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LYSOZYME LEVEL DURING ACUTE INFECTION OF BACTERIUM *Aeromonas salmonicida* SUBSP *Salmonicida* IN HALIBUT AND ATLANTIC SALMON

Devadason C*

Institute of Aquaculture, University of Stirling, Scotland, UK

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ABSTRACT

Lysozme, a humoral defence protein, played an important bactericidal activity in Atlantic salmon and halibut. Turbidometric and bactericidal killing assays were used to determine the lysozyme level and serum killing activity respectively. A challenged experiment in native halibut (*Hippoglossus hippoglossus*) with different doses (10^3,10^5, 10^9) of *Aeromonas salmonidae* (MT423) has been proved that there is no significant relationship between the lysozyme level and bactericidal killing activity ($p>0.005$). Lysozyme level in halibut serum was significantly higher than that of Atlantic salmon serum. Lysozyme activity of serum collected from fish during the summer was found to be significantly higher, ranging from 650 to 850 µg. Halibut serum showed varying level of killing activity (KI) during summer (0.266-0.873) and winter (0.255-1.344) whereas Atlantic salmon had very poor killing activity (0.414 -6.867). There was no correlation between the lysozyme activity of the serum and bactericidal activity in the serum of *A. salmonicida* infected halibut.

* Corresponding author

Department of Zoology, Eastern University, Sri Lanka Chenkalady
E-mail: chand_oo@yahoo.com (Chandravathany Devadason)

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1 Introduction

Fin fish and shell fish farming have been commercialized to fulfill the protein requirement of rapidly arising population and is one of the fastest growing food production sectors in the world, but infections caused by bacteria, viruses, and parasites cost the industry billions of dollars in worldwide losses each year (Ugelvik et al. 2017). Salmon louse infection and economic losses reported by Costello, (2009). Lysozyme is considered to play an important role in humoral defence mechanisms in fish (Lindsay, 1986). These lysozyme are associated with innate immunity, which forms the first line of defense against infections, however, its primary role is lysis of the bacterial cell wall and opsonization (Alhazmi et al., 2014). Lysozyme (mucopeptide-N-acetyl muramylhydrolase) is a low molecular weight cationic protein which hydrolyses N-acetylmuramic-b-1-4-acetyly glucosamine linkages in bacterial cell wall (Ossermann & Lawlor, 1966) particularly sugars moiety of the peptidoglycan of the cell wall of gram (+) ve bacteria causing lysis and seems likely that lysozyme may also play a role in the destruction of some gram (-)ve bacteria (Grinde, 1989). These lysozyme has been detected in blood, mucus and phagocytic cells of fish (Fletcher &White,1973; Studnicka et al., 1986). Further, its presence in the blood of invertebrates was also reported by Barnes (1974). Cheng et al. (1977) used the tubidometric assay to determine the lysoszyme activity in the hemolymph of *Biophalaria glabrate* challenged with heat killed *Bacillus megaterium*. Similarly, Fletcher &White (1973) and Murray & Fletcher (1976) found that lysozyme was consistently present throughout the year in the sera of flat fish, including plaice (*Pleuronectes plattessa* L.), flounder (*Platichthys flesus* L) and turbot (*Scophthalmus maximus* L). Lysozyme levels have been increase to response of microbial attack and physical stress (Ingram, 1980). Mock &Peters (1990) reported reduction in lysozyme concentration in response to stress in rainbow trout. Reddy et al. (2004) and Wang et al. (2005) are employed to the study of lysozyme activity and serum bacterial killing activity (Ourth &Wilson, 1981) and macrophage killing activity (Sharp & Secombes, 1993). Lysozyme isolated from rainbow trout (*Oncorhynchus mykiss*) serum also have bacteriacidal activity against a range of Gram (-)ve bacterial pathogens of fish, including *Vibrio salmonicida*, *Aeromonas salmonicda* and *Yersinia ruckeri* (Grinde, 1989; Rainger & Rowley AF, 1993). *A. salmonicida*, the etiological agent of furnculosis in trout and salmon, has been a worldwide economic threat to intensive culture of the commercial valuable fish for over fifty years (McCarthy & Roberts, 1980). *A. salmonida* subsps *salmonicida* is gram (-)ve bacteria , non-motile, fermentative rods which produce cytochrome oxidase and catalase. It can be differentiated on the basis of their biochemical properties and the ability to produce a brown water-soluble pigment when culture on media containing tyrosine (Austin & Austin, 1987). This study was designed to understand the antibacterial properties of lysozyme against *Aeromonas salmonicida* gram (-)ve virulent bacteria.

