

**STUDIES ON ANTIMICROBIAL FACTORS IN SALMONID EGGS,
WITH SPECIAL REFERENCE TO COHO SALMON
Oncorhynchus kisutch (Walbaum)**

by

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STUDIES ON ANTIMICROBIAL FACTORS
IN SALMONID EGGS, WITH SPECIAL
REFERENCE TO COHO SALMON
Oncorhynchus kisutch (Walbaum)

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ABSTRACT

Immunity in animals is an important physiological mechanism for protection against infection. In lower vertebrates, including fish, 'non - specific' immune factors appear to be particularly important especially during embryogenesis and the initial period of independent existence.

This study demonstrated the presence of at least three immune factors in the eggs of coho salmon *Oncorhynchus kisutch*: viz, lysozyme, an immunoglobulin-like protein and a lectin.

Eggs of coho salmon were shown to contain lysozyme at a concentration in the yolk of approximately 1900 ug ml⁻¹. Purification of the enzyme was achieved using chitin-coated cellulose and gel filtration. One molecular species of lysozyme was present and it was shown by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to have a molecular weight of 14.5 kD. N-terminal amino acid sequence determination and western blot analysis indicated that the enzyme is c-type lysozyme. Similar concentrations of this enzyme were also found in the eggs of chinook salmon *O. tshawytscha* and rainbow trout *O. mykiss*. The antibacterial properties of the purified lysozyme and hen egg-white lysozyme (HEWL) were studied using selected bacterial fish pathogens as the test organisms. Coho egg lysozyme proved capable of lysing cell walls of *Micrococcus lysodeikticus* (the assay substrate), but showed no such activity against

cell wall preparations of the fish pathogenic bacteria. Furthermore, the lysozyme was bactericidal to *Aeromonas hydrophila*, *A. salmonicida*, and *Lactobacillus piscicola* (the causative agents of haemorrhagic septicemia, furunculosis, and pseudokidney disease in salmonids, respectively) at a concentration of 700 ug ml⁻¹, a concentration approximately one third that found in the yolk of most salmonid eggs. In contrast, HEWL was bactericidal only against *L. piscicola*. *Renibacterium salmoninarum* (the causative agent of bacterial kidney disease), however, incubated with as much as 1900 ug ml⁻¹ of either enzyme for 90 min showed no loss of viability. The data presented indicate that the antibacterial role of lysozyme isolated from salmonid eggs should not be equated merely with its ability to lyse microorganisms, and that the lysozyme has a role in preventing the vertical transmission of most bacterial fish pathogens from mother to progeny.

Western blot analysis of the yolk extract of coho salmon eggs revealed the presence of an immunoglobulin - like protein molecule that reacted with antibodies specific for coho serum immunoglobulin (IgM). Sucrose gradient ultracentrifugation analysis of the yolk extract showed that the molecule is closely related to bovine serum immunoglobulin IgG. This indicates that coho salmon eggs contain a low molecular weight form of serum IgM, perhaps a breakdown product, or a precursor of IgM. The precise function of the reported intra-ovum IgM-like protein is unknown.

The yolk of coho salmon eggs also contained a lectin which proved capable of binding to *A. salmonicida* cells, but showed no such activity with *A. hydrophila*, *Vibrio anguillarum*, and *V. ordalii* (the causative agents of vibriosis in salmonids), or *R. salmoninarum*. The interaction between the lectin and *A. salmonicida* was demonstrated by a test in which the protein was adsorbed onto bacterial cells. Boiling the bacterial cells for 1 h did not alter their ability to adsorb the lectin. The lectin was purified by affinity chromatography on D-galactose-Sepharose 4B, and was shown by SDS-PAGE to be a 24.5 kD protein; it was heat stable and its binding property was inhibited by D-galactose, D-galactosamine, and L-rhamnose. Immunofluorescence staining of whole cells, and western blot analysis of cellular fractions from various phenotypes of *A. salmonicida* revealed that lipopolysaccharide's core region and other soluble proteins or glycoproteins were the bacterial lectin-binding sites. The role of the lectin was investigated by studying the interaction of *A. salmonicida*, in the presence or absence of lectin, with rainbow trout macrophages (M ϕ s). Sensitizing A-layer-negative (A⁻) mutants with the lectin greatly depressed their levels of M ϕ association. In contrast, the phagocytic index of A-layer-positive (A⁺) cells was not affected by the presence of lectin. The data presented suggest that the lectin may play a defensive role through blockage of bacterial uptake by salmonid M ϕ s.

DEDICATION

This thesis is dedicated to my wife, Mirfat H. Habib and children, Martin and Ronnie.

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To my parents I extend my thanks for love and support. I offer my love and deepest gratitude to my wife for helping me get started, keep going, and finish.

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SPECIFIC AND NON-SPECIFIC DEFENSE MECHANISMS OF FISH:

GENERAL INTRODUCTION

Immunity is an important physiological mechanism in animals for protection against infection and the preservation of internal homeostasis. It can be either a non-specific, natural immunity, which is an innate defense mechanism rendering the hosts resistant to infection, or an acquired, specific immunity which is induced in response to a foreign agent. Acquired immunity comprises both humoral responses, which are recognized by the production of antibodies, and cellular responses as exemplified by graft rejection and delayed hypersensitivity.

The serum and other fluids of vertebrates contain a number of substances other than immunoglobulins, that affect the growth of micro-organisms. These substances are predominantly proteins, and many of them probably have their counterparts or precursors in the blood and haemolymph of invertebrates. Most have been referred to as 'non-specific' but are in fact highly specific in that they react with unique chemical groups or configurations. However, because the substances with which they react are common, and because they influence the growth of more than one micro-organism, they have been called 'non-specific'.

Fish also contain naturally-occurring, relatively non-specific factors, many of which display, under various conditions, reactivity with foreign materials. Ingram (1980), has reviewed a number of non-immunoglobulin humoral factors thought to contribute to the natural resistance of fish. These factors are reactive against a vast array of different antigenic determinants and exhibit heterogeneity in their physicochemical and biological properties, like immunoglobulins, and are often termed 'natural' or 'spontaneously produced' antibodies in contrast to 'antigen-induced antibodies'. Such naturally-occurring humoral, immune substances include lysozyme, chitinase, interferon, complement, c-reactive protein, lysins, agglutinins, and precipitins. These substances may fulfill several essential functions, including defense against bacterial and viral infections, participation in the recognition process of foreign tissue transplants, and recognition of foreign entities.

At the time of hatching, the lymphoid system of salmonid fish is still developing and not all of the structures or functions present in the adult are present in the fry. In fact, the specific immune system is not fully mature for several weeks after the salmonid has hatched (Van Loon et al. 1981, Tatner and Manning 1983,1985, Razquin et al. 1990). As many diseases affect young fish (fry), it is important for the embryo and young fry to receive humoral substances, of maternal origin, that will confer protection

until the specific immune system matures. A number of these non-specific defense factors have been identified in fish ova. These include: c-reactive protein (Fletcher and Baldo 1976) and lectins (Krajhanzl et al. 1978). Immunoglobulin (Ig) has also been found in a number of fish ova including carp (Van Loon et al. 1981), plaice (Bly et al. 1986), tilapia (Mor and Avtalion 1988,1990), and rainbow trout (Shors and Winston 1989).

This study was undertaken to investigate the presence of defense factors, namely lytic enzymes such as lysozyme, immunoglobulin (IgM) and lectins in the eggs of coho salmon *Oncorhynchus kisutch* (Walbaum) and to study their interaction with bacterial fish pathogens. Its purpose was to develop a better understanding of why only certain fish pathogens (for example, *Renibacterium salmoninarum* (Evelyn et al. 1984)) are vertically transmitted from parent to progeny via the egg while other fish pathogens with equal access to the interior of fish eggs are not vertically transmitted.

CHAPTER I: LYSOZYME

INTRODUCTION

The defense against pathogenic organisms that infect an animal is first provided by the non-specific immune system (Ingram 1980). One humoral component of this system is lysozyme, sometimes referred to as muramidase or N-acetylmuramide glycanhydrolase, a mucolytic enzyme of leucocytic origin which exhibits antimicrobial properties. The enzyme is widely distributed in nature and is found in animals and plants. It occurs in a large variety of animal secretions, such as mucus and saliva, and in many tissues including blood. In plants, it occurs in the cell vacuoles. (Jolles 1969). The enzyme catalyzes the hydrolysis of a structurally important linkage of the peptidoglycan in the cell wall of bacteria, thus solubilizing this complex polymeric substance. The linkage cleaved by the enzyme is the glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine. This linkage is found in most bacterial cell walls (Salton 1957). Lysozyme has optimum activity over a pH range of 6-7, an isoelectric point between pH 10.5-11.0, and an approximate molecular weight of 14,400 (Osserman et al. 1974). In humans, lysozyme can either have a direct bacteriolytic effect on Gram-negative bacteria, or with the aid of complement and antibody, enhance complemented-mediated bacteriolysis. However, the enzyme also attacks structures containing muramic acid, hydrolyses glycol chitin, and has a restricted degrading effect on chitin (a

linear polymer of N-acetylglucosamine) which is a major component of the cell walls of fungi and the exoskeletons of certain invertebrates. Because of these properties, lysozyme may form part of an intrinsic defense system in many species against parasitic and bacterial infections.

Lysozyme from vertebrates has been investigated extensively (Imoto et al. 1972, Osserman et al. 1974). Hence, birds and mammals have been the source of the enzyme for most studies. Lysozyme has been detected in the serum, mucus, and certain tissues of both freshwater and marine fish (Sankaran and Gurnani 1972, Fletcher and White 1973, Fange et al. 1976, Murray and Fletcher 1976). High activities of the enzyme were found against *Micrococcus lysodeikticus* in the lymphomyeloid (haematopoietic) tissues of elasmobranchs and in the lymph and plasma of teleosts. However, lower activities were demonstrated in the spleens and little or no activity was observed in the Atlantic hagfish *Myxine glutinosa* and in cod *Gadus* spp. Recently, the enzyme was reported to occur in the kidney and digestive tissues of rainbow trout *Oncorhynchus mykiss* (Lindsay 1986, Grinde et al. 1988) and in the kidney tissues of Atlantic salmon *Salmo salar* (Lie et al. 1989). However, the presence of the enzyme in the eggs of fish has not been reported.

Several viral diseases of fish, as well as bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum* - a Gram-positive bacterium, are vertically transmitted from

mother to progeny by way of sex products (cited by Barker et al. 1989). Circumstantial evidence has indicated, however, that the major fish diseases caused by Gram-negative bacteria are not vertically transmitted. This is an important observation since the presence of lysozyme in salmonid eggs could help explain why certain bacterial fish pathogens are not transmitted by the egg.

This chapter discusses the isolation, purification, and partial characterization of a lysozyme from the eggs of coho salmon *O. kisutch* and the occurrence of the enzyme in the eggs of chinook salmon *O. tshawytscha* and rainbow trout *O. mykiss*. The antibacterial properties of the purified enzyme were studied with selected bacteria, often present in the ovarian fluid of salmonids, and which represent a direct potential source of infection for ovulated eggs at the time of spawning.

MATERIALS AND METHODS

Source of materials Unfertilized, non-water-hardened eggs were obtained from ripe, apparently healthy coho salmon females (n=7) that had returned from the sea to spawn in the Capilano River near Vancouver, B.C. The eggs were transported on ice to the laboratory and immediately stored at -20°C. Prior to use, one group of eggs was thawed, homogenized in a tissue grinder at room temperature, and the homogenate filtered through cotton cheesecloth. Another group of thawed eggs was transferred onto absorbent pads of filter paper in a petri dish, to remove the ovarian fluid, and the yolk (EY) was removed using a capillary tube. Both the egg homogenate (EH) and the EY were assayed for lysozyme activity by the lysoplate method (Fange et al. 1976, McHenry et al. 1979).

Lysozyme purification Alkaline chitin was prepared by the method of Imoto et al. (1968) using powdered chitin from crab shells (Sigma). The chitin (5 g) was soaked in 50 ml of 42% (w/v) sodium hydroxide solution at 34°C for 3 h under reduced pressure to remove air bubbles and to allow good penetration of NaOH solution into the chitin particles. The suspension was filtered under vacuum and the cake of alkaline chitin mixed with 3.5 times its weight of finely crushed ice. The mixture was kneaded vigorously by pressing the cake against the wall of the beaker with a glass rod until a homogeneous paste was obtained.

Chitin-coated cellulose (cc-cellulose) was prepared using the method of Imoto and Yagishita (1973). Cellulose powder (5 g-Microgranular, Sigma) was mixed with the alkaline chitin cake. Cold distilled water (80 ml) was added slowly with constant stirring followed by 300 ml of 2M glacial acetic acid. The residue was washed several times with cold distilled water and stored as an aqueous suspension at 4°C.

Lysozyme was isolated from coho eggs by stirring 120 ml of cc-cellulose and 40 ml of EH for 1 h at 4°C. The mixture was then centrifuged (5,860 x g for 20 min) at 4°C and the supernatant discarded. The sedimented material was washed in a Buchner funnel with 5 volumes of 0.02M phosphate buffer pH 6.8 containing 0.5M NaCl and the washings discarded. The enzyme was eluted by washing the matrix with 2 volumes of 0.1M glacial acetic acid. The filtered eluate was dialysed against distilled water (48 h) which removed unwanted water-insoluble egg yolk proteins, leaving behind the water-soluble lysozyme. The water soluble fraction was then dialysed against 0.06M phosphate buffer pH 6.0 containing 0.02M NaCl (PBS) for 48 h at 4°C, and the small amount of precipitate that developed was removed by centrifugation (5860 x g for 20 min) at 4°C. The supernatant (partially purified lysozyme=PPL) was stored at 4°C until used in gel filtration.

Gel filtration One ml of PPL was applied to a column (25 x 1.5 cm) of Sephadex G-100 (Pharmacia). The column was equilibrated with PBS. The same buffer was employed for

elution. Fractions of 0.5 ml each were collected and screened for lysozyme activity by the rapid and sensitive lysoplate method. Total protein was measured spectrophotometrically at 280 nm using the Absorbance monitor UA-5 (Instrumentation Specialties Co., U.S.A). Lysozyme-containing fractions were pooled, dialysed against distilled water, and freeze-dried. The purity and molecular weight of the isolated lysozyme were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). In addition, 1.0 ml of PPL was dialysed against 50 mM Tris/HCl buffer pH 5, boiled for 20 min at 100°C, and the supernatant electrophoresed in SDS-PAGE.

Electrophoretic analysis SDS-PAGE procedures for 4.5% stacking and 12.5% or 10-20% linear gradient separating acrylamide gels under denaturing conditions (with 2-mercaptoethanol), and silver staining of proteins were identical with those outlined in Johnstone and Thorpe (1987). Hen egg-white lysozyme, turkey egg-white lysozyme, and human lysozyme were purchased from Sigma (St. Louis, U.S.A), and solubilized in PBS. The reduced lysozyme samples (2.3 ug well⁻¹) were run at a constant current of 25 mA gel⁻¹ (140 x 125 x 1.5 mm) using a large vertical slab gel electrophoresis unit (Se 600, Hoeffler Scientific Instruments, San Francisco, Calif.). Low molecular weight protein standards (BIO-RAD, Richmond, U.S.A) were included in each run.

Western blotting

Preparation of rabbit antiserum: Antiserum against coho egg lysozyme was produced by injection of a New Zealand white female rabbit with the purified lysozyme mixed with AdjuPrime immune modulator (Pierce, U.S.A). AdjuPrime was ground in a mortar and pestle and suspended in the antigen solution to a final concentration of 2.5 mg ml^{-1} . Approximately 70 ug of lysozyme was injected subcutaneously and the rabbit was allowed to rest for 18 days after which it was boosted with 90 ug of the antigen, followed by 18 ug after 34 days. Blood was collected from the marginal ear vein, allowed to clot at 37°C for 1 h and then incubated overnight at 4°C . The serum was collected and kept at -20°C .

The rabbit IgG antibodies against coho egg lysozyme were further purified and concentrated on a column of protein A-AVID gel (Bio-Probe International, Inc. California, U.S.A). Normal rabbit serum was collected before immunization for use as negative control.

Blotting technique: Discontinuous SDS-PAGE of samples was performed as described above. Prestained markers of low molecular mass (BIO-RAD) were included with each gel. Polypeptides were then transferred electrophoretically onto a 0.45 um nitrocellulose (N/C) membrane (BIO-RAD), using a semi-dry transblot apparatus (2117-250 Novablot LKB, Bromma, Sweden) at 0.8 mA/cm^2 for 90 min. All subsequent manipulations were performed at room temperature. The N/C

membranes were washed briefly in Tris buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5), and then blocked for 1 h with 3% solution (w/v) of gelatin in TBS. The N/C membranes were then washed twice in Tween 20-containing TBS (TTBS) and exposed to a solution of rabbit anti-coho egg lysozyme (250 ug affinity purified IgG in 20 ml of 1% gelatin-TTBS= antibody buffer) overnight with gentle shaking. The N/C membranes were washed twice in TTBS and then exposed to a solution of horseradish peroxidase (HRP) conjugated to goat anti rabbit IgG (BIO-RAD), diluted 1:2000 in antibody buffer for 1h with gentle shaking. The solution was then discarded and the N/C membranes were washed twice in TTBS and once in TBS. The N/C membranes were developed by addition of the HRP substrate (4-chloro-1-naphthol, BIO-RAD). Developed blots were kept in the dark until photographed.

Amino acid analysis Total amino acid analysis of the purified protein was obtained by using the major 14.5 kDa protein band separated by SDS-PAGE and electrophoretically transferred to Immobilon (Millipore Corp., Bedford, Mass.). Samples were hydrolyzed in gaseous HCl (165°C, 1 h) and analyzed by using an Applied Biosystems model 420 amino acid derivatizer analyzer. Derivatized proteins were removed from the reaction mixture by overnight incubation (4°C, dark) in the presence of small pieces of Immobilon. The nylon membrane-bound proteins were removed from the mixture, rinsed, air-dried, and stored at -20°C until analysis. β -

Lactoglobulin was used as a standard to confirm the success of the carboxymethylation reaction.

Heat stability test The test was performed as described by Lie and Syed (1986). Briefly, the PPL from the coho salmon eggs was divided into 2 aliquots, one of which was dialysed against 50 mM Tris/HCl buffer pH 5, the other being dialysed against the same buffer at pH 9. Dialysis was performed for 48 h at 4°C. The two aliquots were heated in glass test tubes in a water bath at 100°C for 20 min. Small samples, drawn from each tube before heating and at intervals of 2 min during heating, were placed on ice before being assayed by the lysoplate method.

Lysoplate For assay of lysozyme, 20 ul aliquots of sample were dispensed into wells (3.5 mm dia. x 4 mm deep) cut into 0.5% agarose (Type 1, Sigma) in 15 cm dia. petri dishes. The agarose contained PBS, and *M. lysodeikticus* (0.6 mg freeze-dried cells ml⁻¹, Sigma). After incubation for 20 h at room temperature in a humid chamber, the diameters of zones of *M. lysodeikticus* lysis were measured and compared to those produced by hen egg-white lysozyme (HEWL, Sigma) standards (50-2,000 ug ml⁻¹) (Fange et al. 1976, McHenry et al. 1979).

Adsorption onto cc-cellulose To further characterize the isolated enzyme, the following experiment was conducted: to 30 ml of cc-cellulose, 5 ml of PPL (initial activity = 700 ug ml⁻¹) was added and the reaction mixture was stirred for 90 min at room temperature. Finally, the suspension was

centrifuged (5,860 x g for 20 min) and the supernatant assayed for lysozyme activity by the lysoplate method.

Optimum pH, temperature, and buffer molarity The effect of pH, incubation temperature, and buffer molarity on the lytic activity of coho egg lysozyme were studied using *M. lysodeikticus* (0.2 mg ml⁻¹) as a substrate. This was found to give an absorbance of 0.5 at 540 nm. Each assay of the three parameters, at both time periods, was done in triplicates; the standard errors were in the range 0-2%, i.e. too small to be included in the figures.

To study the effect of pH, the substrate was prepared in 0.04M citrate buffers (pH 3.0, 4.0, 5.0); and 0.04M phosphate buffers (pH 6.0, 7.0, 8.0). Test solution containing lysozyme was prepared by diluting EH (3:1) in an appropriate buffer (pH 3.0 - 8.0). The mixture was vortexed and the supernatant was collected by centrifugation (5,860 x g for 20 min). Test solution (200 ul) was added to the substrate to give a final volume of 2 ml in a spectrophotometer tube. The reaction was carried out at 40°C. Absorbance was recorded at 540 nm at 0 and 45 min.

