15 minutes can cause a decrease in the activity of IgY protein, as this molecule would begin to experience very severe denaturation when the incubation temperature reaches 75°C.

Heat is one of the factors that can cause proteins to be denatured. Changes in kinetic parameters can cause protein denaturation due to heat. Skipnes *et al*. (2008) reported that based on an analysis using the differential scanning calorimetry (DSC) method, heating protein in fish muscles to a temperature of 80°C can reduce the residual denaturation enthalpy (ΔH) to a value of 0.078. Siswoyo (2006) also conducted the same test for other proteins measuring 30 kDa from *melinjo* seeds at 80°C and found that there was no peak temperature or endothermic response with a residual value of ΔH=0. This indicated that the protein from the *melinjo* seeds was also denatured at that temperature. The absence of an endothermic response measured as ΔH indicates that the protein molecule has been damaged and is non-functional due to the absence of

heat being absorbed or released by the system or into the system.

The decrease in the relative activity of the IgY against streptococcal infection due to incubation at 80°C in this study could also be due to changes in the ΔH parameter. This could happen because based on a study conducted by Hatta *et al*. (1993a), the DSC data analysis they conducted on a 5% solution of IgY suspension in distilled water with a heating rate of 1°C per minute showed a single peak as the maximum temperature point for endothermic denaturation (Tmax) for IgY protein at 73.9°C. For comparison, the Tmax for rabbit IgG protein was at a temperature of 77.0°C. These findings indicate that at a temperature of >73.9°C, the IgY molecules will be wholly denatured and lose their biological function.

In this study, there was also a decrease in the P/N ratio at 80°C incubation treatment in minutes 40 and 60. However, the qualitative evaluation through SDS PAGE gel found no change in molecular weight. These results indicate that heat only changed the conformation of the active site of the fragment of antigen-binding (Fab) in IgY, resulting it to become non-functional and unable to attach to the antigens when the ELISA was conducted, and cause the negative results. However, the high temperature did not change or decompose the amino acid residue composition of the protein into smaller residue pieces, therefore, the molecular weights of the heavy chain (70 kDa) and light chain (21 kDa) of IgY could still be detected at their band position. Tilton Jr *et al*. (1992) explained that based on observations of x-ray crystallographic diffraction data, the molecules of a protein would expand with increasing temperature, and this expansion was linear. This development was mainly due to the repackaging of the protein molecular structure based on data analysis of the distribution of the Debye-Waller protein factor, which was observed to expand and shift to a higher value with increasing heating temperature.

The results of the analysis of the relative activity of IgY to the presence of a protease enzyme in the form of pepsin caused a decrease in S/P % of 53.5% during the 60-minute incubation duration, which caused the interpretation of the ELISA test results to be negative or undetectable. These results align with the qualitative evaluation of IgY molecules by using SDS PAGE gel. The qualitative results showed that no bands were found on the molecular weights at 70 kDa and 21 kDa, which are the size of the heavy chain and light chain of IgY protein, respectively.

Pepsin is a kind of acidic proteinase enzyme that works optimally at low pH (Hartley, 1960). Barrett (1980) also classified pepsin as an endopeptidase group in the form of aspartate proteinase, which works optimally in the pH range of 2–7. This indicates that the potential substrate of the enzyme is an aspartate residue. Eisen (1973) described that aspartate proteases, such as pepsin and papain, can cleave IgG

molecules around the disulfide bond region between Fab and Fc and that this binding region is very susceptible to the catalytic activity of the two aspartate proteases. This causes the cleavage of the IgG molecule into separate Fab and Fc fragments. This condition can cause a conformational change of the active site that can be recognized and bound to the second antibody labelled with HRP so that the IgY against streptococcal infection cannot be detected when evaluating it with the ELISA test. Hatta *et al*. (1993b) and Punyokun *et al*. (2013) also found that the highest susceptibility of the IgY molecule to the presence of the pepsin during incubation was at pH 2 with an incubation period of 1 hour.

The insignificant decrease in the IgY against streptococcal infection incubated in the presence of trypsin is thought to occur because of the limited number of target residue of the enzyme in this molecule or lack of target residues at the potential site for binding (Fab or Fc). This did not interfere with the binding of IgY to antigens in the Fab region or binding to secondary antibodies that have been labelled with HRP. Therefore, the IgY against streptococcal infection could still be detected as high by ELISA. The same finding was also reported by Punyokun *et al*. (2013), where no cleavage occurred in the IgY protein chain due to the influence of the trypsin. Trypsin is classified as a serine protease active at pH 7–9 with a target substrate of serine residues (Barrett, 1980). Hatta *et al*. (1993b) stated that the IgY molecule is resistant to the inactivation process conducted by proteolytic enzymes such as trypsin and chemotrypsin but is very susceptible to the presence of pepsin. Moreover, Jaradat and Marquardt (2000) also reported that the trypsin at pH 7.5 had no adverse effect on IgY.

The use of sorbitol, glucose, and sucrose to as much as 30% each was able to protect the decrease in IgY activity due to high temperature (Punyokun *et al*. 2013), and the microencapsulation technique using chitosan-alginate was able to protect the relative activity of IgY against the influence of pH and the presence of enzymes in the digestive tract (Li *et al*., 2007). The whole egg yolk protected IgY activity by 100% when heated to 60°C, but only 7% left behind when heated to 80°C. This indicates that egg yolk as a natural matrix can maintain the stability of IgY activity for temperatures up to 75°C and performs relatively better compared to other protectors, such as various types of sugar and formula milk (Jaratat and Marquardt, 2000). These three methods can be considered for the packaging aspect of IgY so that its stability can remain optimal during the oral administration of fish pellets. By increasing the concentration of IgY up to 2–3 times in the pellet formulation, avoiding heating to 80°C for more than 60 minutes, and using whole egg yolks as a protective natural matrix in additive mixtures, pellets can also be used as alternative efforts in overcoming the disease.

Based on the results could be concluded that the water salinity levels of 0, 15, and 30 ppt and optimum water pH levels in the tilapia aquaculture environment (6.5, 7.5, and 8.5) did not affect the molecular weight of IgY based on the qualitative observations and positive results from ELISA tests obtained. However, heating to 80°C and the presence of pepsin greatly affected the biological function of the IgY molecule and caused the ELISA test result to be negative. However, IgY's molecular weight could still be detected well without any change in molecular weight on the SDS PAGE gel despite its incubation at a temperature of 80°C. The information presented in this study can be used an essential reference in the production of prophylactic and therapeutic pellets that contain IgY which could greatly optimize the potential of fish and aquaculture.

Acknowledgements

The authors would like to thank the Master to Doctorate Program for the Superior Undergraduate (PMDSU) Scholarship Batch IV from the Directorate General of Strengthening of Research and Development, Ministry of Research and Technology/National Research and Innovation Agency of the Republic of Indonesia (Grant Number: 1/E1/KP.PTNBH/2021, Date: March, 08th 2021), which has fully funded this research and Tekad Mandiri Citra, Inc., Bandung, Indonesia for providing some materials during the research. This article is part of a dissertation.

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