2 Materials and Methods

2.1 Collection of Serum and Mucus from healthy fish

The healthy halibut (n=11) and Atlantic salmon (n=11) were obtained from the Scottish office of Agriculture Environment and fisheries department (SOAEFD) field station, Scotland and were lightly anaesthetized by immersion in benzocaine (ethyl-p-aminobenzoate). Samples of mucus were scraped from the dorsal surface using a scalpel and store at -20^{0}C until use. Blood samples were taken from the caudal vein using a 23G needle and syringe and allowed to clot at room temperature (RT). The blood samples were then centrifuged at 4000g for 15 min, the serum collected and stored at -20^{0}C until required for assay.

2.2 Infectivity trial

Two year old halibut, weight range from 250g-300g were reared in seawater in 1 m diameter plastic tanks. They were fed daily on commercial dry pellets and appeared to be healthy with no signs of disease. The effect of intraperitonial injection of three doses of *A. salmonicida* viz., 10^9, 10^5, 10^3 cells/fish was examined. There were four fish per test and control group fish which were injected with sterile phosphate buffer solution (PBS). Each group of fish was maintained in seawater in separate 0.75m diameter circular tanks with continuous aeration. To reduce the risk of cross - contamination, the tanks were covered with polythene sheeting. The temperature of the seawater was monitored throughout the experiment. Prior to the infection of bacteria, the fish in each group were lightly anaesthetized as described above, and a 1 ml sample of blood was taken from the caudal vein using 23G needle and syringe, to enable measurement of pre-infection levels of lysozyme. Each fish was then individually dye-marked using a pan-jet loaded with alcian blue, and injected intraperitonially with 100microlitre of the appropriate dose of *A. salmonicida* for test fish, or sterile PBS for control fish. Blood samples were taken as described above, after 4 and 8 hrs. After 24hrs the fish were killed and bled using a vacutainer and needle (Greiner). The blood samples were allowed to clot at RT, and were then centrifuged at 3000g for 10 min to collect the serum. Samples were stored at 4^{0}C during the trial, and on return to the laboratory, were stored at -20^{0}C until required for assay. The serum samples from individual fish in each group were assayed for lysozyme activity and bactericidal activity using the turbidometric assay and serum bactericidal assay Pech (1985).

2.3 *Turbidometric Assay*

This assay was a modification of the method of Parry et al. (1965), which is based on a decrease in optical density (OD) or

absorbance due to the lysis of bacterial cells by lysozyme. Before this assay was applied, it was optimized. *Micorcoccus lysodeikticus* (80mg/ml in 0.05M sodium phosphate buffer, pH 6.2) was added to varying concentration of hen egg white lysozyme as four replicates in a microtitre plate. Sterile PBS added *Micorcoccus lysodeikticus* was used as a negative control and sterile PBBS alone as a blank. The decrease in OD per minute, measured at 540nm and 370C was recorded using a Dynatech MR 7000 microplate reader. Lysozyme was measured in terms of units, where one unit is equivalent to a decrease in OD of 0.001 per minute (DOD/min =0.001). Lysozyme activity of serum and mucus was determined using this assay. Sterile twofold dilutions of each serum and mucus samples, range from 1:5 to 1:5120, at 100microlitre per well were set up. The *Micorcoccus lysodeikticus* at a concentration of 0.4mg/ml was dispersed by a Dynatech MR 7000 reagent dispenser at 100microliter per well. The plate was shaken for 20 sec and the OD read at 1 min intervals at 540nm and RT over a 5 min period.

2.4 *Culture of Aeromonas salmonicida (MT 423)*

A. samonicida (MT423) , which is highly virulent , A layer possessing form, was obtained from the SOAEFD, Aberdeen , and cultured on tryptone soya broth (TSB, Oxoid) was seeded from the agar culture, and incubated at 22^0C for 16 h to reach log phase growth. The bacterial cells were pelleted by centrifugation at 2000g for 10 min at 22^0C, and washed twice in sterile PBS. The cells were then resuspended in sterile PBS and aggregated bacteria were dispensed by passage through a 26G needle. The bacterial suspension was measured spectrophotometrically and adjusted to give a density of 10^9 cells/ml in PBS (OD=1.0at 540nm).