To study the effect of incubation temperature, the substrate was dissolved in phosphate buffer (0.04M, pH 6.0). Test solution was prepared by diluting EH (3:1) in the same buffer used for the substrate, and the supernatant was collected by centrifugation (5,860 x g for 20 min). Test solution (200 ul) was added to the substrate to give a final

volume of 2 ml in a spectrophotometer tube. The reaction was carried out at different incubation temperatures (10, 20, 30, 40, 50, 60, 70°C). Absorbance was recorded at 540 nm at 0 and 45 min.

Dependence on molarity was studied by preparing the substrate in phosphate buffer (pH 6.0) of various molarities (0.02, 0.04, 0.06, 0.08, 0.1M). Test solution containing lysozyme was prepared by diluting EH (3:1) in the same range of buffers used for the substrate, and the supernatant was collected by centrifugation (5,860 x g for 20 min). Test solution (200 ul) was added to the substrate to give a final volume of 2 ml in a spectrophotometer tube. The reaction was carried out at 40°C. Absorbance was recorded at 540 nm at 0 and 45 min.

Bacteria and growth conditions The bacterial strains were isolated from the following sources: *Aeromonas salmonicida* (strain #76-30 A⁺, A⁺ LPS⁺, the causative agent of furunculosis), and *R. salmoninarum* (strain #384) were isolated from coho salmon *O. kisutch* at the Department of Fisheries and Oceans (DFO) Quinsam River Hatchery, Vancouver Island, B.C. in 1976 and 1979, respectively. *A. hydrophila* (strain #35, S-layer positive, known to cause haemorrhagic septicemia in salmonids) was isolated from pink salmon *O. gorbuscha* at the Robertson Creek Hatchery, B.C. in 1965. *Lactobacillus piscicola* (strain #B270, a Gram-positive coccobacillus that causes pseudokidney disease in salmonids)

was a generous gift of Dr. J. Fryer (Corvallis, Oregon). To increase the range of taxa included in this study, bacteria were also isolated from the ovarian fluid of a single brood coho salmon female. Identification of isolated bacteria was done using standard microscopical and biochemical methods and by reference to Krieg and Holt (1984) and Sneath et al. (1986). Stock cultures of all bacteria were kept at -70°C in tryptic soy broth (TSB, Difco, Detroit, MI), or in KDM-2 broth (Evelyn 1977). Both media were supplemented with 1% gelatin. Propagation of the cells was carried out using brain heart infusion (BHI, Difco) agar at 15°C , except *R. salmoninarum* for which charcoal medium (Daly et al. 1985) that had been made selective (SKDM-C) for the bacterium by the addition of antibiotics described by Austin et al. (1983) was used.

Antibacterial activity determination

Bactericidal assay: The effect of coho egg lysozyme and HEWL on viability of bacterial fish pathogens (*A. hydrophila*, *A. salmonicida*, *L. piscicola*, and *R. salmoninarum*) was investigated. Bacterial cultures were aseptically scraped off agar plates and suspended in sterile PBS. The cells were washed twice by centrifugation ($5,860 \times g$ for 15 min at 4°C) in PBS, and the final suspension was adjusted to an approximate absorbance of 1.0 at 540 nm (equivalent to approximately 5.1×10^8 ; 1.6×10^9 ; 1.9×10^6 ; 1.6×10^9 colony forming units (CFU) ml^{-1} , determined by serial

dilution in sterile peptone 0.1%/saline 0.85% (P/S) and plating onto tryptic soy agar (TSA, Difco), for *A. hydrophila*, *A. salmonicida*, *L. piscicola*, and *R. salmoninarum*, respectively). One ml samples of the suspension were then dispensed into Eppendorf tubes and centrifuged (Micro-Centrifuge, Model 235C, Fisher Scientific) for 10 min at room temperature. The supernatant was discarded and the bacterial pellet was resuspended for 90 min in the fish enzyme preparations (100, 400, 700 ug ml⁻¹) at room temperature. HEWL, however, was used at one concentration of 1900 ug ml⁻¹. Samples (in triplicates) were taken from each suspension and diluted ten-fold in Eppendorf tubes containing sterile P/S. The bacterial suspensions were sampled at the start of the experiment (time=0) to determine CFU ml⁻¹ used in the experiment, and to determine if any reduction in CFU had occurred due to the centrifugation step. At the end of the experiment (time=90 min.), samples (100 ul) were taken from each suspension containing coho egg lysozyme, and inoculated into broth media (TSB/KDM-2) at 15°C, and absorbance was recorded at 540 nm. Sampling was also done every 30 min to determine the effect on CFU brought about by the enzyme. These dilutions were then drop plated (25 ul drop⁻¹ x5) on appropriate agar media, and colonies that survived the treatment were enumerated after 48 h at 15°C. *R. salmoninarum*, however, was drop plated on SKDM-C using the "nurse" culture technique described by Evelyn et al. 1989,

and colonies were counted after 4-6 weeks of incubation at 15°C.

The bactericidal effect of lysozyme was inferred from the difference between CFU in the control (bacteria incubated in PBS) and in the reaction mixtures (bacteria incubated in different concentrations of lysozyme).

The effect of lysozyme on the bacteria isolated from the ovarian fluid was assayed either by placing sterile 5 mm diameter filter paper discs saturated with egg yolk material (EY) that had been diluted (1:3) in PBS or by addition of drops (25 ul) of diluted egg yolk directly onto TSA plates heavily seeded with the live test bacterium. After a period of 48 h incubation, antibacterial activity became visible as a clear region around the discs where the bacterium failed to grow.

Lysis assay: The following lysoplate method was developed to screen *A. hydrophila*, *A. salmonicida*, *L. piscicola*, and *R. salmoninarum* for their susceptibility to lysis effected by coho egg lysozyme. Cells from log-phase cultures were washed twice and suspended in PBS. The suspension was adjusted to an absorbance of 1.5 at 540 nm. This was found to be the absorbance of *M. lysodeikticus* when used as a substrate in the lysoplate method. Agarose (type 1, Sigma) was added to a final concentration of 0.5% (w/v) and the mixture was heated for 10 min with stirring to dissolve the agarose and to kill the bacteria. Five ml of the preparation was poured into

petri plates (35 x 10 mm), and when the agarose had set, wells were made and subsequently inoculated with EY (20 ul/well). The plates were incubated in a humid chamber at room temperature for 20 h and examined for zones of clearance due to bacterial lysis around the wells.

RESULTS

The analysis of homogenized whole specimens of eggs as well as yolk revealed the presence of lysozyme (1,900 and 1,950 $\mu\text{g ml}^{-1}$ for EY and EH, respectively), and lysoplate results (Fig. 1) are the first unequivocal evidence for the presence of lytic activity due to lysozyme in salmonid eggs. The presence of the enzyme at such concentrations in the eggs of coho salmon was not unique. Yolk samples from chinook salmon *O. tshawytscha* and rainbow trout *O. mykiss* were also assayed by the lysoplate method, and the results showed the presence of lysozyme in comparable concentrations to those found in coho eggs: 1,900 and 1,850 $\mu\text{g ml}^{-1}$ of yolk for chinook salmon and rainbow trout, respectively.

The purified lysozyme from coho salmon eggs satisfied the criteria for "true" lysozymes proposed by Salton (1957) and Jolles (1969) because it lysed *M. lysodeikticus* cells (Fig. 1), was readily adsorbed by chitin-coated cellulose (Fig. 2), and was a low molecular weight protein (Fig. 3). In addition, the studies on heat stability (Fig. 4) revealed typical lysozyme features: the enzyme was remarkably stable when heated at 100°C for 20 min at acidic pH, but was inactivated under alkaline conditions. The former property of lysozyme may be useful for removing most of the contaminating proteins present in the lysozyme-containing cc-cellulose eluates (Fig. 3, lane C).

FIGURE 1

Lysozyme activity in whole egg homogenate (A) compared with that of twice-crystallized hen egg white lysozyme (700 ug ml^{-1}) (B). Plate assay (lysoplate) method, with heat-killed *M. lysodeikticus* incorporated in phosphate-buffered (pH 6.0) agarose gel. Samples (20 μl) were placed in wells, 3.5 mm in diameter and 4 mm deep, and the plate was photographed after 20 h at 20–22°C. Dark zones around sample wells are zones of clearing due to bacterial lysis.

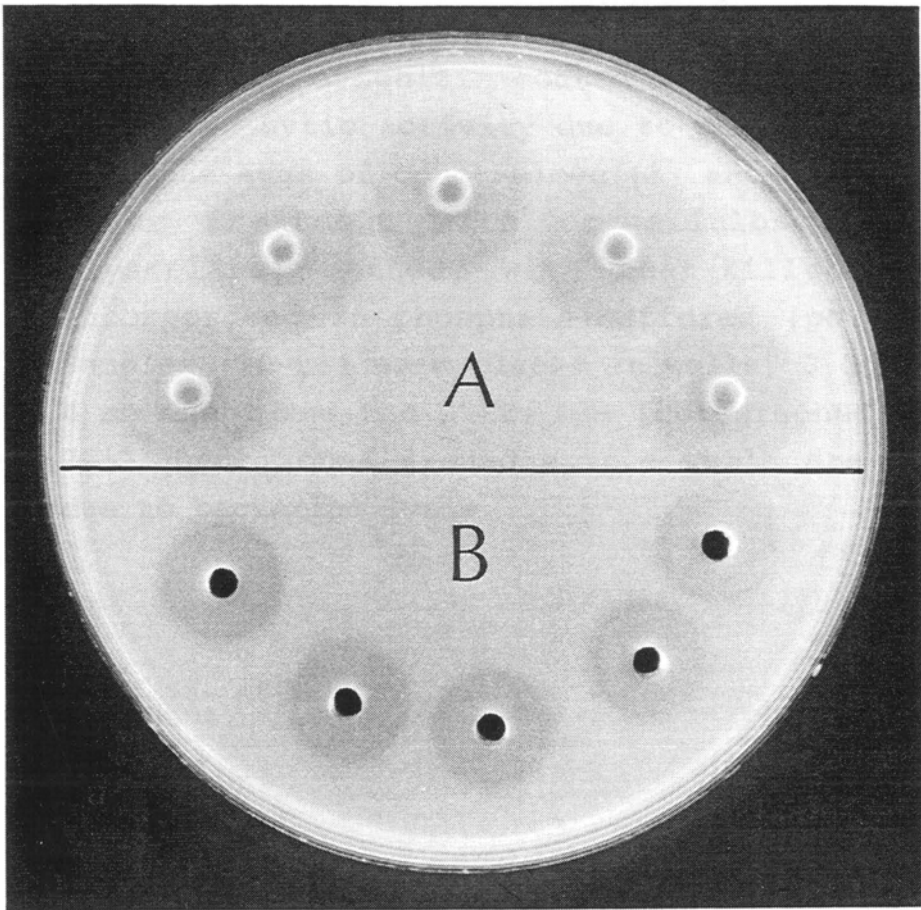


FIGURE 2

The ability of chitin-coated cellulose to adsorb coho egg lysozyme. Lytic activity due to partially purified lysozyme from the eggs of coho salmon (A) and of the same preparation after treatment with cc-cellulose (B). Plate assay (lysoplate) method, with heat-killed *M. lysodeikticus* incorporated in phosphate-buffered (pH 6.0) agarose gel. Samples (20 ul) were placed in wells, 3.5 mm in diameter and 4 mm deep, and the plate was photographed after 20 h at 20-22°C. Dark zones around sample wells are zones of clearing due to bacterial lysis.

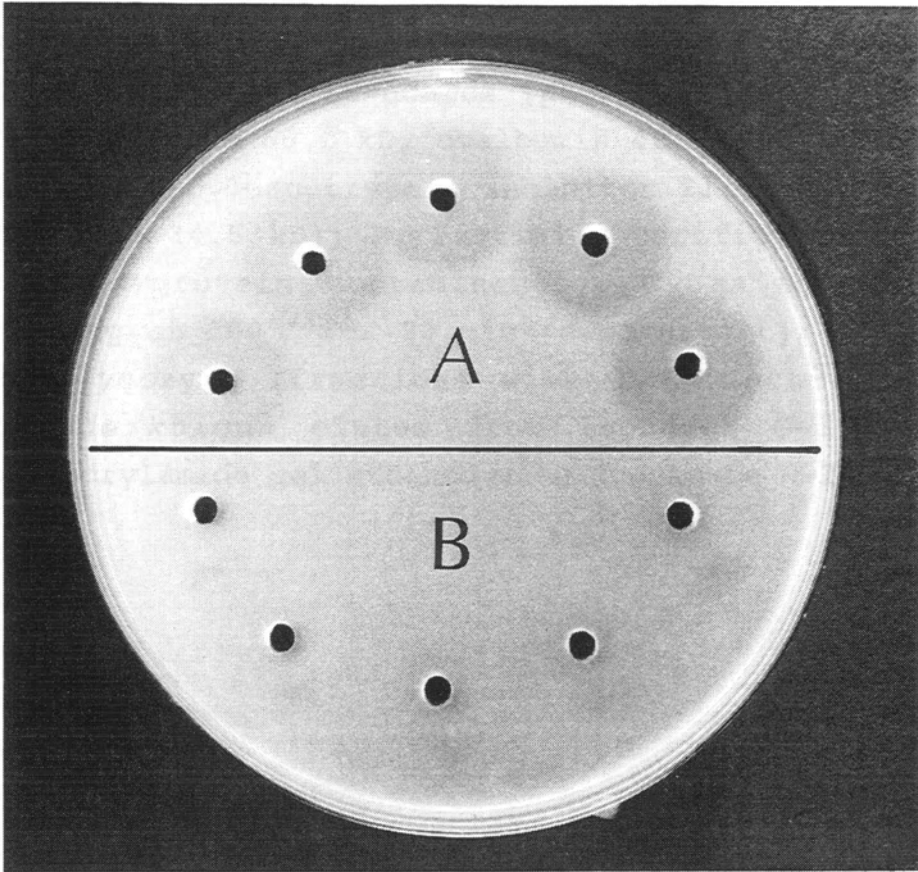


FIGURE 3

SDS-polyacrylamide electrophoretic patterns of A. BIO-RAD Low Molecular Weight Standards (phosphorylase b 97.4 kD, bovine serum albumin 66.2 kD, ovalbumin 42.7 kD, carbonic anhydrase 31.0 kD, soybean trypsin inhibitor 21.5 kD, hen's egg-white lysozyme 14.5 kD); B. Partially purified coho egg lysozyme (arrows=protein contaminants); C. as for (B), but after boiling at 100°C for 20 min (supernatant); D. Purified coho egg lysozyme (fractions with lytic activity against *M. lysodeikticus* eluted from Sephadex G-100 gel). 12.5% polyacrylamide gel stained with Coomassie R-250.

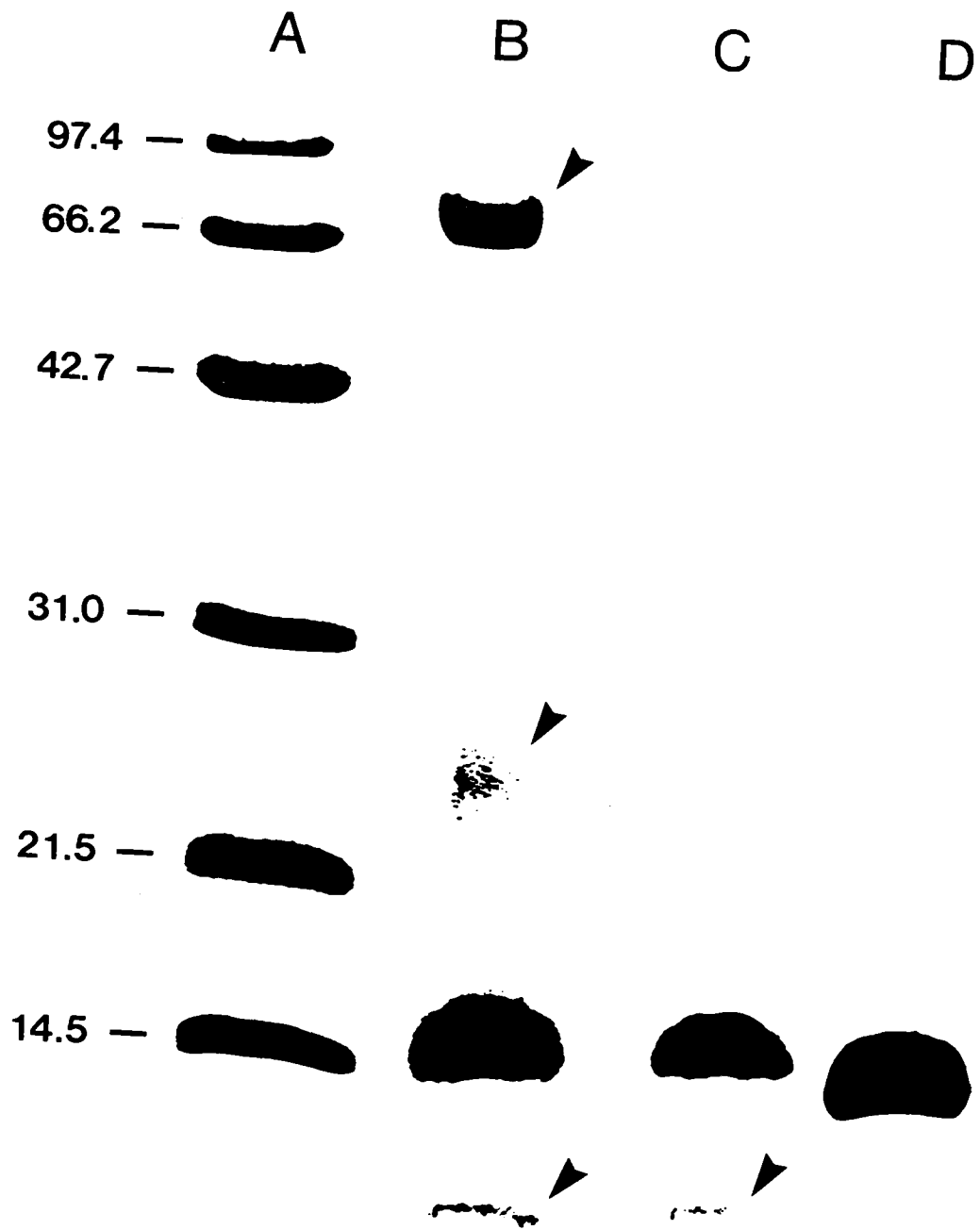
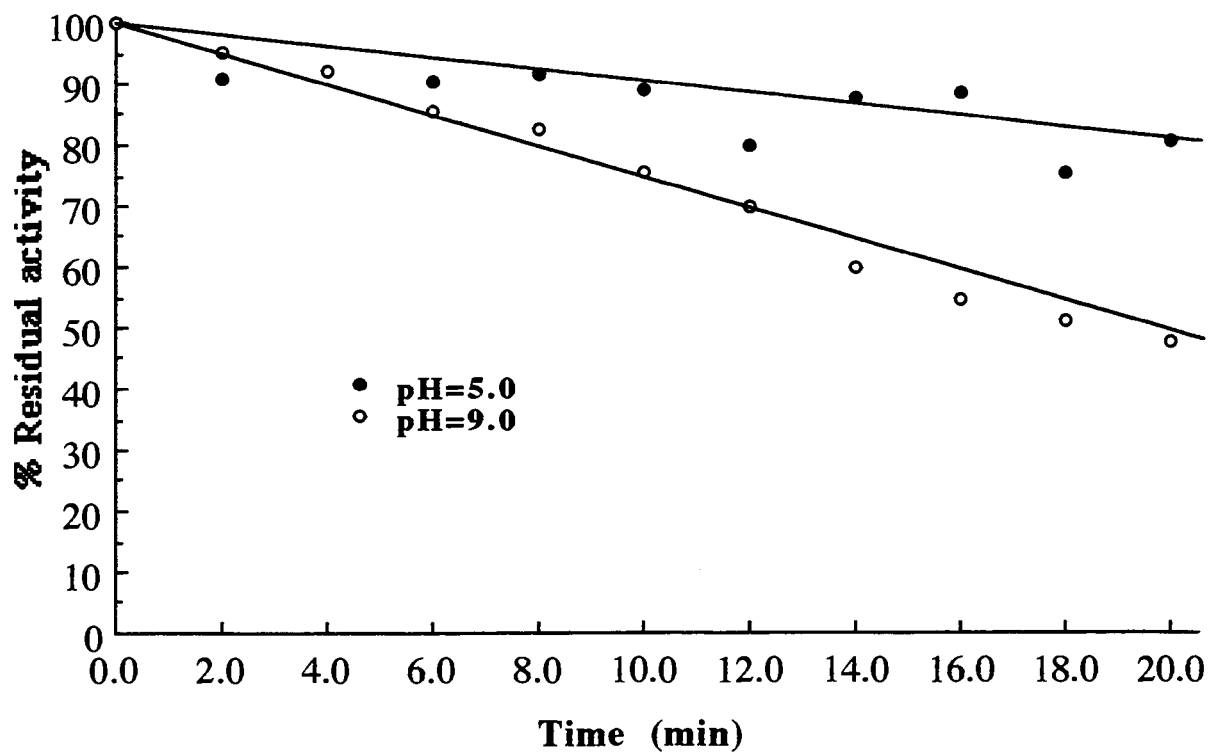


FIGURE 4

Heat inactivation at 100°C of coho egg lysozyme at acidic and alkaline pHs.



