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Extraction and Characterization of Lipopolysaccharide from *Aeromonas hydrophila* and Its Effects on Survival and Hematology of the Carp, *Cyprinus carpio*

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Abstract

A chemical compound was extracted from the outer membrane of the gram-negative fish pathogen, *Aeromonas hydrophila* and subjected to sodium dodecyl sulphate- polyacrylamide gel electorophoresis -PAGE (SDS) analysis. Electrophoretic separation showed ladder-like bands and absence of bands with coomassie staining suggest that the extracted lipopolysaccharide (LPS) was a smooth form of LPS, free from any protein contamination. Intraperitoneal injection of LPS to *A. hydrophila*-infected *Cyprinus carpio* significantly enhanced the survival rate, Total Leucocyte Count (TLC), the percentage of neutrophils and monocytes and the antibody titre. Hence, it appears that LPS induces non-specific as well as specific immune mechanism of carps and thus could be used in aquaculture to prevent the occurrence of diseases.

Introduction

Health management of cultivable organisms in aquaculture is of paramount importance in order to prevent mortality leading to heavy economic loss. Chemotherapy, vaccination and other prophylactic measures are generally adopted in this regard. Utilization of chemotherapeutic agents in aquaculture has been extensively studied (Aoki 1992 and Plumb 1995). Chemotherapeutic agents, however, cause problems such as the development of

antibiotic resistant strains of pathogens, residues in organisms and suppression of immunity in host organisms. (Rijkers et al. 1981, Karunasagar et al. 1991). Utilization of vaccines against pathogens is yet another strategy to protect cultivable organisms from specific infectious diseases (Lillehaug 1989;Karunasagar et al. 1991). However, the immunity provided by vaccines may be brief and are restricted by practical application such as inoculation of individual organisms; furthermore, vaccines are specific and may not protect organisms from all the diseases that prevail under culture conditions.

Recently, some chemical components of pathogens may play a significant role in health management in aquaculture when used as prophylactic measures (Mulero et al. 1998). These compounds stimulate the non-specific immune mechanism of fish and other organisms offering protection against a wide variety of bacterial infections (Siwicki et al. 1994, Mulero et al. 1998). There are about 20 such compounds used as adjuvants, vaccine carriers, immunostimulants and others (Anderson 1992). Among the immunostimulants in vogue, LPS extracted from the outer membrane of gram-negative bacteria may be effective in controlling many infectious diseases (Saeed and Plumb 1986, Salati et al. 1987, AL-Habri and Austin 1992). These previous workers used only crude form of the compounds, which could have been contaminated by other substances, hence the effects could have been minimal. Therefore, there is a necessity to study the pure form of LPS on fish. The present paper reports on the extraction and purification of LPS and its effects on survival and hematology of the widely cultured *C. carpio* infected with *A. hydrophila*.

Materials and Methods

Experimental animals

Experimental fish, *C. carpio* were purchased from Manimutharu dam, Manimutharu, Tirunelveli District, Tamilandu, India (Latitude 8' 46; longitude 75°5) and transported to the laboratory in well-aerated plastic bags. They were allowed to acclimatize to laboratory conditions for 15 days and then used for experiments. The weight of the animals ranged between 30 and 40 g. Experiments were carried out in glass tanks with 150 l capacity filled with fresh and clean tap water and changed on alternate days. The water temperature in the fish holding tank was not controlled and it averaged to 30 ± 2°C. The animals were fed with a pellet feed with 35% crude protein, which was prepared in our laboratory.