2.5 *Serum Bactericidal Assay*

This assay utilizes the reduction of the tetrazolium dye MTT by bacterial dehydrogenases as an indicator of bacterial viability. This reduction is directly proportional to the number of viable bacteria present. Measurement of a decrease in MTT reduction after incubation with serum is an indication of serum killing. Each serum sample to be tested diluted as 1:10 in sterile PBS and heat inactivated samples of each sera (50^0C for 1hr) in duplicates at a volume of 50 microliter were added respective wells in the microtitre plate. Once serum samples were loaded, the microtitre plate was exposed to UV radiation for 1 hr to sterilize the serum and reduce the risk of bacterial contamination. Following this, 100 microliter of the bacterial suspension was added to each test well using the Dynatech MR7000 reagent dispenser and the plate was shaken for 20s. A 100 microliter replicates of 1:10 serum diluted with PBS and 100microliter replicates of bacteria suspension were used as controls. A 150microlitre replicates of sterile PBS was used as blank. For each assay, two identical plates were prepared, plate A 0hr and plate B for 1 hr were incubated at 18^0C. After the set incubation time, the microtitre plate was read at 630nm on a Dynatech MR 7000 microtitre plate reader to measure the background absorbance. Immediately, 10microlitre of MTT (5mg/ml in dH2O) was added to each well, the plate was shaken, and after exactly 15 min , OD was recorded. The reduction in OD after 1 hr is an indication of the killing potential of the serum (Pech, 1985).

The killing index (KI) of each serum was calculated from the equation

$$KI = \frac{\text{MTT reduction after 1h}}{\text{MTT reduction after 0hr}}$$

Where KI is less than1.0, the serum show the killing activity

2.6 Statistical Analysis

Means and standard errors of the means were calculated for each data set and compared using the Student's t test. A one-way analysis of variance was used to analyze the data for degree differences between population data for lysozyme activity. Significance was measured at a $P<0.05$ level.

3 Results

The lysozyme and bactericidal activities of the serum of Atlantic salmon and halibut showed no significant correlation between both fish ($P>0.01$). The lysozyme activity of serum of non-immunized salmon showed variation in serum lysozyme activity between the individuals measured. Of the fish examined (n-11), seven showed low lysozyme activity from 80µg to 128 µg whereas halibut serum collected from the fish during winter showed between 84-276µg. In comparison, the lysozyme activity of serum collected from fish during the summer was found to be significantly higher in all samples measured (n=11) , ranging from 650 to 850 µg(Table 1)

Of the salmon serum tested, only four serum samples showed bactericidal activity (KI<1.0), ranging from 0.90 to 0.40, and the excessively high index may have been due to experimental variation of error within the assay. Halibut serum collected in winter showed bactericidal activity, with killing index of around 0.60 to 0.25 and three samples did not exhibit serum killing. In comparison, serum collected in summer showed bactericidal activity of varying levels, with killing index of from 0.09 to 0.25 (Table 2). At pre-injection level, all fish showed high levels of lysozyme activity of around 750 µg, the control fish showed remained constant 4 hr after injection with sterile PBS and decreased significantly after 2h around 350µg (Table3). The lysozyme activity of serum of the fish infected at a dose of 10^3 cells/fish showed significant increases in lysozyme activity, after

24hr to a level of around 1300µg. In the fish infected at a dose of 10^5cells /fish, the mean lysozyme activity levels indicated that there was no significant difference throughout the 24hr of period of trial. In the lysozyme activity at a dose of 10^9 cells /fish, have some variation between individuals measured. The overall results showed no significant difference in mean lysozyme activity over 24hr period of the trial (Table3).

At pre-infection with sterile PBS, bactericidal activity of serum of control fish was shown with a killing index of around 0.060. The serum of the fish infected at a dose of 10^3 cells/fish showed pre-infection bactericidal activity of varying levels, from killing index of 0.90 to 0.30. Following infection, there was variation in killing potential between the individual fish measured, however, 8hr following infection, all the fish showed bactericidal activity of varying levels from a killing index of 0.85 to 0.25. All the fish infected at a dose of 10^5cells/fish showed bactericidal activity with a killing index from 0.70 to 0.30, however, after a 24h period none of the fish showed significant bactericidal activity. The mean bactericidal activity shows a general upward trend in the killing index over 24hr, indicating a loss of serum killing potential by the fish. The fish infected at a dose of 10^9 cells/fish showed a similar pattern to those infected at 10^5cells/fish. However, the mean levels showed a general reduction in serum killing ability over a 24 hr period (Table 4). There was no observed correlation between the lysozyme activity of the serum and bactericidal activity in the serum of *A. salmonicida* infected halibut.