Affinity purified IgG from the rabbit immunized with purified lysozyme was reacted with yolk extract from coho eggs and lysozymes from human, hen egg-white, and turkey egg-white. Coho egg lysozyme was readily recognized by the antibodies. In addition, the latter also reacted with a number of egg proteins and to a certain extent with lysozymes from the other sources (Fig. 5). This weak cross reactivity of coho egg lysozyme with the other c-type lysozymes could be attributed to structural differences in one or more amino acid residues that leads to alteration in antigenic specificity. Such antigenic variations have been reported even among the most similar c-type lysozymes, the California quail and bobwhite quail lysozymes. These two lysozymes differ from each other only at positions 68 (Arg/Lys) and 121 (His/Gln) and yet they are antigenically different (cited by Jolles and Jolles 1984).

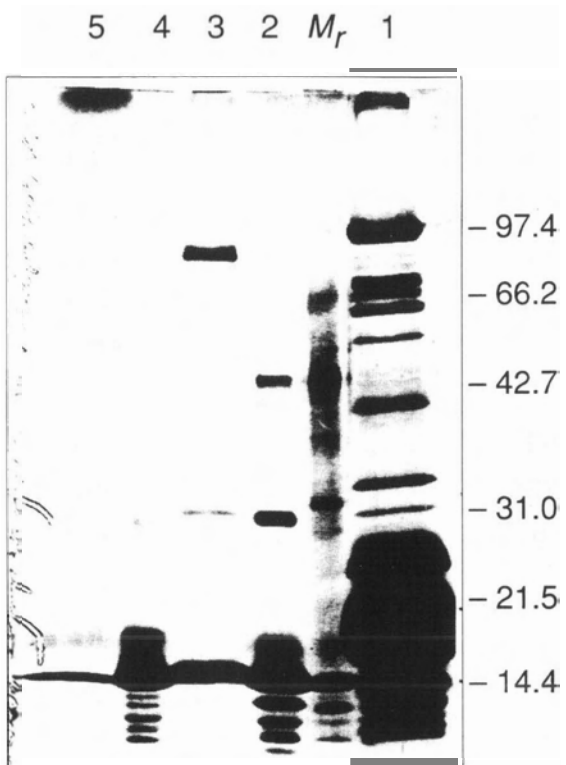
The amino acid composition of coho egg lysozyme is indicated in Table 1. The enzyme lacks the amino acid methionine, and was characterized by a relative abundance of hydrophobic amino acids, such as glycine and leucine. Trials to sequence the protein's N-terminus proved that it was naturally blocked. This is the case with 30-40% of eukaryotic proteins.

Some comments on the lysozyme purification procedure are warranted. SDS-PAGE of the PPL revealed the presence of at least three other proteins with molecular weights of 75 kD,

FIGURE 5

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (A), and western blot (B) of reduced (2-mercaptoethanol) samples of coho salmon *O. kisutch* egg yolk extract (lane 1), hen egg-white lysozyme (lane 2), human lysozyme (lane 3), turkey egg-white lysozyme (lane 4), purified coho egg lysozyme from gel filtration (lane 5). After electrophoresis, protein gels were stained with silver, and nitrocellulose membranes were processed as described in the Materials and Methods. The primary antibody was affinity purified rabbit immune serum raised against coho egg lysozyme. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase. M_r standards are indicated (kDa)

[A]



[B]

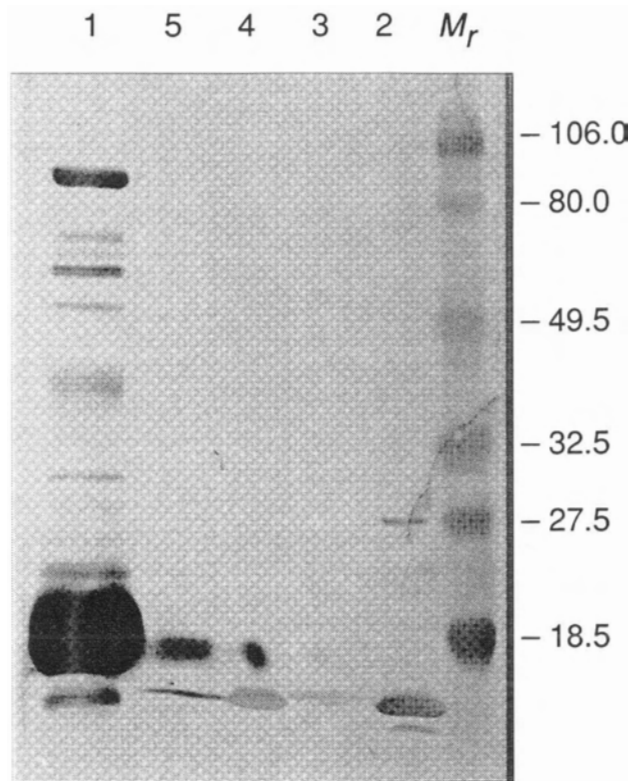


Table 1: Total amino acid analysis of 14.5 kDa coho salmon *Oncorhynchus kisutch* egg lysozyme.

Amino acid residue	No. of residues per lysozyme subunit ^a
Asx	4
Glx	18
Ser	9
Gly	29
His	2
Arg	3
Thr	11
Ala	13
Pro	9
Tyr	1
Val	10
Cys	ND ^b
Met	0
Ile	5
Leu	20
Phe	5
Trp	ND
Lys	1
Total	142

^a The molar ratio of each amino acid was determined from the compositional analysis, and the alanine content was adjusted for a lysozyme molecular mass approximating 14.5 kDa, the apparent molecular mass of the lysozyme subunit as determined by SDS-PAGE.

^b ND, not determined.

24 kD, and less than 14.5 kD, all of which also readily adsorbed to cc-cellulose (Fig. 3, lane B). Tests on these proteins revealed that they possessed no lysozyme-like activity. These contaminating proteins were successfully removed by gel filtration on Sephadex G-100 (Fig. 3, lane D). The typical elution pattern for the egg lysozyme on gel filtration is shown in Figure 6. Lysozyme activity in the sample occurred in fractions 59-71 and was associated with a single protein of molecular weight (MW) 14.5 kD (Fig. 3, lane D).

Absorbance measurements after 45 min incubation at temperatures of 10 and 20°C showed a slight decrease in absorbance, 10% and 26%, respectively (Fig. 7). This indicated that the bacteriolytic activity of the egg lysozyme was not totally inhibited at low temperatures, and that the enzyme was capable of lysing bacterial cells of *M. lysodeikticus*, yet at a slower rate. This was also observed during testing of purified preparations of the enzyme on TSA plates seeded with live *A. hydrophila*, *A. salmonicida*, or *L. piscicola*. Low incubation temperatures (8 - 12°C) had minimal effect on the bactericidal property of lysozyme. As with other enzymatic reactions, lysozyme was much more active when the temperature was elevated. Incubating the reaction mixture at temperatures of 30°C or higher resulted in a substantial increase in the reaction rate (44%). Despite a slight

FIGURE 6

Gel filtration on Sephadex G-100 of 2.0 ml of partially purified lysozyme (PPL). Fractions (0.5 ml) were eluted at 30 ml h⁻¹ from a column 25 x 1.5 cm, with 0.06M phosphate buffer (pH 6.0) containing 0.02M NaCl, at room temperature. Lysozyme activity located in fractions 59-71 (indicated on histogram). The continuous line shows the absorption at 280 nm of the eluted fractions.

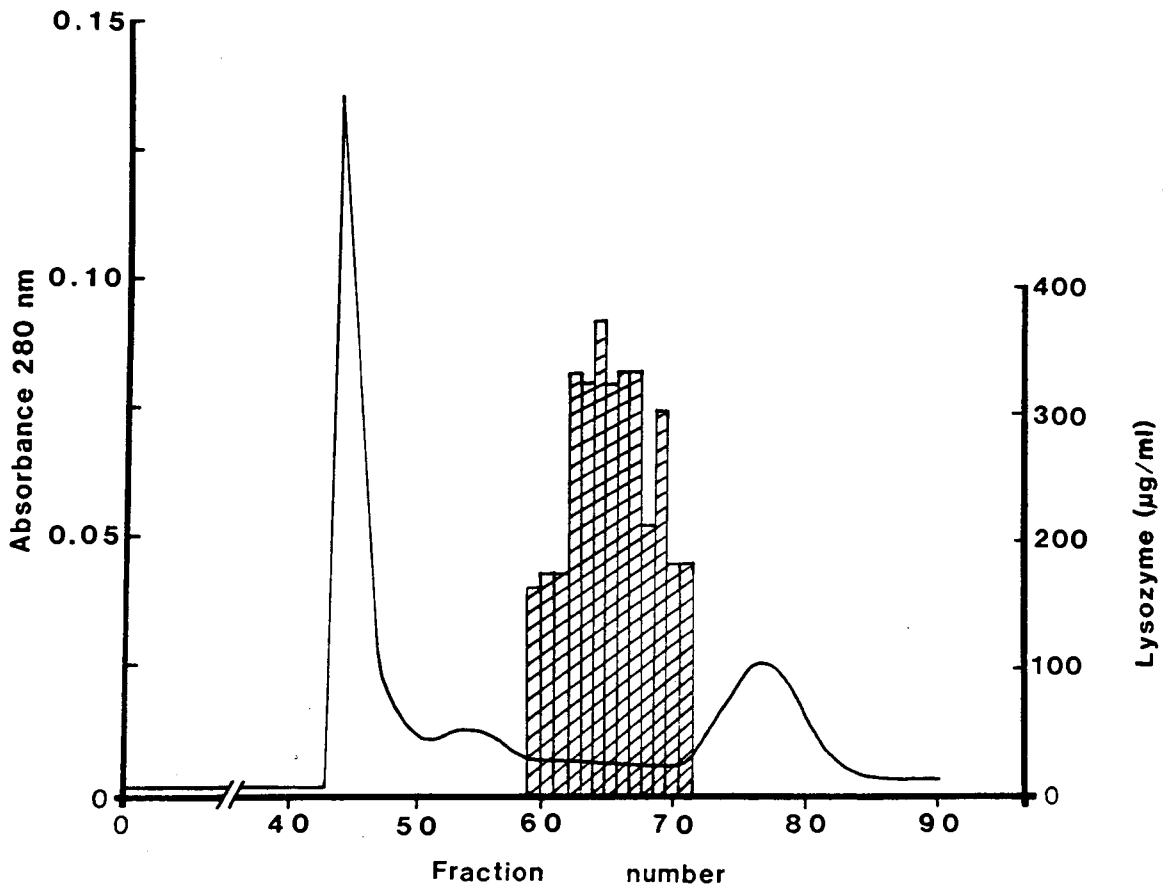
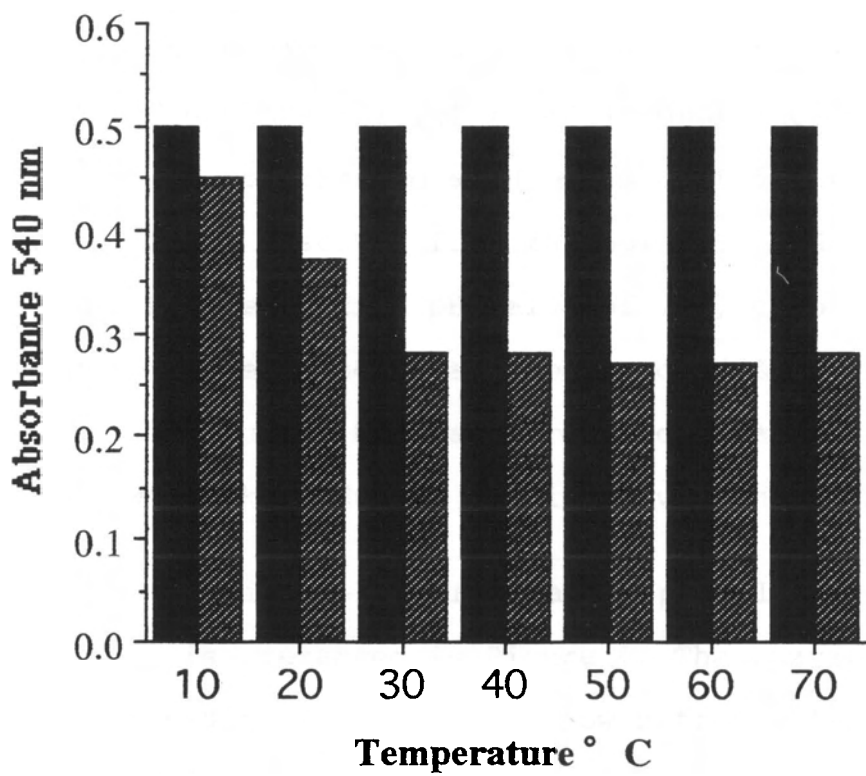


FIGURE 7

The effect of incubation temperature on the bacteriolytic activity of coho egg homogenate (EH). EH (lysozyme concentration=700 ug ml⁻¹) was incubated with *M. lysodeikticus* suspension (0.02%) in a total volume of 2.0 ml of 0.04M phosphate buffer (pH 6.0). The system was incubated at different temperatures for 45 min.



0 min.
45 min.

increase (46%) in lysozyme activity at 50°C and 60°C , no further increase was registered.

The variation of the activity of coho egg lysozyme as a function of pH at constant temperature and buffer molarity was studied. Two buffers (citrate and phosphate buffers) were employed to cover a pH range of 3.0 - 8.0. As depicted in Figure 8, the lytic activity of the enzyme was a function of pH of the incubation medium. After 45 min, at an acidic pH value of 6.0 the initial (0 min) absorbance decreased by 46%. This is in contrast to a pH optimum of 5.5 that has been reported for lysozyme from rainbow trout (Grinde et al. 1988). At the neutral pH value of 7.0, coho egg lysozyme produced a decrease of absorbance of 40%, whereas at the basic pH value of 8.0, a decrease of 20% was registered, which was likely due to the inactivation of the enzyme.

The activity-buffer molarity profiles of coho egg lysozyme is presented in Figure 9. The enzyme showed best lytic activity (54%) at very low buffer molarity of (0.02M); a 5-fold increase of buffer molarity (M=0.1) produced a slightly lower lytic activity (34%).

Much work has been done on the action of lysozyme on bacteria. The effects produced may be divided into killing without lysis (bactericidal activity), killing with demonstrable lysis (bacteriolytic activity), and inhibition of growth (bacteriostatic activity) - depending upon the

FIGURE 8

The effect of pH on the bacteriolytic activity of coho egg homogenate (EH). EH (lysozyme concentration=700 ug ml⁻¹) was incubated with *M. lysodeikticus* suspension (0.02%) in a total volume of 2.0 ml of 0.04M citrate/phosphate buffer of varying pHs. The system was incubated at 40°C for 45 min.

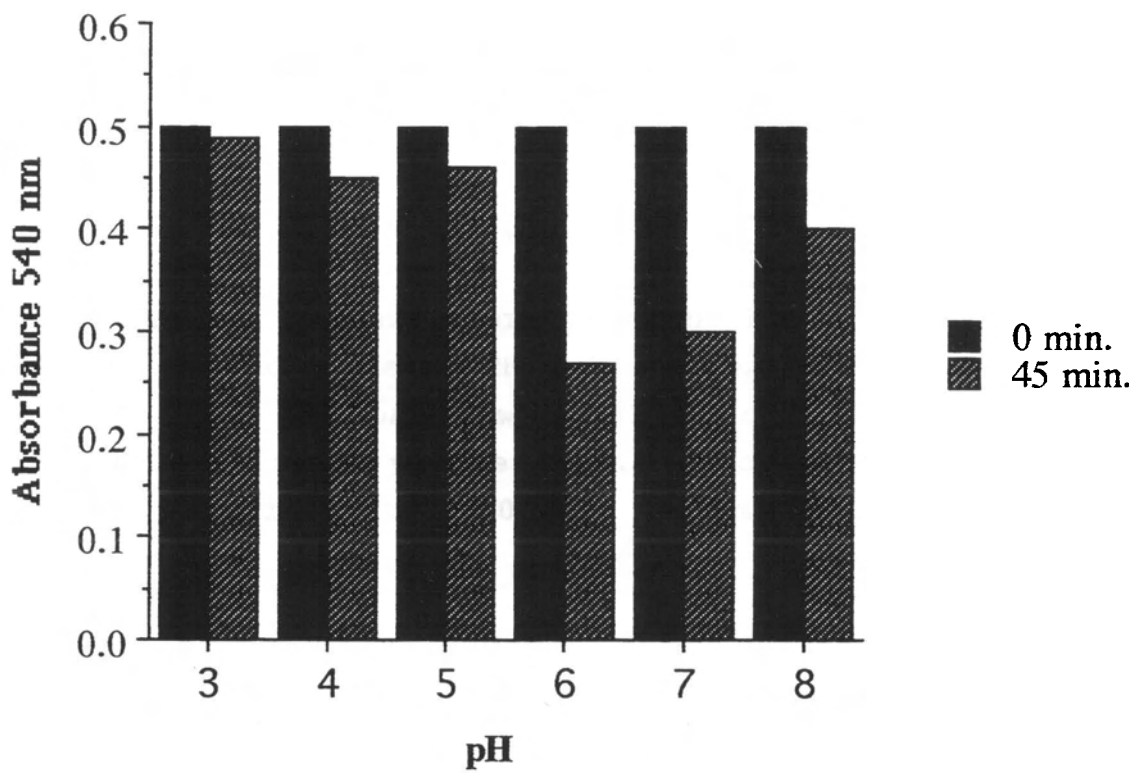
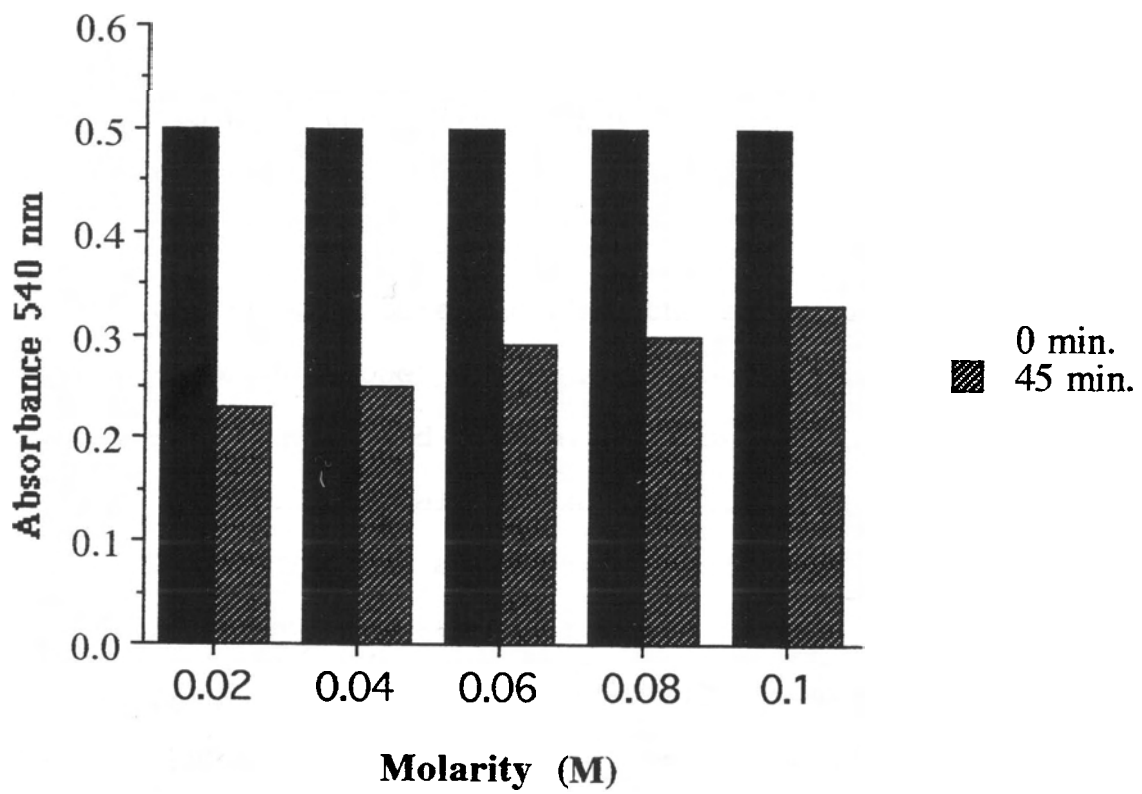