Experimental compound

LPS was prepared by following the method of Kido et al. (1990). Virulent strain of A. *hydrophila* was inoculated into 250 ml of Luria-Bertain broth (LB) and incubated for 24 hrs at 30°C on a shaker at 250 rpm. The culture was harvested by centrifugation at 10000 rpm for 10 min at 4°C

(Kokusan, Japan). The bacterial pellet was suspended in 16.6 ml of Tri Acetic acid EDTA buffer TAE buffer (40 mM Tris-acetate, (pH.8.5) 2mM EDTA) and mixed with 33.2 ml alkaline solution [containing 3 g of SDS, 0.6 g of Trizma (Sigma), 160 ml of 2N NaoH in 1000 ml of water]. The suspension was heated at 55 to 60°C for 70 min and then mixed with phenol and chloroform in the ratio of 1:1 (V/V). The mixture was spun at 10000 rpm for 10 min at 4°C and the supernatant obtained was mixed with 33.2 ml of water and 8.3 ml of 3 M sodium acetate buffer (pH 5.2). LPS was precipitated by adding twice the volume of ethanol. The precipitate was dissolved in 33.2 ml of 50 mM Tris-HCl (Sigma) (pH 8.0), 100 mM sodium acetate mixed well, and was reprecipitated with twice the volume of ethanol. The final precipitate was dissolved in 8.3 ml of water.

Electrophoresis

SDS-PAGE

To check the purity of LPS, it was subjected to the SDS-PAGE (Laemmli 1970). It included a 4% stacking gel and a 12.5% separating gel. Trisglycin buffer (pH 8.3) was used as electrode buffer. The electrophoresis was carried out using Mini Protean II apparatus (Hoefer, USA). LPS (10ml) sample was mixed with an equal volume of loading buffer which consisted of 0.1M Tris- HCl (pH7.0), 4% SDS, 40% (W/V) sucrose, 2% (V/V) mercaptoethanol and 0.02 % bromophenol blue. The mixture was heated at 100°C for 5 min and 10 to 20 ml sample was loaded into sample well. Electrophoresis was performed at 100V current until the dye front reached the bottom of the gel. Subsequently, the gel was removed and cut into two portions - one portion was used for silver staining to characterize LPS and the other portion was used for Coomassie staining for the detection of any protein contamination.

Detection of LPS by silver staining

Silver staining was carried out according to Tsai and Frasch (1982). After performing electrophoresis, the gel was kept overnight in a fixative containing 40% ethanol and 5% acetic acid in clean plastic box. Next, the fixative was replaced with 0.7% periodic acid in 40% ethanol and 5% acetic acid and oxidized the LPS for 5 min. Subsequently, three 15 min washes were performed using 500 to 1000 ml of double distilled water. Finally, the gel was immersed in fresh staining reagent (150 ml) for 30 min. The staining reagent was prepared as follows: Two ml of concentrated ammonium hydroxide was added to 28 ml of 0.1 N sodium hydroxide and to this 5 ml of 20% silver nitrate (W/V) was added. Transient brown precipitate was formed but it disappeared within seconds. Then 115 ml of double distilled water was added to make up to 150 ml of staining reagent. After staining, three 10 min washes in water were performed. Later, the gel was immersed in 200 ml of formaldehyde developer solution (containing 50 mg of citric acid and

0.5 ml of 37% formaldehyde solution). The LPS in the gel stained dark in 2 to 5 min and the development was stopped by adding water.

Coomassie staining

After electrophoresis, one portion of the gel was removed carefully and stained for 2 to 3 h in Coomasie brilliant blue stain with gentle shaking. After staining, the gel was destained using destaining solution I (50% methanol, 10% acetic acid) followed by destaining with solution II (7% acetic acid, 5% methanol).

Compound application

Thoroughly acclimated animals were taken from the stock and divided into four groups of 10 fish each. Three groups were used for application of compound and one group served as control. The pure LPS was suspended in phosphate buffered saline (PBS, pH 7.2) and heated at 75°C for 30 min to reduce its toxicity. Each test fish was given intraperitoneal injection of pure LPS on the 1st, 7th and 14th days. The experimental groups received 10, 50, and 100 µg respectively of LPS per fish in 0.1 ml of PBS. The mortality rate was recorded daily up to 7 days by counting the number of dead animals in each treatment and control.