4 Discussion

This study was designed to understand the antibacterial properties of lysozyme against *Aeromonas salmonicida* gram (-)ve virulent bacteria. As lysozyme can be stress related factor, handling, transporting, anasthetising and withdrawal of blood can induce changes in levels of lysozyme. In control fish lysozyme activity decreases possibly due to stress. Mock &Peters (1990) have shown that atypical response to stress in rainbow trout is a reduction in lysozyme concentration, as it was observed in control halibut. However, when bacteria were injected into the peritonial cavity of halibut, the complement cascade is activated by the antigens and lysozyme activity in serum increases. The variation in lysozyme levels in challenged fish may be due to the function of the non-specific humoral responses. The serum bactericidal activity shows no correlation with lysozyme activity in this study. High level of killing activity may be due to the activation of complement proteins due to alternative pathway. Lysozyme activity also gradually decreases throughout the experimental period suggesting that the lysozyme was being metabolized during the infection. It has been noted that 10^9 cells/fish of *Aeromonas salmonicida* is a lethal dose for halibut (Bricknell et al. 2006) and there was no mortality over the 24hr period in halibut. At 10^9 cells/fish injected, possibly due to bacterial toxins suppressing the

Table 1 Lysozyme level (µg) and the serum bactericidal activity (KI) in halibut ±serum collected during winter and summer.

Number	Winter Season		Summer Season	
	Lysozyme (µg)	KIndex (KI)	Lysozyme (µg)	K. Index (KI)
1	91±05	0.507	748±07	0.722
2	276±04	1.344**	732±03	0.658
3	276±.02	0.552	756±05	0.357
4	268±00	1.166**	648±02	0.573
5	126±10	0.403	728±04	0.873
6	119±11	0.336	640±06	0.266
7	244±01	0.627	756±12	0.426
8	84±04	1.152**	736±15	0.717
9	142±06	0.365	704±13	0.293
10	119±07	0.357	732±19	0.329
11	144±12	0.255	652±22	0.656

Value presented mean± SD, ** indicates the non-killing activity of serum, K<1 -Killing activity of serum

Table 2 Lysozyme level (Microgram) and serum bactericidal activity (KI) of naïve Atlantic salmon serum

Fish	Lyoszyme level(µg)	Killing Index(KI)
1	13±08	0.414
2	19±12	1.727**
3	15±03	0.723
4	12±04	1.000**
5	12±08	1.689**
6	120±23#	1.553**
7	112±16#	4.442**
8	17±07	6.867**
9	23±12	1.131**
10	80±24	1.420**
11	128±27#	1.325**

# indicates that only three fish show high level of lysozyme and the sign (**) indicates that 9 fish of total 11 fish show the non-killing activity

Table 3 The changes of lysozyme level (microgram) during the different doses of *Aeromonas salmonicida* (MT432) challenged experiment in naïve halibut

Number of bacteria challenged	Lysozyme (µg) (0hr)	Lysozyme (µg)04hr	Lysozyme (µg)08hr	Lysozyme (µg)24hr
1.Control	740±08	700±20	551±193	345±03
2.10^3 dose	693±28.9	613±95.2	1070±199	1290±38
3.10^5 dose	732±10.7	1043±484	856±211	959±197
4.10^9 dose	737±41.7	842±221	689±68.2	572±88.9

Control (PBS only injected), Lysozyme level was measured every 4 hours interval, Value presented as mean±SD

Table 4 Serum bactericidal activity (KI) of halibut serum collected from the fish that received different doses of *Aeromonas salmonicida* (MT423) challenged with intraperitoneally

Number of bacterial challenged	K-Index(KI)-0hr	K-Index(KI)-04hr	K.Index (KI)-08hr	K.Index(KI)-24hr
1.Control	0.690±0.032	1.220±0.231	0.554±0.242	0.595±0.01
2.10^3	0.517±0.135	1.359±0.524	0.620±0.134	1.130±0.187
3.10^5	0.441±0.096	0.866±0.117	0.577±0.042	1.084±0.090**
4.10^9	0.670±0.101	1.046±0.103**	0.612±0.093	1.839±0.330**

The serum collected at every 4 hours interval, ** indicates the non-killing activity of serum, Value presented as mean±SD

immune system, the lysozyme activity decreases and the bactericidal activity also decreases. As far as virulent action of *A.salmonicda* (MT 243) is concerned, two virulent factors such as casinolyitc protease and a haemolysin have so far been identified among the extra cellular products (ECP) from MT423(Ellis,1990). If the level of ECP reached lethal levels, the ECP might neutralise or inactivate the lysozyme and other non-specific proteins in serum.

Ellis (1981) pointed out that serum inactivation of ECP was not due to complement activation but rather to a protease inhibitor present in fish serum. Moyner (1993) noted that the protease activity is present in salmon sera and increased during acute furunculosis as it was observed better bactericidal activity in 10^5 cells/fish was injected even though it showed low lysozyme level. Further, Sakai (1983) pointed out complement mediated bactericidal action against *A. salmonicida* is through the alternative pathway. The gradual increase of lysozyme in serum of fish injected with 10^3 cells and sudden increased of lysozyme in 10^5 cells /fish injected fish are suggested that it could be due to activation of lysozyme by stimulation of macrophages due to opsonization.