FIGURE 9

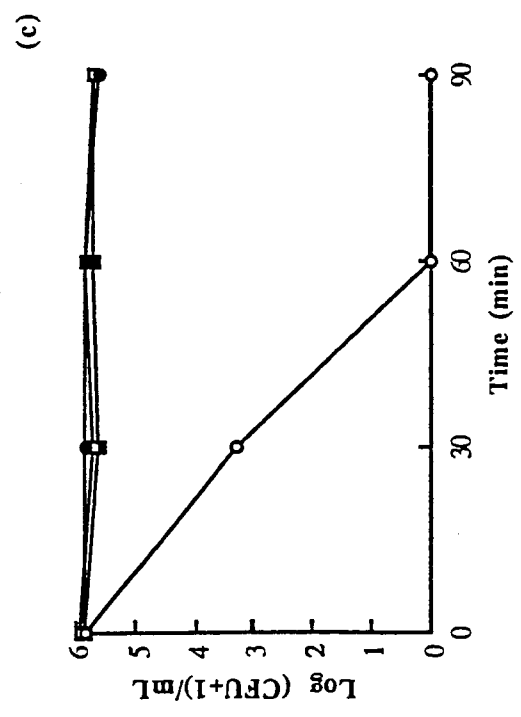
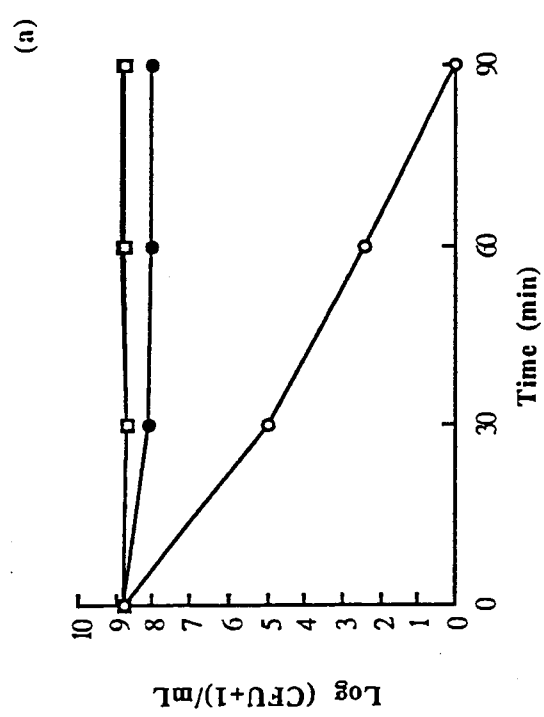
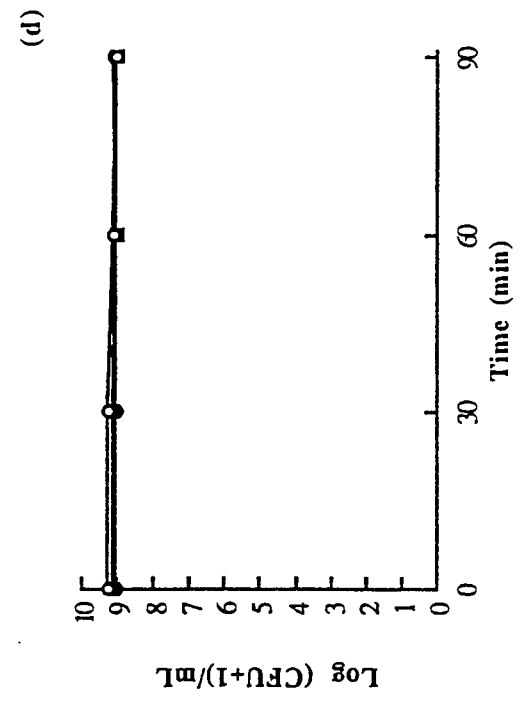
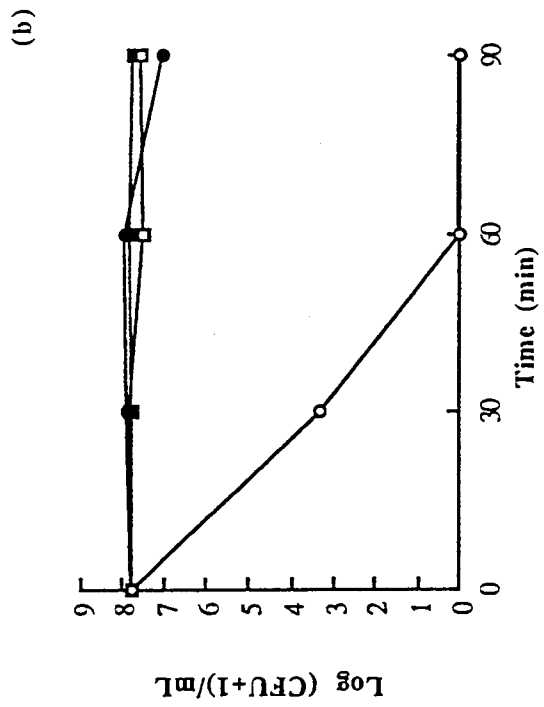
The effect of buffer molarity on the bacteriolytic activity of coho egg homogenate (EH). EH (lysozyme concentration=700 ug ml⁻¹) was incubated with *M. lysodeikticus* suspension (0.02%) in a total volume of 2.0 ml of phosphate buffer of varying molarity at pH 6.0. The system was incubated at 40°C for 45 min.



concentration of lysozyme and the resistance of the particular organism to its action. In this study, the antibacterial (bactericidal and bacteriolytic) effect of the enzyme was tested on four species of bacteria pathogenic to fish. The CFU ml⁻¹ were determined after 90 min of exposure to three different concentrations of lysozyme: 100, 400 and 700 ug ml⁻¹. The bactericidal effect of coho egg lysozyme was found to be concentration and time dependent. While lower concentrations of the enzyme had no effect on *A. hydrophila*, total (100%) killing of the bacterium was caused by 700 ug of enzyme ml⁻¹ after an incubation period of 90 min (Fig. 10a). Similar results were obtained when the extract of the "fertilization envelope" of rainbow trout and *Tribolodon hakonensis* was incubated with *A. hydrophila* (Kudo and Inoue 1986). *A. salmonicida* cells were found to be equally susceptible to lysozyme at 700 ug of enzyme ml⁻¹, however, total killing occurred after a shorter period of treatment (Fig. 10b). This result supports previous observations made over many years that the causative bacterium of furunculosis is not vertically transmitted (Bullock and Stuckey 1987). The possible role of lactobacilli as the cause of disease in salmonid fish has been discussed (Ross and Toth 1974, Cone 1982, Hiu et al. 1984). This Gram-positive bacterium is found most frequently in fish which have suffered some form of stress, such as that which occurs at spawning. Figure 10c depicts the effect of coho egg lysozyme on *L. piscicola*. Once

FIGURE 10

Effect of coho egg lysozyme on bacterial cell viability. *Aeromonas hydrophila* (a); *A. salmonicida* (b); *Lactobacillus piscicola* (c); *Renibacterium salmoninarum* (d) were incubated in the absence (PBS ■-■) or presence of lysozyme (100 □-□, 400 ●, 700 o-o ug ml⁻¹). Samples were taken at the start (time=0) and every 30 minutes, diluted in P/S and drop plated onto agar media. CFU ml⁻¹ were counted after the plates were incubated at 15°C.



again, 700 ug of lysozyme per ml resulted in total killing of the bacterium after 60 min of incubation. Lysozyme had no effect on *R. salmoninarum*, as preincubation of the cells with as much as 700 ug ml⁻¹ of the enzyme for 90 min did not affect bacterial viability (Fig. 10d). This prompted a further investigation of the *in vitro* effect of the enzyme at a higher concentration of 1,900 ug ml⁻¹, a concentration found in most salmonid eggs (Yousif et al. 1991). Once again, CFU counts on two different agar media:SKDM-C and KDM-2 (Evelyn et al. 1990) showed that the bacterium is completely resistant to coho egg lysozyme (Table 2). While HEWL was ineffective against *A. hydrophila*, *A. salmonicida* and *R. salmoninarum*, it exhibited substantial bactericidal activity against *L. piscicola* after 30 min (Fig. 11).

Pigmented and coryneform bacteria are considered as part of the microflora of salmonid eggs and ovarian fluids (Yoshimizu et al. 1980, Sauter et al. 1987, Barker et al. 1989). In this study, two types of bacteria were isolated from the ovarian fluid of coho salmon (Table 3). One of these resembled *Flavobacterium* (Gram-negative, non-motile rods, aerobic, oxidase- and catalase-positive), while two other closely related bacteria were identified as members of the genus *Corynebacterium* (Gram-positive irregularly-stained rods, non-motile, facultatively anaerobic, oxidase-negative and catalase-positive). Direct application of antimicrobial substances on TSA seeded with live bacteria does not provide enough information regarding the mode of action of the

Table 2: The effect of coho salmon *Oncorhynchus kisutch* egg lysozyme on *Renibacterium salmoninarum*. Bacterial cells were incubated in the absence (PBS=control), or the presence of 1,900 ug ml⁻¹ of lysozyme for 90 min. at room temperature (20 - 22°C). Samples were taken at the start (0 min.) and every 30 min., diluted in P/S, and drop onto agar media. CFU ml⁻¹ were determined after 4 weeks of incubation at 15°C.

MEDIA	DILUTION	COHO EGG LYSOZYME (1,900 ug ml ⁻¹)				CONTROL			
		0 min.	30 min.	60 min.	90 min.	0 min.	30 min.	60 min.	90 min.
SKDM-C ¹ + NURSE CULTURE TECHNIQUE ²	10 ⁻⁴	NC*	NC	8.4x10 ³	NC	NC	1.0x10 ⁴	1.1x10 ⁴	1.0x10 ⁴
	10 ⁻⁵	1.2x10 ³	1.6x10 ³	1.1x10 ³	1.0x10 ³	1.5x10 ³	1.0x10 ³	1.2x10 ³	1.0x10 ³
	10 ⁻⁶	4.0x10 ¹	1.2x10 ²	1.2x10 ²	8.0x10 ¹	1.6x10 ²	1.2x10 ²	1.6x10 ²	4.0x10 ¹
	X ⁺	8.0x10 ⁷	1.4x10 ⁸	1.2x10 ⁸	9.0x10 ⁷	1.6x10 ⁸	6.7x10 ⁷	1.3x10 ⁸	7.9x10 ⁷
	10 ⁻⁴	7.3x10 ³	7.4x10 ³	4.4x10 ³	3.8x10 ³	8.1x10 ³	5.8x10 ³	3.2x10 ³	2.9x10 ³
KDM-2 ³ SUPPLEMENTED WITH SPENT KDB ⁴	10 ⁻⁵	6.0x10 ²	8.8x10 ²	4.0x10 ²	3.6x10 ²	6.4x10 ²	4.4x10 ²	3.6x10 ²	3.2x10 ²
	10 ⁻⁶	4.0x10 ¹	4.0x10 ¹	4.0x10 ¹	0	4.0x10 ¹	4.0x10 ¹	4.0x10 ¹	4.0x10 ¹
	X	5.8x10 ⁷	6.7x10 ⁷	4.1x10 ⁷	3.7x10 ⁷	6.2x10 ⁷	4.7x10 ⁷	3.6x10 ⁷	3.4x10 ⁷

*NC, not counted (too numerous and coalescing)

+X, CFU ml⁻¹

1 (Austin et al. 1983, Daly et al. 1985)

2 (Evelyn et al. 1989)

3 (Evelyn 1977)

4 (Evelyn et al. 1990)

FIGURE 11

Effect of hen egg-white lysozyme on bacterial cell viability. *Aeromonas hydrophila* (\diamond - \diamond); *A. salmonicida* (o-o); *Lactobacillus piscicola* (\square - \square); *Renibacterium salmoninarum* (Δ - Δ) were incubated in PBS (control=filled symbols) or presence of lysozyme (1900 ug ml^{-1}). Samples were taken at the start (time=0) and every 30 minutes, diluted in P/S and drop plated onto agar media. CFU ml^{-1} were counted after the plates were incubated at 15°C .

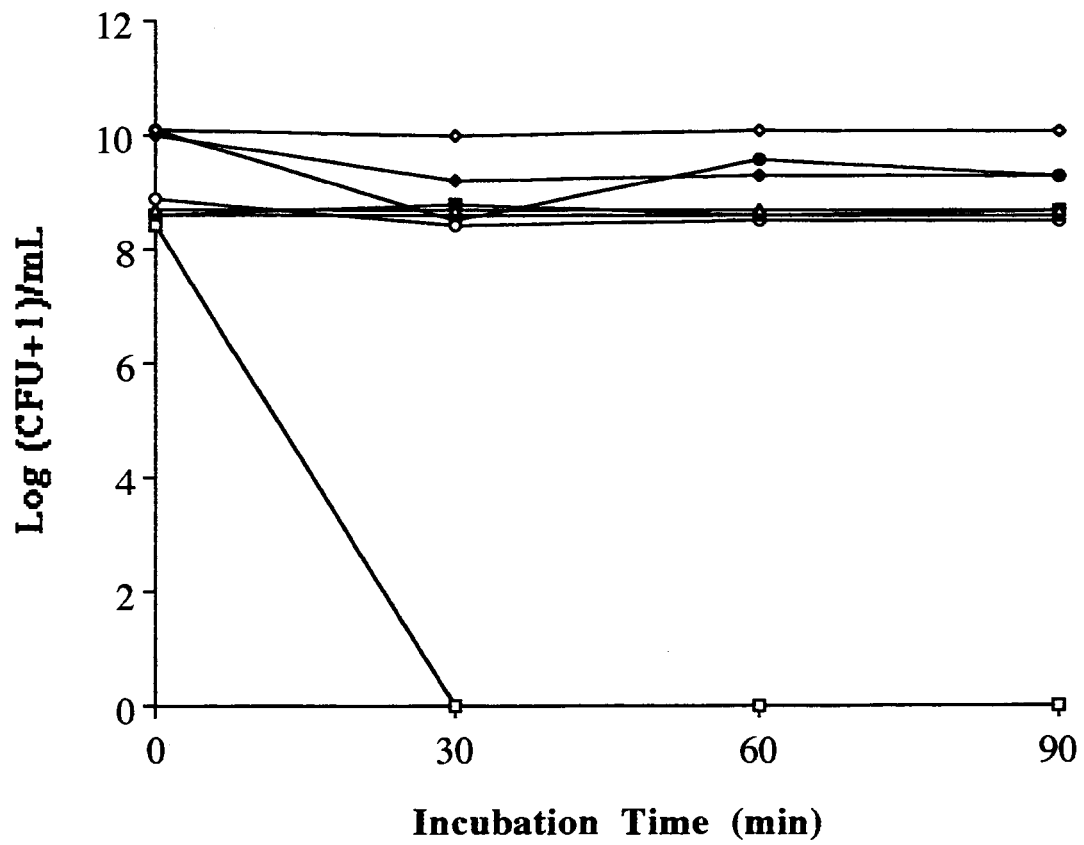


Table 3: Biochemical characteristics of 3 bacterial isolates from the coelomic fluid of a female brood coho salmon *Oncorhynchus kisutch* in British Columbia, Canada.

Character	<i>Flavobacterium</i> sp.	<i>Corynebacterium</i> sp.	<i>Corynebacterium</i> sp.
Gram reaction	-	+	+
Motility	-	-	-
Morphology	rod	rod	rod
Production of yellow pigment	+	+	-
Endospore	-	-	-
Acid-fast	-	-	-
Growth at 37°C	-	-	-
Growth on TSA ^a + 7.5% NaCl	-	-	-
Growth on nutrient agar	+	+	+
Growth at room temp. (18-22°C)	+	+	+
Hydrolysis of:			
Gelatin	+	+	+
Agar	-	-	-
Acid from:			
glucose	+	+	-
sucrose	+	-	-
lactose	-	-	-
Methyl red	-	-	-
Simmons citrate	-	+	+
Indole production	-	-	-
H ₂ S production	-	-	-
Urease	-	-	+
Nitrate reduction	-	+	+
Catalase	+	+	+
Oxidase	+	-	-
Growth on MacConkey agar	-	-	-
Voges-Proskaur	-	-	-
Oxidative-fermentative	O ^b	O	O

^aTSA, Tryptic soy agar

^bO, Oxidative

substances i.e. bacteriolytic, bactericidal or bacteriostatic. Nevertheless, EY application to TSA seeded with the bacterial species isolated from the coelomic fluid resulted in growth inhibition of both isolates of *Corynebacterium*. *Flavobacterium*, however, showed complete resistance. Similarly, Grinde (1989), reported that *Flavobacterium* sp. cells were resistant to HEWL and type I lysozyme, an enzyme present in rainbow trout kidney tissues.

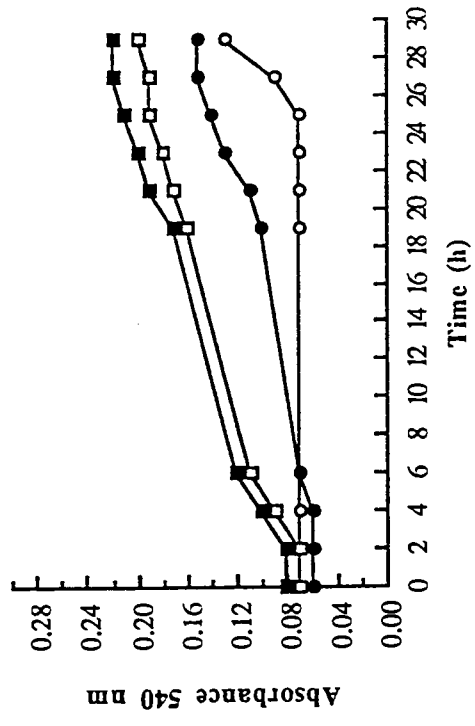
Data obtained by measuring changes in the turbidity of bacterial suspensions, after exposure to lysozyme, are included (Fig. 12). Indeed, treatment of susceptible bacterial cells of the species tested in 700 ug of enzyme for 90 min substantially affected viability of the cells. However, a few viable bacterial cells that escaped detection by culturing on agar media gave detectable turbidity in broth media after 2 days incubation at 15°C (Fig. 12a & b). Untreated controls as well as bacteria treated with lower concentrations of lysozyme produced detectable growth when inoculated into broth medium (Fig. 12).

Clearing due to bacterial lysis was not detected with any of the four bacterial fish pathogens when they were exposed to EY in lysoplates.

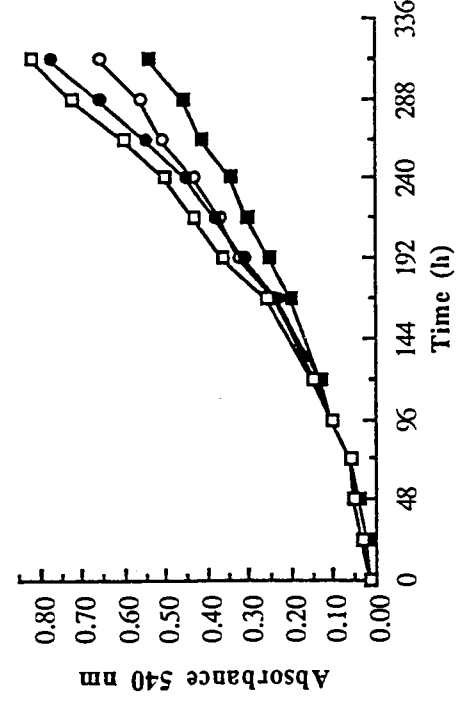
FIGURE 12

Growth curves of *Aeromonas hydrophila* (a); *A. salmonicida* (b); *Lactobacillus piscicola* (c); *Renibacterium salmoninarum* (d). The bacteria were incubated in the absence (PBS■-■) or presence of coho egg lysozyme (100□-□, 400 •-•, 700 o-o ug ml⁻¹) for 90 min. Pretreated bacteria were then inoculated in broth media (TSB/KDM-2) at 15°C, and absorbance was recorded at 540 nm.