Experimental pathogen

The infected fishes were collected from a fish farm at Manimutharu dam, Manimutharu, Tirunelveli district, Tamilnadu, India. They were found with characteristic symptoms of dropsy in the abdomen, blisters, abscesses and hemorragic septicemia particularly in the gills, vent and abdomen. Experimental pathogen from the infected fish was isolated, according to Shome and Shome (1999), by taking swab from gills, liver, heart, kidney and abdominal fluid and streaked on Tryptone soya agar, which was then incubated at 37°C for 24 h. Predominately cream colored, mucoid, round, elevated colony was picked out and reinoculated into LB broth for further biochemical characterization using standard procedure. Based on the comparative biochemical test, the isolated bacterium was identified as *Aeromonas hydrophila* (Table 1). To fulfill the Koch postulate, the pathogenecity test was conducted by intraperitoneal inoculation of live *A. hydrophila* at a concentration of 2.11 x 10^7·CFU·ml into the fish using PBS as control. The same types of clinical signs were observed after two days of inoculation. *A. hydrophila* was then used to find out the LD50 concentration by bioassay method (Saeed and Plumb 1986).

Thoroughly acclimated experimental animals were divided into five groups each containing 10 animals; five different concentrations of pathogen in 0.1 ml of PBS was administered intraperitoneally to animals and mortality was observed for 72 hrs. Bacterial density in each concentration was measured colorimetrically at 620 nm and the number of bacteria present in each concentration was determined by pour plate method.

Challenge study

After determining the LD50 concentration, *A. hydrophila* (2.94 x 10^7·CFU·ml) was used to challenge the experimental animal. To find out the efficacy of the compound against the pathogen, all test fish were infected intraperitoneally with 0.1 ml of known LD50 concentration of *A. hydrophila* on 17th day and the mortality rate was recorded daily for the next 7 days.

Hematological and serological study

To know the effect of LPS on blood cells and serum protein changes of the test animal, hematological and serological studies were done. The blood was collected for study on 0, 7th, 14th and 17th days. Blood sample was collected by cutting the fish with sharp knife at caudal peduncle and a drop placed directly on glass slide for preparation of blood smear, and also blood was collected in watch glass containing EDTA, and used for Total Leucocyte Count (TLC). Blood sample was collected by cutting the fish with sharp knife at caudal peduncle and the serum was separated by centrifugation at 4000 rpm for 10 min and used for serum protein determination by Bradford method (1976).

Table1. Comparison of biochemical characters according to Bergey's Manual of known culture and bacterium isolated from infected animals

Biochemical characters	Known Characters	Present isolated response
Motility	+	+
Indole production	+	+
MR	-	-
VP	+	+
Citrate utilization	D	D
Nitrate reduction	+	+
Casein hydrolysis	D	+
Starch hydrolysis	+	D
Gelatin liquification	+	+
Urea hydrolysis	-	-
Acid from		
Adonitol	-	-
Cellobiose	D	V
Dulcitol	-	+
Fructose	-	ND
Galactose	+	-
Glucose	+	+
Inositol	-	+
Lactose	V	+
Maltose	+	+
Mannitol	+	ND
Raffinose	-	+
Salicin	+	ND
Sucrose	+	ND
Trehalose	+	+
Xylose	-	-
Sorbitol	-	-

V – Variable result, D – Doubtful, ND – Not detected

Vaccine preparation, immunization and serum antibody level determination

Four groups of fish were used for serum antibody level determination. A whole cell *A. hydrophila* vaccine was prepared at a concentration of 2.11 x 10^7·CFU·ml and it was subjected to heat inactivation at 65 ºC for about 1 hr and the heat-inactivated vaccine was mixed with equal volume of Freund's incomplete adjuvant (Sigma USA). The experimental groups which received 10, 50, 100 µg of the LPS on 1st, 7th and 14th days and the control groups were injected with the heat inactivated vaccine on day 17 and the booster dose was given on day 24.