As lysozyme is believed to play an antibacterial role during infection, causing lysis of bacterial cell walls and opsonization of macrophages, it acts specifically against gram (+) ve bacteria. The results of the present study found that hen egg white lysozyme did not have any bactericidal effect upon Gram (-)ve bacteria *Aeromonas salmonicida* . However, Grinde (1989) demonstrated bactericidal activity of rain bow trout lysozyme upon *A.salmonicida* . As Gram(-)ve bacteria possess LPS membrane around the cytoplasmic membrane. Withholt et al. (1978) stated that lysozyme penetrates the outer membrane in Gram (-)ve bacterium under mild osmotic shock. The outer membrane of gram (-)ve bacteria external to and covalently linked to the peptidoglycan and serves as a permeability barrier. Because of this diffusion barrier, lysozyme would not be able to gain access to the peptigycan. However, the degree of susceptibility under an optimum situation has generally been used as an index of the lysozyme potential as an antibacterial agent (Studnicka et al. 1986). In the present study, turbidometric method was applied to measure the lysozyme level in units and it was found to be the most accurate and convenient method among other methods used. It was found that when the bacteria is lysed by lysozyme activity, the turbid nature of the reaction mixture decreases and, therefore the absorbance decreases. The decrease in optical density gives a measurement of the lysozyme activity (OD/min). Of the fish serum examined, Atlantic salmon shows significantly low level of lysozyme activity in comparison with the lysozyme activity of halibut serum ($P<0.001$) and the lysozyme activity of individual fish from within a single species varied considerably. Lie et al. (1986) pointed out the differences in lysozyme activity between individuals is probably attributable to the different enzyme concentration rather than to the specific activities of different types of enzymes. It appears that lysozyme activity of halibut serum is lower in winter than in summer. Along with other non-specific humoral proteins, the lysozyme levels respond to physical and environmental changes (Ellis,1981). The higher levels of lysozyme activity in halibut in the summer could be due to exposure to high temperatures, which may have reached the upper thermal limit of halibut. With regard to the bactericidal activity of serum against *A.salmoinicida* (MT423), there was shown to be no significant correlation with the lysozyme level in serum.

In Atlantic salmon and halibut, Atlantic salmon showed very poor bactericidal activity (KI>1) whereas in halibut, four fish of eleven halibut did not show bactericidal activity eventhough it posses high levels of lysozyme activity. It is explained why serum did not show effective killing activity against the bacterium *A.salmonicida* which is a virulent and sophisticated salmonid pathogen. It has been suggested that A-layer could act as a major virulence factor (Ellis, 1988) and *in vitro* studies have shown that the surface structure of *A.salmonicida* provides good protection against complement activity. Both the A-protein and long polysaccharide O-antigen chains of the LPS contribute to this protection, presumably by low levels of the non-specific activation and non-specific humoral proteins or by minimizing access of the various components of complement to their target. One important role for the A-layer in virulence appears to be protection of *A.salmonicida* from the bactericidal activity of complement (Munn et al., 1992).

The MIT bactericidal assay (Peck, 1985) used during the study to evaluate serum killing potential has several advantages over other methods. There are considerable savings in time and materials and accuracy is proved when compared with conventional colony counting. The tetrazolium dye, MIT (3-4-5-di-methylthioazoyl-2-5-diphenyltetrazoilum bromide), is reduced by dehydragenases from the bacteria and forms a purple formazan that can be easily

quantified by measuring optical density. At 0h, reduction of the MIT is produced by large numbers of living bacteria. This is compared with reduction after 1h incubation, by which time any cell death should have occurred. This is very sensitive assay to detect the growth of bacteria, especially in serum killing and macrophage mediated killing experiments. From this trial it could be proposed that lysozyme was not involved in bactericidal activity against *A.salmonicida* (MT 423) and halibut are most resistance to *A.salmonicida* infection. Bactericidal activity may be due to acute phase proteins or other humoral proteins in serum of halibut. The humoral proteins are reported to be found in blood and tissue fluid of shrimp species. So, these assays could be used to determine the lysozyme activity in commercial shrimp species and also will be worthwhile study to understand the resistance shrimp species which show resistance to wide range of bacterial infection. In addition, lysozyme is a stress related factor, it will be useful to know the environmental and physical stress effect in the culture shrimp species using the changes of lysozyme level.

Conflict of Interest

The authors declare that they have no conflict of interests.

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