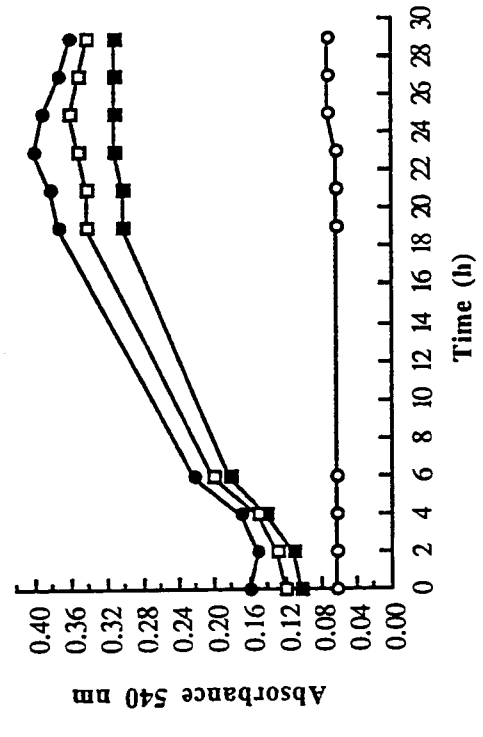
(b)



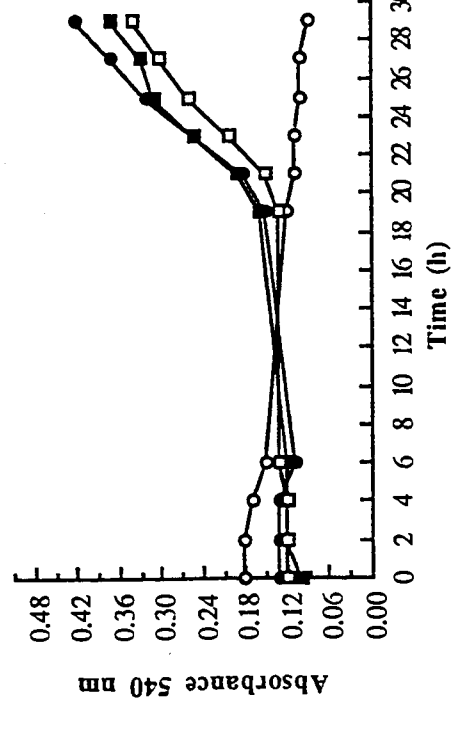
(d)



(a)



(c)



DISCUSSION

In salmonids, the ovarian fluid of the brood females may become infected with high concentrations of bacteria at the time of spawning. This fluid bathes the eggs following ovulation and serves as a source of infections for salmonid eggs (Evelyn et al. 1984). In addition, internal egg contamination by bacteria may occur at an early stage of development (Bruno and Munro 1986), or perhaps during water-hardening when eggs themselves actively take up water (Barker et al. 1991). Viability in the egg, however, depends on the individual bacterium's ability to resist antimicrobial substances present within the egg.

R. salmoninarum, the causative agent of bacterial kidney disease in salmonids enters the egg through the micropyle, and remains in the yolk from which location it infects the developing embryo where it multiplies (Evelyn et al. 1984, Evelyn et al. 1986). Other Gram-positive and Gram-negative bacteria, that are motile and small enough to pass through the micropylar canal, e.g. the fish pathogens *A. hydrophila*, and *A. salmonicida* are also likely to enter eggs. These bacteria as well as others have been reported to occur in the ovarian fluid of mature brood females (Cone 1982, Evelyn 1984, Sauter et al. 1987, Barker et al. 1989). However, evidence for the vertical transmission of these bacteria is lacking. This is perhaps because they tend to occur in low numbers in the ovarian fluid, thus adversely affecting their

chances of establishing egg infections (see Evelyn et al. 1984). Alternatively, their failure to readily infect eggs is due to physical or chemical factors on or in eggs that resist intra-ovum infections. In contrast, Sauter et al. (1987) found a large range of bacteria within chinook salmon *O. tshawytscha* eggs. This may possibly be a consequence of their particular method of 'enrichment' recovery. More likely, however, it was due to the stock of highly stressed fish that served as the source of Sauter et al.'s eggs. Sauter et al.'s results differed from those of others (Yoshimizu et al. 1980, Barker et al. 1989, 1991).

Natural antibacterial activity of various body fluids and tissues has been subjected to numerous investigations in several species during the last century. However, not all the inhibitory factors described have a relationship with lysozymes. Since the discovery and the designation of lysozyme by Fleming (1922), it has been possible to classify them as being either bacteriolytic or non-lytic. Years later, studies with hen and duck egg-white lysozymes as well as with human lysozymes have led to the following definition of these enzymes (Jolles 1969) i.e. they are: (a) basic proteins; (b) low molecular weight MW (15,000); (c) stable at acidic pH and at fairly high temperature; (d) labile at alkaline pH values; (e) lyse suspensions of *M. lysodeikticus*; and (f) their action on an appropriate substrate liberates compounds which can be detected by reagents for reducing sugars and amino sugars. This definition has been subjected to

subsequent modification because of the discovery of several types of lysozymes with different behavior (see Jolles et al. 1974 for a review). Some of these lysozymes differ radically, e. g. hen egg white lysozyme (chicken type, c-type) and the goose egg white lysozyme (g-type). The g-type has a molecular weight of approximately 21,000 instead of 14,000 for the c-type, and does not cross-react immunologically with the latter (Jolles and Jolles 1984 - review). The results presented in this chapter clearly indicate that coho egg lysozyme is a true lysozyme that corresponds to the classical type (c-type) lysozyme found in birds and mammals (Jolles and Jolles 1984) as well as in fish kidneys (Lie et al. 1989).

The marked differences between HEWL and coho egg lysozyme indicate an evolutionary adaptation towards species-specific microbes as suggested by Jolles and Jolles (1984). Such variations in antibacterial activity of c-type lysozymes from different sources have been reported elsewhere (Iacono et al. 1980, Grinde 1989).

The source of the lysozyme present in coho eggs is unknown. It seems likely, however, that it is released from the kidneys and other lysozyme-rich tissues and transported to the developing eggs via the serum. Studies indicate that lysozyme activity is associated with tissues rich in leucocytes (kidney, spleen, alimentary tract). The leucocytes probably contribute to the serum lysozyme activity since their number increases concomitantly with serum lysozyme

levels (Fletcher and White 1973). Observations of seasonal variation in lysozyme activity in carp serum indicate significant differences in the enzyme concentration in fish of different ages, the highest levels of the enzyme occurring in spawners (Studnicka et al. 1986). The occurrence of the enzyme in such high concentrations outside the digestive tract (i.e. in eggs as well as in cells of the immune system) strongly suggest that it has an important defensive function. Furthermore lysozyme alone or in conjunction with complement and antibody is thought to contribute to bacteriolytic mechanisms in vertebrates, including fish (Vladimirov 1968, Glynn 1969).

The Lysoplate method, as opposed to the turbidimetric method, has always been considered as the technique of choice for determining bacteriolytic activity (Osserman and Lawlor 1966). The generated zone of clearance in a positive test can only be accounted for by lysis and solubilization of the cells. In the turbidimetric method, agglutination of bacterial cells brought about by lysozyme (Salton 1957) (which results in a decrease in turbidity due to the settling of aggregates) could easily be mistaken for lysis. Although the lysoplate test was included in this study, clearing due to lysis was not detected in the four tested bacterial pathogens after addition of EY to the wells.

The specific substrate of lysozyme has been alluded to earlier. Susceptibility of microorganisms to the lytic

property of lysozyme depends on accessibility of the enzyme to its peptidoglycan substrate and the tightness of the peptidoglycan network. Tightness of the substrate is a function of the frequency with which the polysaccharide chains are substituted by peptide subunits and the degree to which these peptides are, in turn, interlinked. In *M. lysodeikticus* which is particularly susceptible to lysozyme action, the specific substrate is apparently located in a position directly accessible to the enzyme. In other organisms, especially Gram-negative bacteria, the substrate may be present but inaccessible, because of surface lipids, proteins, and other polysaccharides or additional surface structures.

Gram-negative cells are surrounded by an envelope which consists of an inner cytoplasmic membrane, a murein layer (peptidoglycan), and an outer membrane. Data on the properties of cell surface components expressed by virulent cells of *A. salmonicida* indicate, for example, that the pathogen possesses an additional protein layer (A-layer) which is surface localized (Kay et al. 1981). This barrier allows the penetration of required nutrients and the excretion of waste products. Furthermore, studies have shown that A-layer appears to cover most of the surface of virulent *A. salmonicida*, although some lipopolysaccharide (LPS) may be exposed (Kay et al. 1981).

The mechanism by which coho egg lysozyme exerts its bactericidal activity, especially against those cells that are known to have surface barriers is unclear. However, recent evidence suggests that A⁺ cells are not always armored, and alteration in A protein conformation does occur (W.W.Kay-personal communication). Being a sphere with a diameter of 3.2 nm (Phillips 1967, Harte and Rupley 1968), lysozyme may readily attach to the LPS, or it may be small enough to penetrate through the A-layer and attach to the outer membrane.

The fact that some of the microorganisms were killed but not lysed by the enzyme is consistent with what has been proposed about lysozyme's different mechanisms for bactericidal and lytic activity (Iacono et al. 1980). A possible explanation for these results could be related to properties of lysozyme distinct from its muramidase function. It is apparently able to bind to the lipopolysaccharide of Gram-negative bacteria (Bladen and Mergenhagen 1964, Day et al. 1978), to attach to other cell structures like the cytoplasmic membrane, and to activate an autolytic peptidoglycan hydrolase (Shockman et al. 1979). Binding to lipopolysaccharide or the cytoplasmic membrane may result in loss of viability without obvious lysis, especially when the enzyme is present in high concentrations (Ridley 1928).

In a systemic disease such as bacterial kidney disease, the successful pathogen must have properties which allow it

to avoid, withstand, or overcome the nonspecific and immunospecific defense mechanisms of the host. In addition to the 57 kD protein, a common antigen found in all isolates of *R. salmoninarum* (Getchell et al. 1985), two other cell wall components have been found to be characteristic of the type strain of this bacterium: a unique peptidoglycan, that constitutes 14 to 27% of the cell wall material (Fiedler and Draxl 1986), and an unusual cell wall polysaccharide with galactose, rhamnose, N-acetylglucosamine, and N-acetylfucosamine as constituents (Kusser and Fiedler 1983). This peptidoglycan may be responsible for *in vitro* resistance to many antimicrobial agents including lysozyme (Fryer and Sanders 1981) as well as intracellular survival, particularly in those cells e.g. the macrophages (Young and Chapman 1978) and eggs in which lysozyme, based on the high concentrations present, may be the major antimicrobial substance present.

Although the bacteriolytic property of the enzyme against bacteria tested in this study was not confirmed, our results clearly demonstrate that coho egg lysozyme is an effective bactericidal agent at 700 ug ml⁻¹ - a concentration one third below physiological levels, and indicate that the vertical transmission of *A. hydrophila*, *A. salmonicida* and *L. piscicola* by eggs to coho salmon progeny is doubtful. These results are consistent with previous work on lysozyme from rainbow trout. Grinde (1989), reported that the enzyme has a substantial antibacterial activity not only against Gram-positive bacteria but also against Gram-negative bacterial

fish pathogens. It should also be noted that the effect of lysozyme *in vivo* is most probably potentiated by the presence of other factors, implying that the present results with *Flavobacterium* sp. tend to underestimate the importance of lysozyme, and that other physical or chemical mechanisms for the prevention of intra-ovum infection in salmonids may exist.

CHAPTER II:

IMMUNOGLOBULIN-LIKE PROTEIN

INTRODUCTION

The salmonid embryo has little or no ability to display specific immunity due to immaturity of its lymphoid tissues (Ellis 1977). In contrast to mammals and fowl in which the embryo is protected by materno-foetal or biophysical barriers during the entire period of embryogenesis, embryonic development in oviparous fishes continues after hatching, at which stage the embryo is in direct contact with a harmful environment. It is well known that the mother-to-offspring transfer of antibodies is mainly accomplished through the yolk sac, the placenta, or the neonate gut, depending on the animal species (Brambell 1970). The pathway of this transfer of immunity from mother to offspring has been well studied in the fowl. Antibodies (IgG) in the blood of the mother are transferred to the oocyte via the IgG receptors on the oocyte (Buxton 1952, Kramer and Cho 1970, Rose et al. 1974).

Because fish larvae hatch into a hostile environment when their immunological capacity is still severely limited, it is reasonable to suggest that a mechanism of specific protection of maternal origin has also been developed in fishes. However, the actual information about this subject in fish is very limited. Preliminary results have been reported recently indicating that a maternal transfer of immunity may occur in some fishes (Van Loon et al. 1981; Bly et al. 1986; Mor and Avtalion 1988, 1990; Shors and Winston 1989) through the yolk sac, as in birds.

The purpose of the study reported here was to examine the eggs of coho salmon *O. kisutch* for maternal immunoglobulin (Ig).

MATERIALS AND METHODS

Eggs and preparation of yolk extract Unfertilized, non-water-hardened eggs were obtained from ripe females of coho salmon *O. kisutch* that had returned from the sea to spawn in the Capilano River near Vancouver, British Columbia, Canada. The eggs were transported on ice to the laboratory and immediately stored at -20°C . Prior to use, the eggs were thawed at room temperature and processed as follows. Approximately 40 ml of eggs were washed with 3 changes of phosphate buffer saline (PBS, pH 7.1) to remove as much of the ovarian fluid as possible. The eggs were then homogenized in a tissue grinder at room temperature, and the homogenate filtered through cotton cheesecloth. To 25 ml of the filtrate (Yolk), 75 ml of precooled (-20°C) isopropyl alcohol were added with mixing at 4°C for 20-30 min. The precipitate was allowed to settle for 5 min before the supernatant was decanted. For complete removal of lipids, this procedure was repeated three times with the alcohol and twice with precooled acetone. The final residue was collected in a Buchner funnel (filter paper no. 1, Whatman U.K), washed with a small amount of acetone, and air dried. The dry powder was extracted with 75 ml of PBS with stirring for 1 h at room temperature. The supernatant (yolk extract=YE) was collected following centrifugation ($12,100 \times g$) and stored at 4°C . Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE), and immunoblotting

The proteins of ammonium sulphate-precipitated normal coho serum and YE were separated on SDS-PAGE (separating gel: linear gradient 5-12.5%, stacking gel: 4.5% at 25 mA for 6-7 h) under a denaturing system (with 2-mercaptoethanol) as outlined in Johnstone and Thorpe (1987). Upon completion of electrophoresis, half of the gel was silver stained (Wray et al. 1981) to ensure proper separation of proteins, and the other half was equilibrated for 1 h in the transfer buffer: 25 mM Tris - 192 mM glycine (pH 8.3) with 20% methanol. The separated proteins were then transferred electrophoretically on to a nitrocellulose (N/C) membrane (BIO-RAD, Richmond, U.S.A), using a transblot apparatus (2117-250 Novablot LKB, Bromma, Sweden) at 0.8 mA/cm² for 2 h. All subsequent manipulations were performed at room temperature. After blocking unreacted sites of the N/C membrane by immersion in a 3% solution (w/v) of gelatin in Tris buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h with gentle shaking, the N/C membrane was washed twice in Tween 20-containing TBS (TTBS). It was then exposed to a solution of rabbit anti-coho IgM (diluted 1:250 in 1% gelatin-TTBS=antibody buffer) for 2 h with gentle shaking. Control N/C membranes were treated with a similar dilution of preimmune rabbit serum. The N/C membranes were washed twice in TTBS and then exposed to a solution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (diluted

1:2000 in antibody buffer) for 1 h with gentle shaking. The solution was then discarded and the N/C was washed twice in TTBS and once in TBS. The N/C membrane was finally developed by addition of the HRP substrate (4-chloro-1-naphthol, BIO-RAD). Developed blots were kept in the dark until photographed.

Antisera Antiserum to coho serum immunoglobulin (IgM, purified by the method of DeLuca et al. 1983) was induced in rabbits and generously provided by M.I.Velji. The purity of coho IgM and the specificity of the induced antiserum were determined by comparing the number of protein bands that developed, when a sample of ammonium sulphate-precipitated coho normal serum was run in SDS-PAGE and silver stained, to those bands that developed when the same sample was electroblotted, following SDS-PAGE, on to a N/C membrane, and the latter was probed with the rabbit antiserum. Briefly, the immunoglobulin in 5 ml of normal coho serum was precipitated by addition of 1.25 g of $(\text{NH}_4)_2\text{SO}_4$ (1.95M), incubated for 20 h at room temperature followed by centrifugation at 2,500 x g for 30 min at 4°C. The precipitate was resuspended in PBS, and the process of precipitation was repeated twice before the suspension was finally dialysed against PBS at 4°C for 2 days to reduce the salt content. The preparation was finally stored at 4°C until used. HRP-conjugated goat anti-rabbit IgG was obtained from BIO-RAD.

Absorption of antibody activity Heat-killed bacterial suspensions (100 ul, absorbance = 5.0 at 540 nm) of *Aeromonas salmonicida*, *A. hydrophila*, *Vibrio anguillarum*, *V. ordalii* or *Renibacterium salmoninarum* were combined into Eppendorf tubes, and centrifuged (Micro-Centrifuge, Model 235C, Fisher Scientific) for 10 min at room temperature. The bacterial pellet served as a solid phase for absorption of the antibody activity in the YE. This absorption was assessed by two successive incubations (14 h at 4°C, each) of 100 ul YE (650 ug total protein) with the bacterial pellet. Following centrifugation (10 min at room temp.), the supernatant was run in SDS-PAGE and silver stained.

Sucrose gradient centrifugation A linear gradient of 5 to 42% sucrose (12 ml) was formed in 14x95 mm polyallomer tubes (Beckman, Calif., U.S.A) using a gradient maker. A sample of 100 ul of YE (500 ug ml⁻¹ total protein) was gently layered on top of the sucrose gradient. As markers, Bovine serum IgG and Bovine serum IgM (Sigma) were prepared in PBS at a final concentration of 500 ug ml⁻¹, and 100 ul of each preparation was layered on top of the sucrose gradient. The tubes were then centrifuged for 24 h at 38,000 RPM in a Beckman ultracentrifuge (model L2-65B, with SW-40 rotor) at 4°C. Fractions of 0.5 ml were collected from the bottom of the tube with the aid of a peristaltic pump and a fraction collector. The refractive index of all fractions was determined using a refractometer (Carl Zeiss, Germany), and the readings were converted to sucrose concentration by

reference to the International Critical Tables (1926) of sucrose. Proteins in the fractions were detected using SDS-PAGE.

RESULTS

Figure 13 depicts the separation of coho salmon serum and YE proteins in linear gradient SDS-PAGE. Our preliminary trials with yolk extract suggested the use of a linear gradient (5-12.5%), or a separating gel whose upper portion is $\leq 7.5\%$ acrylamide in order to reveal all the proteins present in coho eggs. This always had to be preceded by total elimination of lipophilic substances and carotinoids present in salmonid eggs. Removal of these agents from the yolk contents seemed to be critical during preparation of YE samples for SDS-PAGE because of their tendency to coagulate upon heating in the reducing buffer.

The specificity of the rabbit antiserum for coho IgM was indicated by the appearance of two major bands on the N/C membrane when the latter was probed with the polyclonal antiserum in western blot technique (Fig. 14). By virtue of their size, and therefore their mobility in SDS-PAGE, these two bands were considered to be the heavy and light chains of the fish tetrameric immunoglobulin molecules in serum.

When electroblotted proteins of the YE were reacted with rabbit anti-coho IgM, the antiserum recognized ,with different degree of reactivity, 3 bands on N/C membrane (Fig. 15, A₁ - A₃). In addition to two broad bands, one narrow band appeared at the upper part of the membrane. This band corresponded to a low mobility protein (≥ 200 kD, the largest

FIGURE 13

Electrophoretic patterns of coho salmon *Oncorhynchus kisutch* yolk extract (A); normal serum (B). MM= BIO-RAD Low Molecular Weight Standards. Reduced (2-mercaptoethanol) samples (10 ug total protein/lane) were run on a 5-12.5% gradient SDS-polyacrylamide gel, and silver stained.