On the 31st day, a blood sample was taken and serum was separated and used for antibody determination by direct slide agglutination followed by antibody titer determination. Fifty µl of serum was taken and serially diluted in 96 well flat bottom micro titer plate using PBS as the diluent (50 ml·well). Live *A. hydrophila* of 50 ml (~ 2.11 x 10^7·CFU·ml) was added to each well. The plate was gently agitated and left for 30 to 60 min at room temperature. The titer was determined using a microscope (45 X). The control consisted of diluents and the sera from unimmunised fish.

Results and Discussion

Isolation and characterization of LPS

The compound LPS used in the present study was extracted from *A. hydrophila* and characterized by silver staining and Coomassie brilliant blue staining, subsequent to SDS-PAGE analysis. The electrophoretic separation of LPS, when observed by silver staining, exhibited a profile with ladder-like bands. No bands appeared upon Coomassie brilliant blue staining. Previously, several authors have extracted LPS from different types of bacteria like *Salmonella typhimurium*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *E. ictaluri* and cytophage-like bacteria (Ingram and Alexander 1980; Saeed and plumb 1986, Salati et al. 1987, Baba et al.1988, AL-Harbi and Austin 1992). LPS could be identified as either smooth or rough type based on the presence or absence of ladder like structure (Tsai and Frasch 1982, Hitchcock and Brown 1983, Hendrick and Sequira 1984, Kido et al. 1990, Sledjeski and Weiner 1991). According to the above authors, the rough form of LPS does not possess a ladder-like structure due to the lack of 'O' specific chain containing repeating units of oligopolysaccharides. On the other hand, the smooth form of LPS has a typical ladder-like structure. The electrophoretic separation of LPS from *A. hydrophila* in the current study exhibited ladder-like bands, which is typical of smooth form of LPS. Further, the staining of SDS-PAGE with Coomassie blue did not show any bands indicating that the LPS extracted from *A. hydrophila* was not only smooth but also free from protein contamination (Fig. 1).

Experimental pathogen

Based on the various biochemical tests performed (Table 1), the organism was identified as motile, gram negative, non-spore forming bacteria.

Survival of carp challenged with A. hydrophila

The test fish treated with LPS showed significantly more survival rate than the control fish. The degree of survival depended upon the dose of the compound. The percentage of survival significantly increased with higher concentrations like 50 or 100 µg of LPS per fish. Control fish recorded 40% mortality; test fish treated with 10 µg exhibited 20% mortality, while 50 or 100 µg treatments did not register any mortality after the challenge. The increased survival of *C. carpio* treated with LPS might be due to protection offered by the compound against *A. hydrophilla.* Lower doses were less effective for *C. carpio* where as doses above 50 mg per fish are required to achieve a survival rate over 50%. Many previous workers have observed increased survival of fish treated with LPS. Baba et al. (1988) reported that carp could be protected against *A. hydrophila* after bath immunization using crude LPS. Intraperitoneal injection of crude LPS given to turbot produced better survival against infection with cytophage like bacteria (CLB) (Al-Harbi and Austin 1992). Salati et al. (1987) found that intramuscular injection of crude LPS produced enhanced survival, antibody titer and phagocytic activity when compared with control animals. Multiple injections of crude LPS to catfish *Ictalurus punctatus*, brown trout *Salmo trutta* produced increased survival and antibody titer over single injection (Saeed and Plumb 1986, Ingram and Alexander 1980). As crude form of LPS was used in all the fore-mentioned studies, the effectiveness of pure LPS was not ascertained. Through the use of a highly purified preparation of the LPS, the impact of LPS on immune protection of fish is thus validated in the current study.

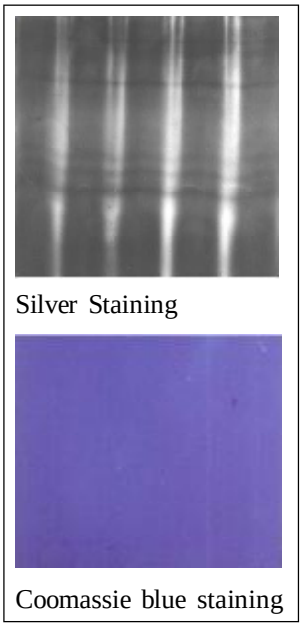

Fig. 1. Detection of LPS by silver staining and Coomassie blue staining after separation by SDS-PAGE.