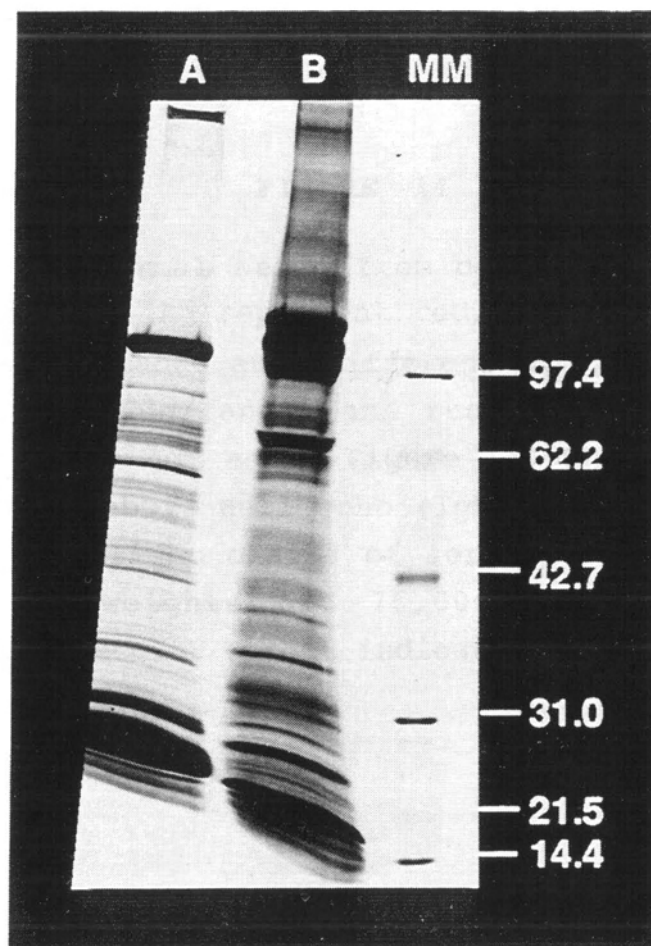
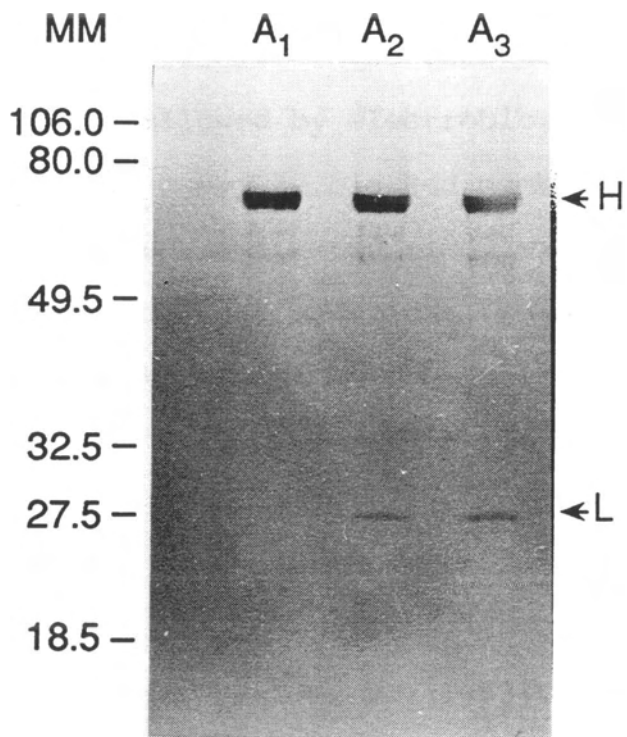


FIGURE 14

Western blot of normal serum from coho salmon *Oncorhynchus kisutch*. Lanes A₁ - A₃ represent reduced (2-mercaptoethanol) samples of serum loaded at 3 different protein concentrations (20.0, 25.0, 35.0 ug/lane), and run on a 5-12.5% gradient SDS-polyacrylamide gel as in Figure 13. The blot was probed with polyclonal rabbit anti-coho (IgM). (H) & (L) correspond to the heavy and light chains of serum immunoglobulin (IgM) with molecular weights of $\approx 76,000$ and 29,000 daltons, respectively. Molecular weight indicated in kilodaltons.

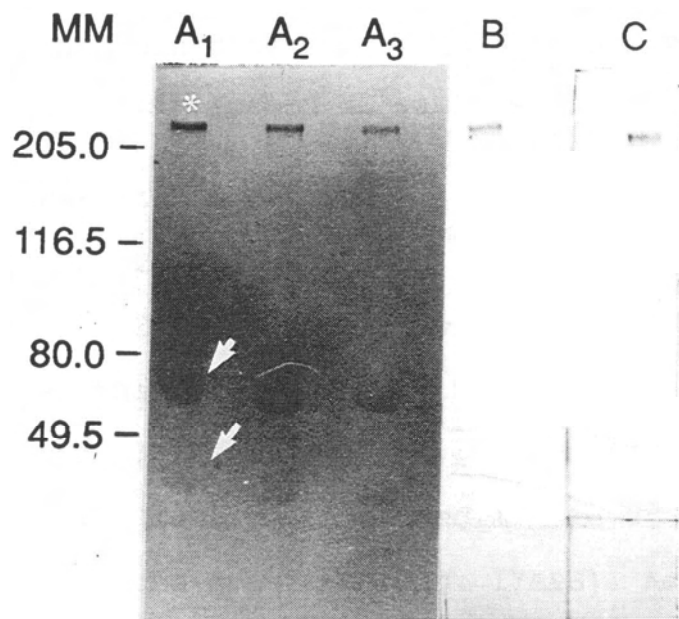


molecular mass marker used) that appeared near the origin of the gel in SDS-PAGE (Fig. 13, lane A). The three reacting proteins of YE were initially considered to be due to either (a) a low molecular weight form of serum IgM and its breakdown products, or (b) to be nonspecific factors such as lectins which are known to bind to horseradish peroxidase and serum globulins (Gilboa-Garber et al. 1977a, Lis and Sharon 1977). In order to resolve these questions, YE samples were run in SDS-PAGE, followed by electroblotting of the proteins on to the N/C membrane. The blocked membrane was cut into lanes and each lane was treated (1 h, with gentle shaking) in one of the following test solutions (TS= D-galactose, L-rhamnose, D-mannose, or N-acetyl D-galactosamine, each at 0.3M), and subsequently processed as outlined before. The test solutions were prepared in the antibody buffer at a concentration that has been shown to inhibit a lectin from coho eggs (see Chapter III-Lectin), as well as a lectin from other salmonid eggs (Ozaki et al. 1983).

The positive reactivity recorded at the upper part of the N/C membrane was not influenced by any of the sugars tested. However, the lower two broad bands were equally susceptible to N-acetyl D-galactosamine and D-mannose, and their non-specific reaction with the reagent(s) used in the western blot was substantially suppressed (Fig. 15, lanes B&C). It is noteworthy, that N/C membranes treated with pre-immune serum did not react with the secondary antibody,

FIGURE 15

Western blot of coho salmon *Oncorhynchus kisutch* egg-yolk extract. Reduced (2-mercaptoethanol) samples of protein (A₁, A₂, A₃=35.0, 25.0, 20.0 ug/lane, respectively) were run on a 5-12.5% gradient SDS-polyacrylamide gel as in Figure 13. The blot was probed with polyclonal rabbit anti-coho IgM. Coho egg immunoglobulin (star) and lectin-like proteins (arrows) are indicated. B and C as for A₁, but after treatment with N-acetyl-D-galactosamine, and D-mannose, respectively. Molecular weight indicated in kilodaltons.



subsequently no visible bands were seen on the blots upon staining. On the basis of these results it was concluded that the high molecular weight activity was likely due to an Ig-like protein, whereas the low molecular weight activity, suppressed by treatment with monosaccharides, was due to lectin-like proteins. Furthermore, the preliminary efforts to absorb the antibody activity in the eggs with selected species of bacterial fish pathogens were unsuccessful, and the Ig-like protein band was visible in SDS-PAGE of the supernatants from absorbed YE samples (results not shown).

Ultracentrifugation analysis of YE in sucrose gradient resulted in fractionation of the proteins, and revealed the presence of the Ig-like protein which was localized at the protein layer that sediments at the 17.0-20.0% sucrose area of the gradient (Fig. 16A&B). Bovine serum IgG molecules were also located in fractions that sedimented at the 20.0-21.5% sucrose area of the gradient (Fig. 17A&B). As expected bovine serum IgM molecules were located in areas of a higher sucrose concentration (33.0-34.5% sucrose, Fig. 18A&B).

FIGURE 16(A)

Electrophoretic analysis of coho egg-yolk extract = YE (A). 100 ul of YE (50 ug total protein) was layered on top of the sucrose gradient (linear, 5-42%) followed by centrifugation. Fractions (B₁ - B₂₃) were collected from the bottom of the tube and run on a 5-12.5% gradient SDS-polyacrylamide gel (with 2-mercaptoethanol), and silver stained. Location of the immunoglobulin (Ig)-like protein in fractions 14 - 16 is indicated (stars). MM= BIO-RAD Low (left) and High (right) Molecular Weight Standards.

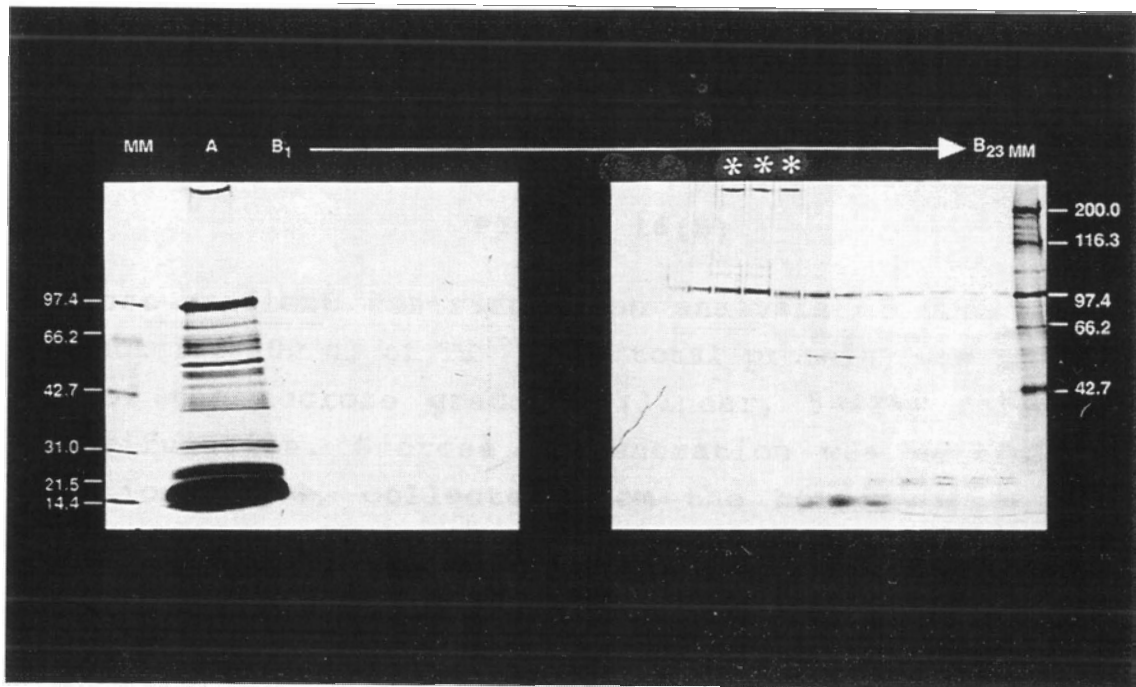


FIGURE 16(B)

Sucrose-gradient centrifugation analysis of coho egg-yolk extract=YE. 100 ul of YE (50 ug total protein) was layered on top of the sucrose gradient (linear, 5-42%) followed by centrifugation. Sucrose concentration was determined in fractions 1-23, collected from the bottom of the tube. Arrows=immunoglobulin (Ig)-like protein molecule in fractions 14, 15, 16 (= 20.0, 18.5, 17.0% sucrose), respectively.

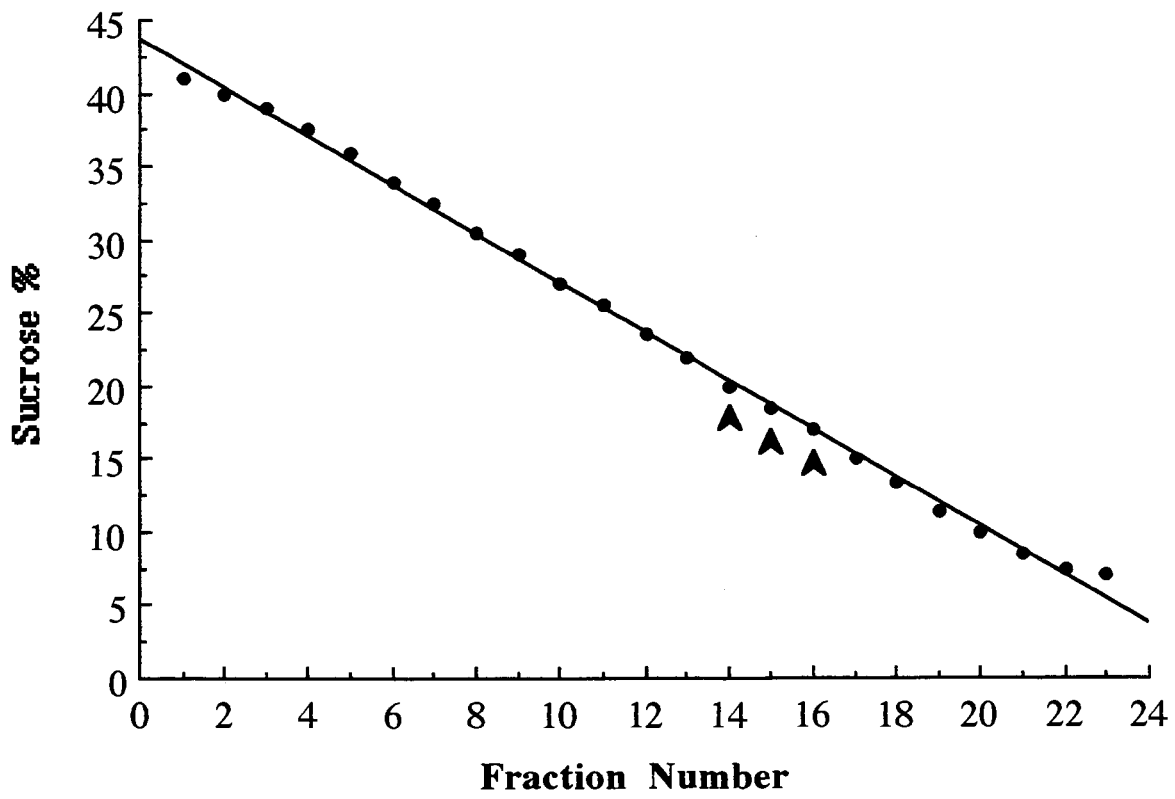


FIGURE 17(A)

Electrophoretic analysis of bovine serum immunoglobulin IgG (A). 100 ul of the protein (equivalent to 50 ug protein) was layered on top of the sucrose gradient (linear, 5-42%) followed by centrifugation. Fractions (B₁ - B₂₃) were collected from the bottom of the tube and run on a 5-12.5% gradient SDS-polyacrylamide gel (with 2-mercaptoethanol), and silver stained. Location of the protein in fractions 14 - 15 is indicated (stars). MM= BIO-RAD Low Molecular Weight Standards.

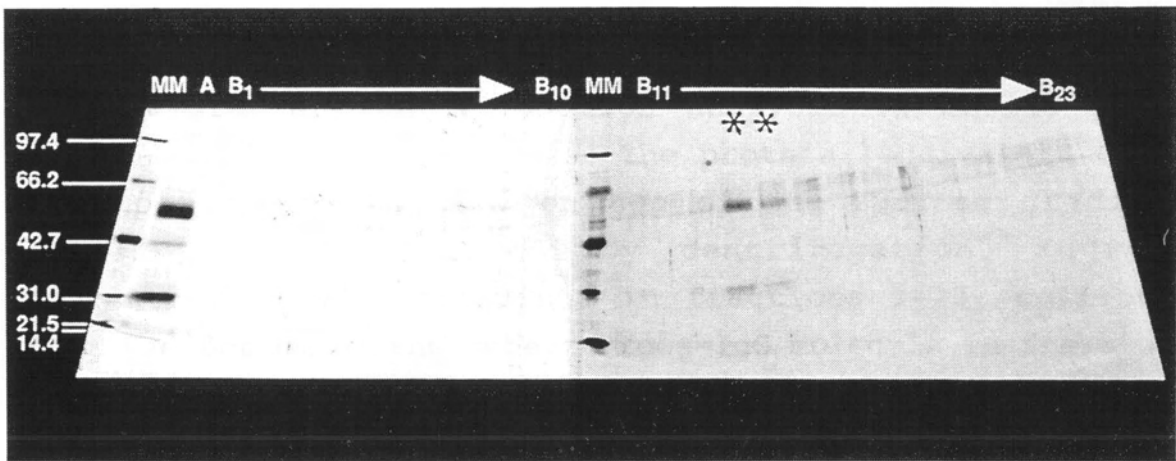


FIGURE 17(B)

Sucrose-gradient centrifugation analysis of bovine serum immunoglobulin IgG. 100 ul of the protein (equivalent to 50 ug protein) was layered on top of the sucrose gradient (linear, 5-42%) followed by centrifugation. Sucrose concentration was determined in fractions 1-23, collected from the bottom of the tube. Arrows=IgG molecule in fractions 14 & 15 (= 21.5 & 20.0% sucrose), respectively.

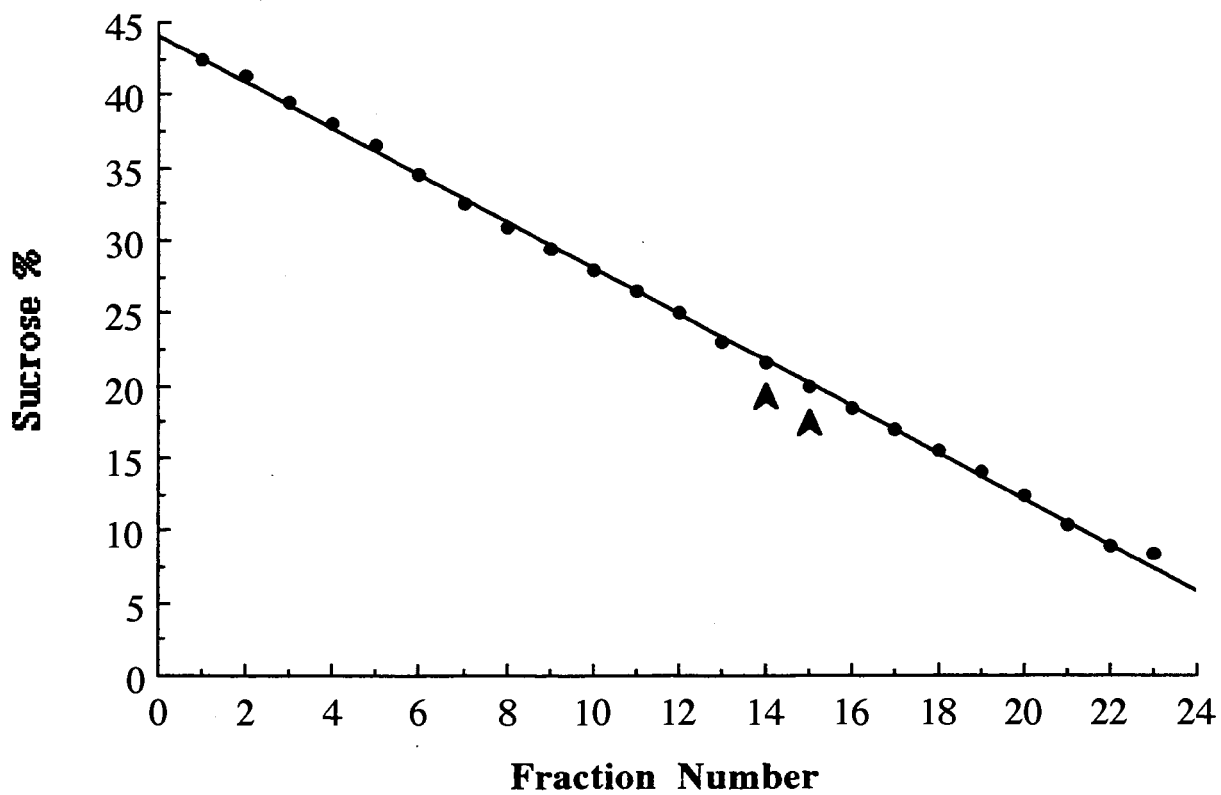


FIGURE 18(A)

Electrophoretic analysis of bovine serum immunoglobulin IgM (A). 100 ul of the protein (equivalent to 50 ug protein) was layered on top of the sucrose gradient (linear, 5-42%) followed by centrifugation. Fractions (B₁ - B₂₃) were collected from the bottom of the tube and run on a 5-12.5% gradient SDS-polyacrylamide gel (with 2-mercaptoethanol), and silver stained. Location of the protein in fractions 6 & 7 is indicated (stars). MM= BIO-RAD Low Molecular Weight Standards.

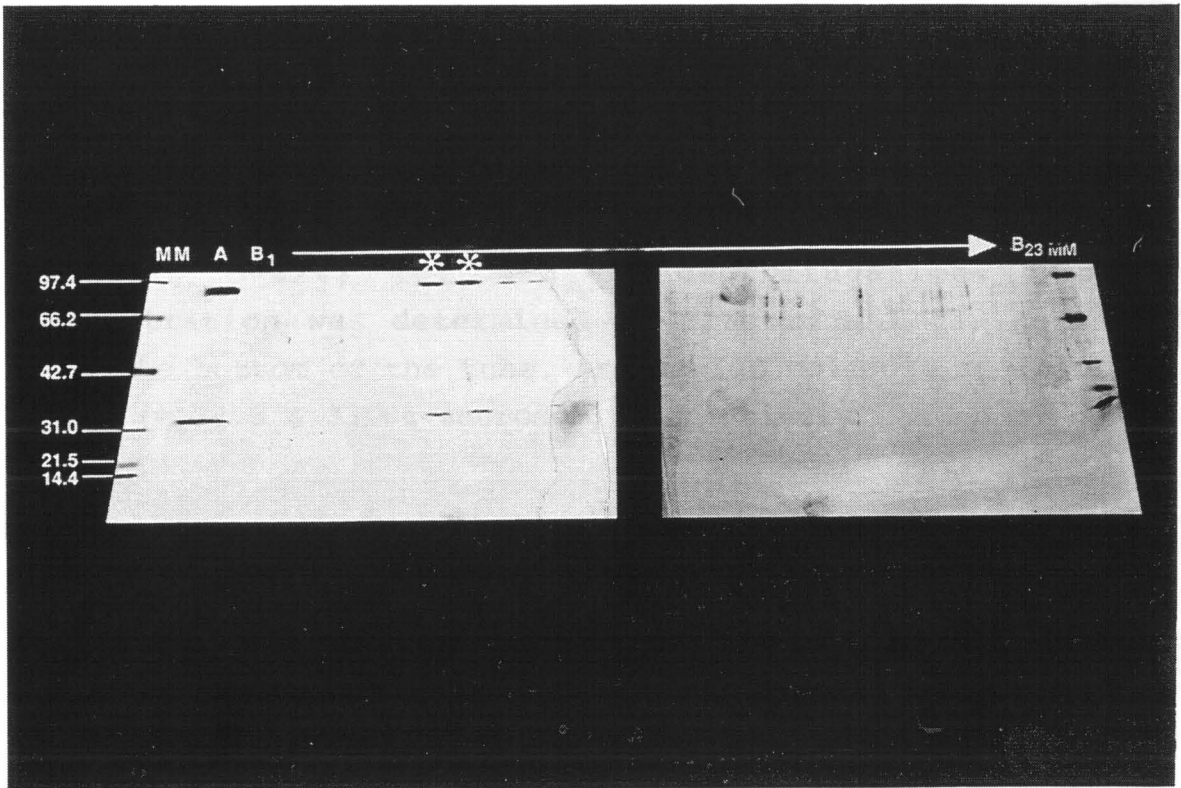
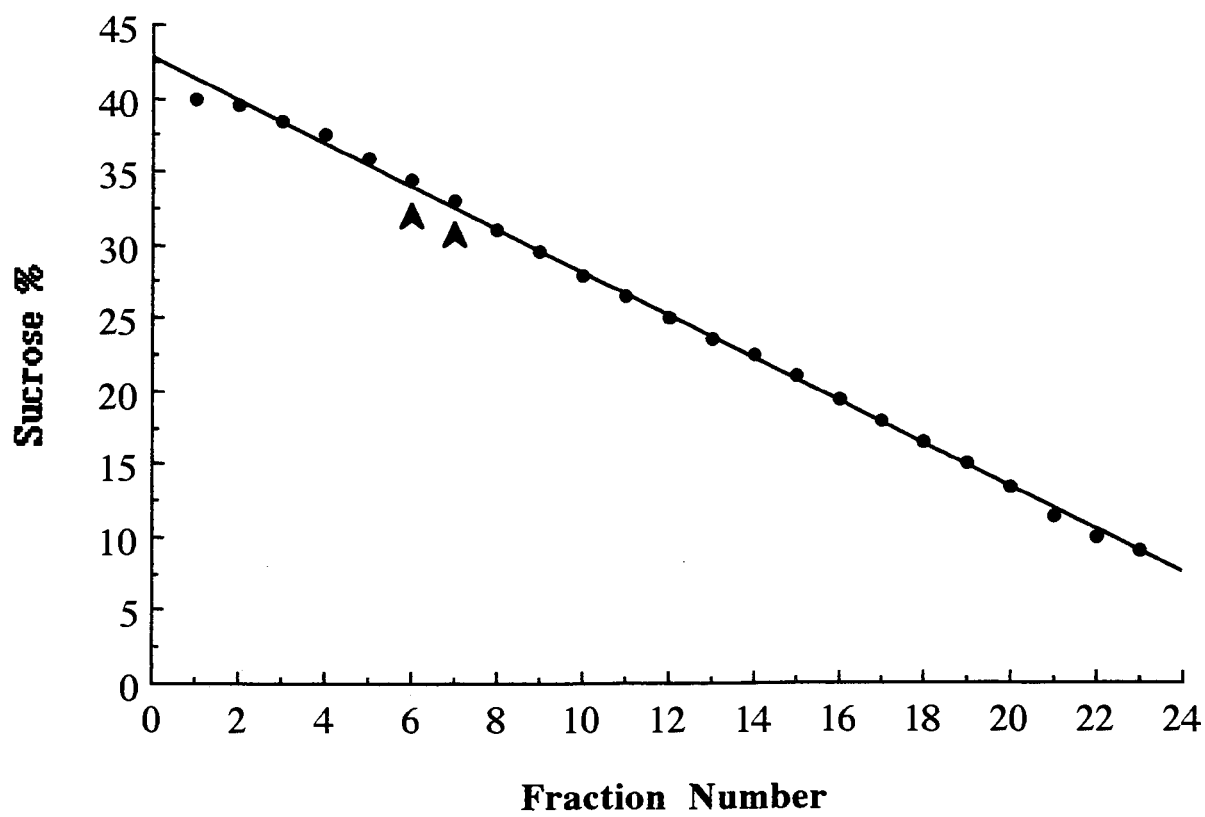


FIGURE 18(B)

Sucrose-gradient centrifugation analysis of bovine serum immunoglobulin IgM. 100 ul of the protein (equivalent to 50 ug protein) was layered on top of the sucrose gradient (linear, 5-42%) followed by centrifugation. Sucrose concentration was determined in fractions 1-23, collected from the bottom of the tube. Arrows=IgM molecule in fractions 6 & 7 (= 34.5 & 33.0% sucrose), respectively.



DISCUSSION

A combination of the high resolving power of the polyacrylamide gel technique with the specificity of antibodies constitutes a valuable research tool. Inside the gel, however, protein detection is limited because probes can not easily penetrate the gel matrix. Because access is restricted, the information obtained from a gel is usually limited to the molecular weight and purity of a moiety. This problem has been at least partially overcome by the development of the western blot technique, where proteins are separated by SDS-PAGE and electrophoretically transferred to a sheet of porous material e.g. nitrocellulose which can then be stained with labeled antibodies. This technique has considerable advantages over alternatives such as double immunodiffusion and crossover immunoelectrophoresis. These features include its high sensitivity and the ability to detect protein concentrations at the nanogram levels. In addition, the system uses minimum amounts of reagents, thus making it a suitable and yet feasible tool in immunology.

Under normal circumstances fish are exposed to environmental antigens. In salmonids, a single class of immunoglobulin (IgM) molecules is produced. These specific antibodies can be detected in the serum and mucus of fish (Ellis 1982).

In higher bony fishes (osteichthyes) the IgM is a 13-16S tetramer with a molecular weight of 600 - 630 kDa (Acton et al. 1971). However, under reducing conditions of SDS-PAGE, coho serum IgM dissociates into heavy (H) and light (L) chains with estimated molecular weights of 76 kDa (H) and 29 kDa (L) (Cisar and Fryer 1974, Groberg 1982). Thus it resembles the stingray *Dasyatis americana* IgM (M_r = 820 kDa) as determined by Johnston et al. (1971).

In this study, transfer of antibodies from mother to offspring in coho salmon was investigated using SDS-PAGE and western blot techniques. The results indicate the presence of an immunoglobulin (Ig)-like protein in unfertilized eggs of coho salmon, and the presence of two low molecular weight proteins that behaved like lectins because of their reactivity with sugars. Vertical transfer of protective substances such as those just mentioned would not be surprising in view of finding of immunoglobulin in the eggs/embryos of carp (Van Loon et al. 1981); plaice (Bly et al. 1986); tilapia (Mor and Avtalion 1988,1990); and rainbow trout (Shors and Winston 1989).

Egg homogenates of coho salmon separate into 3 different layers upon centrifugation: an upper fatty layer, a PBS soluble fraction, and a pellet of broken egg shells (chorions). In this study, broken shells were removed by filtration through cotton cheesecloth, and the filtrate (fatty layer and PBS soluble fraction) was subjected to a

process of simultaneous precipitation and extraction using isopropyl alcohol and acetone. The PBS extract of the precipitate proved to contain an Ig-like protein. Similarly, Mor and Avtalion (1990) showed that most of the antibody activity in the eggs of tilapia *Oreochromis aureus* was located in the PBS soluble fraction rather than in the upper fatty layer.

Sucrose gradient separation of the YE proteins, followed by SDS-PAGE showed that the Ig-like protein sedimented at sucrose concentrations relatively similar to those of bovine serum IgG (Fig 16B, 17B). Mor and Avtalion (1988), showed that some of the antibody activity in tilapia eggs sedimented at a sucrose concentration similar to that of rabbit immunoglobulin (IgG). While direct evidence for the presence of coho immunoglobulin in the egg extract is not yet available, the antibody activity reported in this study strongly supports the assumption that a maternal transfer of humoral immunity mediated by an immunoglobulin-like protein occurs. Because this antibody-like protein sedimented in a manner analogous to IgG rather IgM, it is concluded that a low molecular weight form of serum IgM, perhaps a breakdown product or precursor of the IgM molecule is transferred from the mother to egg in coho salmon.

Failure to absorb the antibody activity in the eggs with heat killed bacterial cells of *A. salmonicida*, *A. hydrophila*, *V. anguillarum*, *V. ordalii* or *R. salmoninarum* suggests that

the reported Ig-like protein in coho eggs was likely induced in the mother fish as a result of exposure to some other antigen(s).

CHAPTER III: LECTIN

INTRODUCTION

Living organisms possess a large variety of proteins that have the ability to specifically bind different compounds. Most important among these are enzymes that combine specifically with substrates and inhibitors, and antibodies that bind antigens. A third class of proteins with specific combining sites, found mainly in plants, also occurs: the lectins. Boyd and Shapleigh (1954), coined the term "lectin" -from the latin for "choose"- to describe a group of plant proteins with common properties, such as the capacity to agglutinate certain erythrocytes.

Through their sugar-combining sites, lectins interact specifically with polysaccharides and glycoproteins to form precipitates. This reaction is similar to the precipitin reaction between antibody and antigen, in that it is specific, exhibits concentration dependence on both lectin and polysaccharide (or glycoprotein), and may be inhibited specifically by low molecular weight "haptens" - compounds identical with or derived from sugar(s) for which the lectin is specific.

Lectins are thus similar in many respects to antibodies. There are, however, also striking differences between the two classes of compounds. First and foremost is the fact that the antibodies are the product of the immune system of animals, where they are formed as a response to an antigenic stimulus.

In contrast, lectins are formed without the requirement of such a stimulus because most of them are found in organisms (e.g. plants and bacteria) which do not possess the capacity for an immunologic response. Secondly, although each antibody is specific for the antigen (e.g. a protein, carbohydrate, or a glycoprotein) that elicited its formation, lectins are specific for carbohydrates, both simple and complex. Thirdly, in contrast to antibodies that are structurally similar, lectins are diverse, their only common feature being that they are all proteins. In this last property, lectins are similar to enzymes although they are devoid of catalytic activity.

Lectins have gained an important niche in the analysis of microbes. One important explanation is that they interact with several surface structure components of microorganisms, including teichoic and teichuronic acids, peptidoglycans, lipopolysaccharides, glycoproteins, and polysaccharides. Therefore, lectins offer several advantages in applications in the diagnostic microbiology laboratory. They can be used in very low concentrations. When mixed with microorganisms, they may lead to spontaneous agglutination of the cells. They also adhere to plastic, polystyrene, and latex particles. In such bound forms, lectins can be configured for use in rapid diagnostic assays.

In fish, the presence of lectins has been recorded in the ova of many species of the family *Salmonidae* (Uhlenbuck

and Prokop 1967, Anstee et al. 1973, Voak et al. 1974, Krajhanzl et al. 1978, Ozaki et al. 1983, Kamiya et al. 1990). The function of these lectins is not yet known. However, in lower vertebrates, which lack a highly developed immune system, they are thought to serve antibody-like functions in defense against foreign entities, including bacteria (Prokop et al. 1968, Voss et al. 1978, Kudo and Inoue 1986, 1989).

Studies reported in this chapter describe the occurrence, purification, and partial characterization of a lectin from the eggs of coho salmon *Oncorhynchus kisutch*. The opsonic role of the lectin was investigated using rainbow trout *O. mykiss* macrophage cultures. The interaction of the lectin with bacterial fish pathogens, particularly *A. salmonicida*, is also discussed.

MATERIALS AND METHODS

Eggs and preparation of yolk extract Unfertilized, non-water-hardened eggs were obtained from ripe females of coho salmon that had returned from the sea to spawn in the Capilano River near Vancouver, British Columbia, Canada. The eggs were transported on ice to the laboratory and immediately stored at -20°C . Prior to use, the eggs were thawed at room temperature and processed as follows.

Approximately 40 ml of eggs were washed with 3 changes of phosphate buffered saline (PBS, pH 7.1) to remove as much of the ovarian fluid as possible. The eggs were then homogenized in a tissue grinder at room temperature, and the homogenate filtered through cotton cheesecloth. To 25 ml of the filtrate (Yolk), 75 ml of precooled (-20°C) isopropyl alcohol were added with mixing at 4°C for 20-30 min. The precipitate was allowed to settle for 5 min before the supernatant was decanted. For complete removal of lipids, this procedure was repeated three times with the alcohol and twice with precooled acetone. The final residue was collected in a Buchner funnel (filter paper no. 1, Whatman U.K), washed with a small amount of acetone, and air dried. The dry powder was extracted with 75 ml of PBS with stirring for 1 h at room temperature. The supernatant (yolk extract=YE) was collected following centrifugation ($12,100 \times g$) and stored at 4°C .

Bacterial strains Bacteria used in the experiments were: *A. salmonicida* [strain #76-30 A⁺, (virulent, A⁺ LPS⁺)]; *Renibacterium salmoninarum* (strain #384); *A. hydrophila* (strain #35); *Vibrio ordalii* (strain #74-48), isolated from sockeye salmon *O. nerka* with vibriosis at the Pacific Biological Station, Nanaimo, B.C.; and *V. anguillarum* (strain #R20), isolated from chinook salmon *O. tshawytscha* with vibriosis in 1989 at Read Island, B.C. *A. salmonicida* strains A450 (virulent, A⁺ LPS⁺); A450-1 (avirulent, A⁻ LPS⁻); and A450-3 (avirulent, A⁻ LPS⁺) were kindly provided by W. W. Kay, University of Victoria, Victoria, B.C. Stock cultures of all bacteria were kept at -70°C in tryptic soy broth (TSB, Difco, Detroit, MI) with or without salt, or in KDM-2 (Evelyn 1977). Both media were supplemented with 1% gelatin or 15% glycerol. Propagation of the cells was carried out using brain heart infusion (BHI, Difco) agar, with or without salt at 15°C, except *R. salmoninarum* for which charcoal medium (Daly et al. 1985) that had been made selective (SKDM-C) for the bacterium by the addition of antibiotics described by Austin et al. (1983) was used.

Treatment of the bacteria Bacterial cultures were aseptically scraped off agar plates, suspended and washed twice in sterile PBS by centrifugation (5,860 x g for 15 min at 4°C), and the final suspension was adjusted to an absorbance of 5.0 at 540 nm before being heated at 100°C for 1 h. The bacterial cells were then washed three more times in PBS and the suspensions were finally stored at 4°C.

Adsorption test Adsorption of coho egg lectin onto bacterial cells was examined by incubating bacterial cells (cell pellets from 100 ul of bacterial suspension of each species or a combination of all species) with 100 ul of YE (total protein=650 ug) for 14 h with occasional shaking at 4°C. Following centrifugation, the supernatants were removed and examined for the presence of lectin using gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bacterial pellets were then washed in PBS (1.5 ml), and bound lectin was eluted by resuspending the pellet in 100 ul of 0.2M D-galactose or L-rhamnose for 2 h at room temperature followed by centrifugation. The supernatants were dialysed extensively (2 d) against distilled water and kept at 4°C.

Effect of saccharides, 2-mercaptoethanol, and heat The inhibitory effects of saccharides and 2-mercaptoethanol on the binding of the lectin to heat-killed *A. salmonicida* (strain #76-30 A⁺) cells were studied as follows. To 100 ul of one of the test solutions (TS= L-rhamnose, D-galactose, D-mannose, D-glucose, N-acetyl D-galactosamine, D-mannosamine, D-galactosamine or 2-mercaptoethanol, each at 0.2M, Sigma, St. Louis, U.S.A) 100 ul of YE was added. The mixtures were incubated for 2 h at room temperature. Each bacterial suspension (100 ul) was centrifuged for 10 min at room temperature and the supernatant was discarded. The pellet was then resuspended in the YE-TS mixture and the suspension incubated for 14 h at 4°C. Finally, the supernatant was collected by centrifugation and examined for the presence of

lectin using gradient SDS-PAGE. The effect of heat on the lectin's ability to bind to the surface of *A. salmonicida* was examined by incubation of YE at 100°C for 15 min. Following heating and centrifugation, the residual adsorption activity of the lectin in the supernatant was tested as outlined before.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein preparations: Electrophoresis procedures for SDS 4.5% stacking and linear gradient (5-20%, 10-20%) separating acrylamide gels under denaturing conditions (with 2-mercaptoethanol) and silver staining were identical with those outlined in Johnstone and Thorpe (1987). The samples were run at a constant current of 25 mA/gel (140 x 125 x 1.5 mm) using a large vertical slab gel electrophoresis unit (Se 600, Hoeffer Scientific Instruments, San Francisco, Calif.).

Bacterial preparations: *A. salmonicida* strains #76-30 A⁺, A450, A450-1, and A450-3 were grown statically in BHI for 48 h at 15°C. Bacteria were centrifuged for 15 min at 5,860 x g and whole cell (WC) lysates were prepared in sample buffer as described by Laemmli (1970) using a concentration of 1.0 g (wet weight) of bacteria to 20 ml of sample buffer. A portion of the WC lysate was also treated with proteinase K (PK) according to the procedure of Hitchcock and Brown (1983). Membrane preparation (MP) of A450 was kindly provided by W. W. Kay, University of Victoria, Victoria, B.C.

Electrophoresis of bacterial preparations (WC, PK, MP) was carried out according to the method of Laemmli (1970)

using a 4% stacking gel and a 12% separating gel 1 mm thick. WC lysates (15 ul/5 mm well), PK digested lysates (30 ul/ 5 mm well) and MP (7.4 ug total protein) were separated on the BIO-RAD mini-Protean II Slab Cell at 25 mA constant current. After electrophoresis the gels of WC lysates and MP were stained for proteins with Coomassie brilliant blue R 250. The gels of PK digested lysates were silver stained, as described by Tsai and Frasch (1982), to visualize LPS.

Low molecular weight protein standards (BIO-RAD, Richmond, U.S.A) were included in each run. All chemicals used in electrophoresis were purchased from Sigma (St. Louis, MI).

Western Blotting

Preparation of rabbit antiserum: Antiserum against coho egg lectin was produced by injection of a New Zealand white female rabbit with the purified lectin mixed with AdjuPrime immune modulator (Pierce, U.S.A). AdjuPrime was ground in a mortar and pestle and suspended in the antigen solution to a final concentration of 3.3 mg ml^{-1} . Approximately 112 ug of lectin was injected subcutaneously and the rabbit was allowed to rest for 2 w after which it was boosted with 98 ug of the antigen. Blood was collected from the marginal ear vein, allowed to clot at 37°C for 1h and then incubated overnight at 4°C . The serum was collected by centrifugation and kept at -20°C . Normal rabbit serum was collected before immunization for use as negative control.

Blotting technique: Discontinuous SDS-PAGE of samples was performed as described above. Prestained markers of low

molecular mass (BIO-RAD) were included with each gel. Polypeptides were then transferred electrophoretically onto 0.45 μ m nitrocellulose (N/C) membrane (BIO-RAD), using a semi-dry transblot apparatus (2117-250 Novablot LKB, Bromma, Sweden) at 0.8 mA/cm² for 1-2 h. All subsequent manipulations were performed at room temperature. The N/C membranes were washed briefly in Tris buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5), and then blocked for 1 h with 3% solution (w/v) of gelatin in TBS. The N/C membranes were then washed twice in Tween 20-containing TBS (TTBS) and exposed to a solution of rabbit anti-coho egg lectin (diluted 1:50 in 1% gelatin-TTBS= antibody buffer) overnight with gentle shaking. The N/C membranes were washed twice in TTBS and then exposed to a solution of horseradish peroxidase (HRP) conjugated to goat anti-rabbit IgG (BIO-RAD), diluted 1:1500 in antibody buffer for 1h with gentle shaking. The solution was then discarded and the N/C membranes were washed twice in TTBS and once in TBS. The N/C membranes were developed by addition of the HRP substrate (4-chloro-1-naphthol, BIO-RAD). Developed blots were kept in the dark until photographed. In those experiments with bacterial cell whole lysates, proteinase K digest and membrane preparations, the N/C membranes were treated first with either purified lectin (255 μ g in 16.5 ml PBS) or YE (20.0 ml, total protein=126 mg) overnight with gentle shaking. The N/C membranes were then probed with the antisera as described above.