Total leucocytes count (TLC)

Hematological studies showed that test fish treated with LPS have enhanced TLC than control fish. The total number of leucocytes in control fish did not show significant variation during the experimental period and it averaged to 22.0, 24.8, 27.7 and 28.5 x $10^3 mm^3$ on 0, 7, 14 and 17^{th} day respectively. The number increased markedly in the fish treated with LPS compared to control fish. The highest TLC has occurred on the 17th day in fish injected with 100 mg per fish and it averaged to 44.25 x $10^3 mm^3$. At a concentration of 10 mg

per fish, TLC was 22.0, 29.0, 32.8 and 34.3 x $10^3 mm^3$ on 0, 7, 14 and 17th day respectively. The number increased with increase in duration after challenge in all concentrations and it was due to an increase in neutrophil and monocytes population (Fig. 2). Using glucan, Jorgenson et al. (1993) found that increased total number of leucocytes was due to an increase in the number of macrophages and neutrophils.

Differential count

The differential count did not vary in the control fish throughout the experiment. Monocyte population showed statistically significant change ($t = 5.15$, $n = 6$; $p < 0.01$) between the control and 10 µg LPS treated fish on the 7th day itself. However, the neutrophils showed significant change ($t = 2.35$, $n = 6$; $p < 0.05$) on the 7th day in the highest concentration (100 µg). Among the leucocytes, neutrophil and monocyte were the predominant cell types that showed variation in response to LPS injection. The neutrophil showed the maximum change. These two types of leucocytes are involved in first line of defense mechanism for processing and presenting of antigen in addition to the main function of phagocytosis by neutrophils. Administration of azadirachtin to *Tilapia* increased the population of macrophage and granulocytes (Logambal and Dinakaran Michael 2000). Other leucocytes like eosinophils and basophils declined considerably in all treatments. The lymphocyte count did not show much variation in relation to LPS concentration and period of exposure (Table 2).

Serum protein

In the present study, a reduction in the serum protein level was observed in *C. carpio* treated with LPS. The serum protein of uninfected fish averaged to 3.12 g·100 ml; protein level of control fish, which received 2.33, 1.97, 2.18 g·100 ml on 0, 7th, 14th, and 17th days, respectively. Protein concentration of test fish receiving 10, 50, or 100 mg of the compound was 2.31,

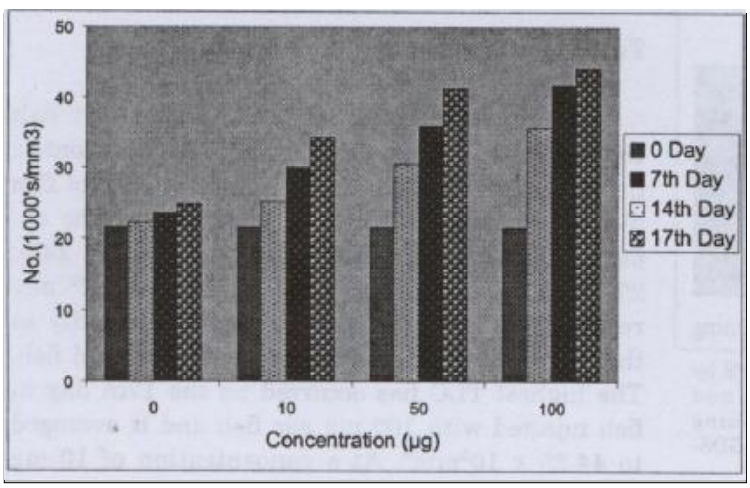

Fig. 2. Effect of intraperitoneal injection of LPS on leucocyte count (no. x10^3· mm^3) in experimental fish *C. carpio*. Each value is the average of ($X \pm SD$) of three observations.

2.74, 2.74 on 7th day, 1.84, 1.96, 1.38 on 14th day and 2.21, 2.32 and 2.06 g·100 ml on 17th day respectively (Table 3). Other authors have also observed such decrease in protein level after LPS treatment and the decrease in the protein level might be due to handling and injection stress (Ingram and Alexander 1980).

Antibody titer

Antibody titer in LPS treated *C. carpio* showed an increase compared to the control fish. The antibody titer was significantly different between the control and experimental fish. The antibody titer in the control fish was 1:8, whereas the titer in experimental fish treated with 10, 50 or 100 mg of compound per fish was 1:32, 1:32 or 1:64 respectively (Table 3). This observation coincides with the results obtained from previous workers. Ingram and Alexander (1980) and Saeed and Plumb (1986) have reported that injection of LPS to brown trout *Salmo trutta* and catfish *Ictalurus punctatus* showed increased titer of antibody. Anderson et al. (1979) reported that in dip immunization of animals, rainbow trout with *Yersina* 'O' antigen or *Aeromonas*'O' antigen exhibited increase in antibody producing cells. The present study reveals that LPS treatment in fish induced both non-specific as well as specific immune mechanisms in carps.

Table 2. Effect of intraperitoneal injection of LPS on differential cell counts (%) in the conntrol and experimental *C. carpio*. Each value is the average of (x±SD) of three observations

Concentration (µg) and day of sampling		Types of Leucocytes (%)				
		Neutrophil	Basophil	Eosinophil	Monocyte	Lymphocyte
Control	0	21.0±0.816[a]	18.30±0.471	17.0±0.816	20.3±0.471[a]	21.66±1.247
	7th	22.3±2.86[d]+	17.0±0.816	16.7±0.471	20.7±0.471[c]*	22.0±0.816
	14th	25.0±3.84[d]+	14.7±0.471	14.0±0.816	23.0±0.816[c]*	22.33±0.942
	17th	25.3±3.29[d]+	12.0±0.816	12.7±0.816	23.6±0.471[c]*	23.66±0.942
10	7th	26.3±2.86[d]+	7.3±1.24	10.0±1.63	32.6±3.26[c]*	22.7±2.49
	14th	26.7±2.49[d]+	4.0±0.816	4.3±1.63	32.6±2.06[c]*	24.7±2.49
	17th	28.3±2.49[d]+	0.3±0.461	1.3±0.47	32.3±2.62[c]*	25.0±4.32
50	7th	30.0±5.09[d]+	1.7±1.24	7.3±1.24	33.6±2.49[c]*	26.0±1.63
	14th	28.3±3.39[d]+	1.0±0.816	1.7±1.24	36.3±4.10[c]*	26.7±2.49
	17th	29.7±1.88[d]+	0	0.7±0.47	39.0±3.74[c]*	25.7±4.02
100	7th	30.3±3.85[b]*	1.0±0.816	4.30±2.60	32.6±2.62[c]*	22.3±2.05
	14th	30.5±2.06[d]+	0	0.7±0.471	36.0±2.44[c]*	23.3±2.49
	17th	31.7±3.29[d]+	0	0	39.3±5.35[c]*	26.3±1.24

a vs b * -significant at 5% level; a vs c * - significant at 1% level; a vs d^+ not significant

Table 3. Effect of intraperitoneal injection of LPS on serum protein (%) and antibody titer in *C. carpio*. Each value is the average of (X ± SD) of three observations

Concentration (µg)	Days			Antibody titer
	Seventh	Fourteenth	Seventeenth	
Uninfected	3.12±0.820	3.12±0.82	3.12±0.820	- -
Control	2.33±0.135	01.96±0.311	2.18±0.077	1:8
10	2.31±0.154	1.84±0.172	2.21±0.094	1:32
50	2.75±0.154	1.96±0.311	2.32±0.145	1:32
100	2.74±0.450	1.37±0.455	2.06±0.016	1:64

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