Purification of egg lectin

Production of galactose affinity medium: 10 g of damp Sepharose 4B (Sigma) was washed with 500 ml of distilled

water and dried to dampness by suction on a glass-fretted funnel. Sepharose was then resuspended in a solution containing 10 ml butanedioldiglycidyl ether (DGE) and 10 ml 0.6N NaOH containing 0.6% NaBH₄. The gel was left to react overnight on a horizontal shaker at room temperature and then washed with 1.5 L of distilled water as before. Coupling with carbohydrate was achieved by resuspending the epoxyactivated Sepharose in a 50 ml solution of 1.5M D-galactose in 0.5M Na₂HCO₃ at pH 11. The mixture was kept in suspension overnight on a horizontal shaker held at 50°C. This was followed by washing the gel with 1.5 L of distilled water as before. The gel was then resuspended in 50 ml of 0.5M ethanolamine for 1 h with shaking at room temperature. This was necessary to block any of the remaining activated epoxy groups. The gel was washed with distilled water as before and the slurry was equilibrated in PBS, and packed into a 10-cm-long column (internal diameter=1.6 cm).

Chromatography procedure: Coho egg lectin was purified from yolk materials by affinity chromatography using a column (1.6x5.2 cm) of galactose substituted epoxyactivated Sepharose 4B. 10 ml of YE (total protein=90 mg) were applied onto the column at a flow rate of 15 ml h⁻¹. This procedure was repeated three times with the eluate, and then the column was washed with PBS until absorbance at 280 nm was zero. Bound protein was eluted with 0.2M D-galactose in PBS and fractions of 2 ml were collected. The eluted protein fractions were pooled, dialysed extensively (2 d) against distilled water at 4°C, and lyophilized.

The purity and molecular weight of the purified lectin were determined electrophoretically using SDS-PAGE.

Protein quantification Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

Amino acid analyses N-terminal sequence was determined by using an Apple Biosystems model 470A gas-phase sequencer with on-line phenylthiohydantoin analysis. The N-terminal amino acid sequence was confirmed using the major 24.5-kDa protein band that had been separated by SDS-PAGE and electrophoretically transferred to Immobilon (Millipore Corp., Bedford, Mass.) The Immobilon-bound protein was stained with silver, cut from the membrane, and sequenced directly (LeGendre and Matsudaira 1988). Comparisons of the N-terminal sequence of the purified lectin with proteins listed in the GenBank data base (release number 64), SWISS-PROT (release number 14), and GENPEPT (release number 63) were made using the BLAST program.

Total amino acid analysis of the purified protein was obtained on samples hydrolyzed in gaseous HCl (165°C, 1 h) and analyzed by using an Applied Biosystems model 420 amino acid derivatizer analyzer. Derivatized proteins were removed from the reaction mixture by overnight incubation (4°C, dark) in the presence of small pieces of Immobilon. The nylon membrane-bound proteins were removed from the mixture, rinsed, air-dried, and stored at -20°C until analysis. β -

Lactoglobulin was used as a standard to confirm the success of the carboxymethylation reaction.

Isolation and culture of anterior kidney M ϕ s The anterior kidney of a single male rainbow trout (~300 g) was removed through a ventral incision and dissociated mechanically into single cells (by passing through nylon mesh 20 μ m pore size) in Leibovitz's L-15 medium (Gibco, U.S.A) containing glutamine, glucose and heparin at a final concentrations of 300 mg/L, 40 g/L and 10 I.U/ml, respectively. The M ϕ s were isolated from the rest of the cells by centrifugation in a self generating gradient of Percoll (Pharmacia, Uppsala, Sweden) according to the method of Garduno and Kay (1992). The isolated M ϕ s were then washed by centrifugation (300 x g at 4°C for 10 min) in Hanks' balanced salt solution (HBSS, pH 7.9). The cells were counted in a Neubauer hemocytometer after 1:2 dilution in 0.4% trypan blue in PBS, and subsequently diluted with L-15 containing glutamine and glucose to give approximately 2.0×10^6 (M ϕ) ml⁻¹. Cover slip cultures were prepared by seeding 250 μ l of the diluted cell suspension (5.0×10^5 M ϕ) onto 22 x 22 mm cover slips which were kept in 35 x 10 mm petri dishes (Garduno and Kay 1992). Similarly, plate cultures were prepared by seeding 250 μ l of the diluted cell suspension in 24-well plates (Corning, U.S.A) at 5.0×10^5 M ϕ /well. Cultures were incubated at 14°C for 2 h, and non-adherent cells were removed by gentle aspiration. Adherent M ϕ s on coverslips and wells were rinsed with HBSS and cultured for 48h or 72h in L-15 containing glutamine, glucose, 10% heat inactivated bovine calf serum (Sigma) and antibiotic-

antimycotic solution (Sigma:100 U/ml penicillin, 100 ug/ml streptomycin, 0.25 ug/ml amphotericin B) with media changes every 24h. The medium was then washed off with HBSS and the M ϕ s were prepared for immunofluorescence localization of lectin receptors, and the association experiments with bacteria.

Immunofluorescence Smears of heat-fixed *A. salmonicida* strain # 76-30 A⁺ were prepared by air-drying cells onto glass slides followed by heat fixing. Circles (1 cm dia) were drawn on the smear using a Tek pen (Manostat, New York). The area within the the circle was then treated with coho egg lectin (170 ug ml⁻¹) for 1 h. This was followed by 1:20 dilution of rabbit anti-coho egg lectin antiserum for 30 min, 1:100 dilution of affinity purified goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma) for 30 min. After each incubation in a humid chamber at room temperature, the slides were rinsed three times in PBS and then incubated in the same buffer for 15 min. However, after the fluorescein-labeled antibody the slides were rinsed in Na₂CO₃/Na₂HCO₃ buffer (pH 9.5) and incubated in the same buffer for 15 min. Mounting medium (80% glycerol in Na₂CO₃/Na₂HCO₃ buffer) was added to blot-dried slides before microscopical examination.

Head kidney M ϕ s cultured on cover slips for 72 h were rinsed with HBSS, air-dried, and fixed in 100% methanol for 5 min. Indirect immunofluorescence localization of lectin's receptor(s) on the surface of the M ϕ was performed following the same staining sequence outlined for *A. salmonicida*. However, lectin was used at a concentration of 30 ug ml⁻¹.

Photomicrographs were taken using a Zeiss Axioplan binocular microscope fitted with an epifluorescence attachment, and a Plan-Neofluar 100x/1,30 oil immersion objective.

Sensitization of bacteria and bacterium-M ϕ association assay *A. salmonicida* strains # A450 and A450-3 were grown on TSA for 48 h at 22°C. Bacterial colonies were aseptically scraped off, and washed twice in sterile PBS, and the PBS-cell suspension was adjusted to an absorbance of 1.0 at 650 nm. Stock suspensions contained approximately 7.5×10^8 CFU ml⁻¹ [determined by serial dilution in sterile peptone 0.1%/saline 0.85% (P/S) and plating on TSA]. Prior to their use with M ϕ s 600 μ l of the stock suspensions from both strains were centrifuged (300 x g at 4°C for 10 min) and the pellets were resuspended in 1.0 ml of PBS containing 60 μ g or 20 μ g of purified lectin for 2 h at 4°C with occasional shaking. The medium was removed from the M ϕ s plate cultures and HBSS was used for rinsing and maintaining M ϕ cultures prior to the phagocytosis experiment. Duplicate wells were inoculated with 100 μ l of bacterial suspensions in lectin (4.5×10^7 CFU well⁻¹) and phagocytosis was allowed to proceed for 30, 60, or 90 min at 14°C. Control wells received bacteria that had been incubated in lectin-free PBS, and were incubated for the same time periods.

Following incubation of the M ϕ s with the bacterial suspensions, the M ϕ s were washed twice in HBSS, drained and air dried, fixed in 100% methanol for 5 min at room temperature and stained with an aqueous solution (1:50) of Giemsa stain (Sigma). The bottom of each well was removed,

using a motorized cork borer, and mounted on a glass slide for direct microscopy counts on monolayers. 100 Mφs were counted randomly from populations in duplicate cultures of each treatment. The phagocytic index (number of bacteria associated with 100 Mφs) was determined by multiplying the percentage of phagocytosing Mφs by the mean number of bacterial cells per phagocytosing Mφs.

The growth inhibitory effect of lectin on strains 76-30 A⁺, A450, A450-1, and A450-3 was investigated by placing sterile 5 mm diameter filter paper discs saturated with the purified lectin (170 ug ml⁻¹) onto TSA plates heavily seeded with the live test bacterium. The plates were examined for zones of inhibition after 48 h incubation at 15°C.

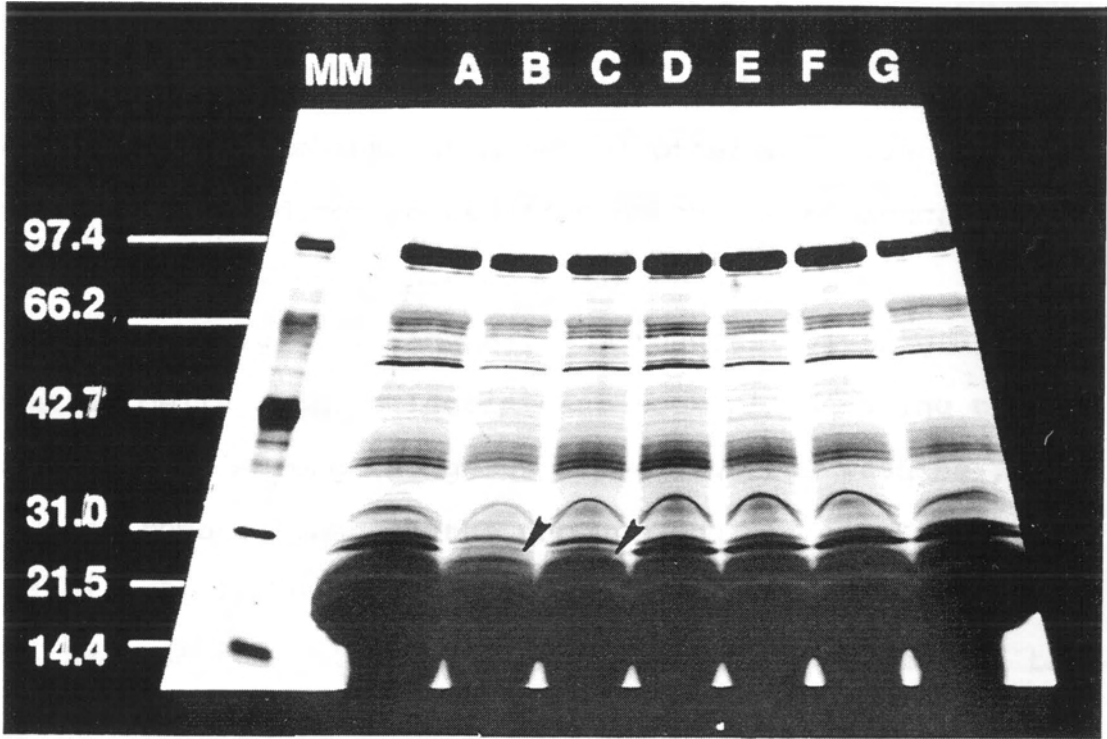
RESULTS

Preliminary SDS-PAGE runs of YE revealed the presence of a large number of egg yolk proteins capable of entering a gel of $\geq 10\%$ acrylamide. However, standard (5.0%, 7.5%, 12.5% acrylamide) gels did not produce satisfactory separations because of their limited porosity. In this study, linear gradient polyacrylamide gels were therefore used. The latter proved efficient, in the range used, for separating egg proteins into discrete bands corresponding to individual proteins and their subunits. An electrophoretic profile of the YE of coho salmon eggs is shown in Figure 19, lane A. The profile consisted of a considerable number of PBS-soluble proteins and glycoproteins, most of which have a molecular weight (M_r) within the range of the protein standards (97.4 - 14.5 kDa).

Because of their specificity for certain sugar moieties, lectins are usually defined in terms of their ability to agglutinate erythrocytes in haemagglutination tests. Those reacting with the erythrocytes do so by virtue of the presence on the latter of carbohydrate configurations or antigenic determinants of the human blood group substances (Springer 1970). Indeed, many kinds of microorganisms, including bacteria, share surface structure components with vertebrate erythrocytes. In the present study, a modified adsorption test in which SDS-PAGE , as opposed to the

FIGURE 19

Electrophoretic patterns of the yolk extract used in the adsorption test. Unadsorbed (A); adsorbed with: a combination of C-G (B), *Aeromonas salmonicida* strain # 76-30 A⁺ (C), *A. hydrophila* (D), *Vibrio ordalii* (E), *V. anguillarum* (F), *Renibacterium salmoninarum* (G). MM= BIO-RAD Low Molecular Weight Standards. Reduced (2-mercaptoethanol) samples were run on a 5-20% gradient SDS-polyacrylamide gel, and silver stained. Arrows indicate adsorbed lectin.



conventional haemagglutination test, was used to detect the protein in adsorbed and unadsorbed samples of YE. In the adsorption test, heat-killed cells of five different bacteria (including four phenotypes of *A. salmonicida*) were incubated with the yolk extract at 4°C. Of all bacteria tested, only *A. salmonicida* cells exhibited substantial ability to absorb a single protein molecule from the yolk extract (Fig. 19, lane C; Fig. 20, lanes A-D). This ability to adsorb the lectin, however, was not demonstrated by the other bacteria also tested, and seemed not to be affected by the presence, in the reaction mixture, of bacterial cells other than *A. salmonicida* (Fig. 19, lane B). The resulting linkage between bacterial cells and the lectin that took place during the adsorption was strong enough to allow rigorous washing, with PBS, that removed most of the unbound egg proteins (Fig. 21, lanes A₁ - A₁₀). This was followed by addition of D-galactose or L-rhamnose that eluted the lectin from the surface of the bacterial cells in a semipurified form (Fig. 20, lanes F-I & 21, lanes B-C). The upper protein bands ($M_r = 97,400$ & $66,000$) that occurred in sugar eluates were considered contamination by egg proteins because PBS washes contained these proteins.

To characterize the binding specificity of the lectin, various sugars were tested for their ability to inhibit lectin binding in the adsorption test. Figure 22 depicts the effect of sugars and denaturing agents on the lectin.

FIGURE 20

Electrophoretic patterns of the yolk extract (YE) used in the adsorption test. Adsorbed YE with *A. salmonicida*: strain # 76-30 A⁺ (A), strain # A450 (B), strain # A450-1 (C), strain # A450-3 (D), unadsorbed (E). F-I represent D-galactose eluates from the same bacterial strains, respectively. Reduced (2-mercaptoethanol) samples were run on a 10-20% gradient SDS-polyacrylamide gel, and silver stained. M_r standards are indicated (kDa).

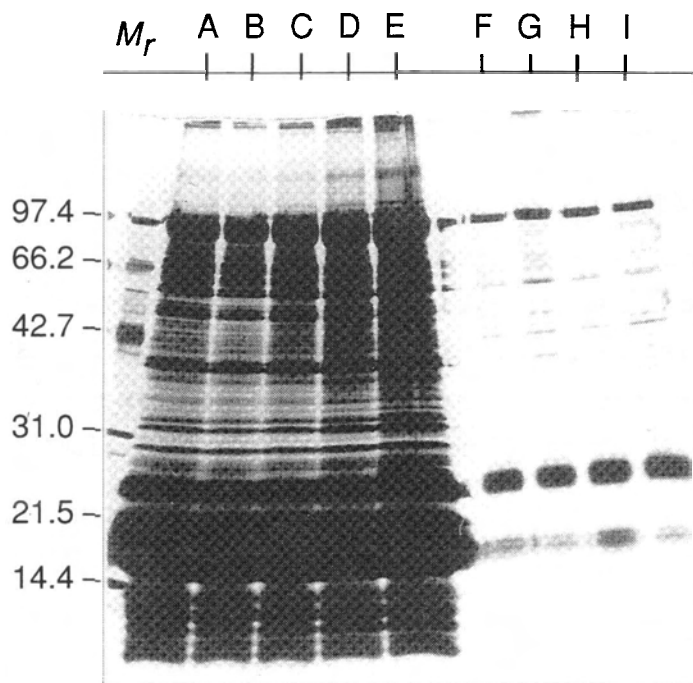


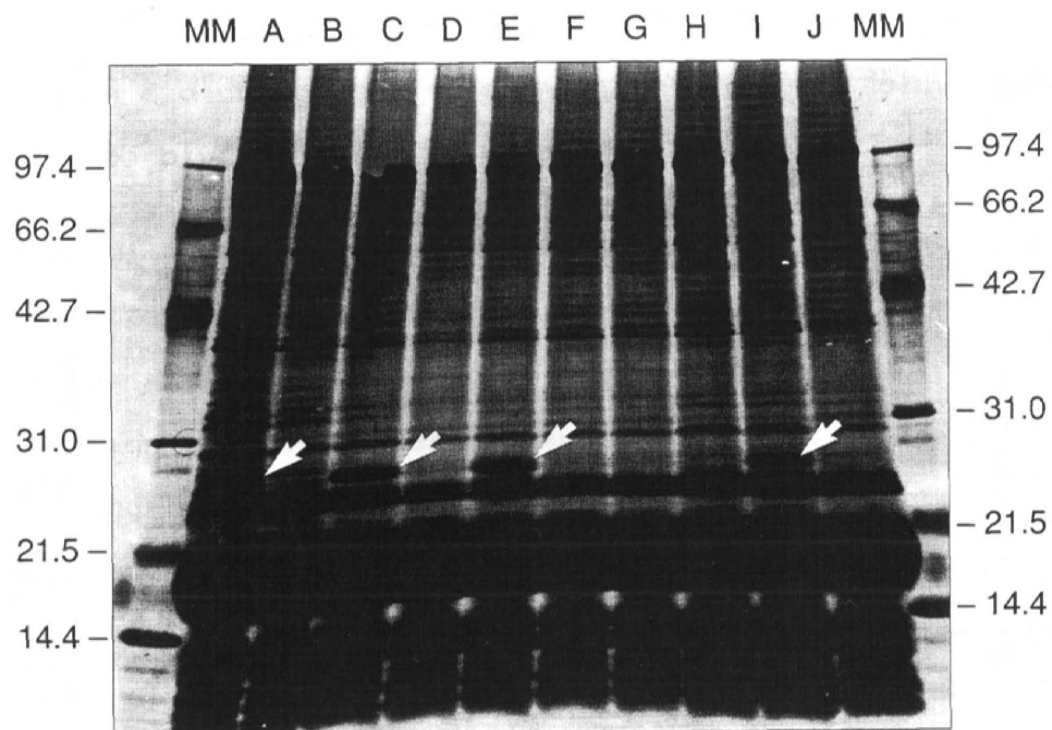
FIGURE 21

PBS washes (A_1 - A_{10}) of *A. salmonicida* cells following incubation with the yolk extract of coho salmon eggs. The lectin (arrows) was eluted by D-galactose (B) or L-rhamnose (C). MM= BIO-RAD Low Molecular Weight Standards. Reduced (2-mercaptoethanol) samples were run on a 10-20% gradient SDS-polyacrylamide gel, and silver stained.



FIGURE 22

Electrophoretic patterns of coho salmon egg yolk extract (YE) (A); YE interaction with *A. salmonicida* (strain # 76-30 A⁺) in the absence (B) and the presence of L-rhamnose (C), the presence of D-mannose (D), the presence of D-galactose (E), the presence of D-glucose (F), the presence of N-acetyl D-galactosamine (G), the presence of D-mannosamine (H), the presence of D-galactosamine (I), the presence of 2-mercaptoethanol (J). MM= BIO-RAD Low Molecular Weight Standards. Reduced (2-mercaptoethanol) samples were run on a 10-20% gradient SDS-polyacrylamide gel, and silver stained. Arrows indicate unadsorbed lectin.



Addition of simple sugars (D-galactose, D-galactosamine or L-rhamnose) to the yolk extract before the latter was incubated with cells of *A. salmonicida* resulted in total inhibition of the lectin and prevented any adsorption of the molecule to bacterial cells. On the other hand, D-mannose, D-glucose, N-acetyl D-galactosamine, D-mannosamine or 2-mercaptoethanol had no inhibitory effect on the binding property of the lectin. Furthermore, heating the YE at 100°C for 15 min did not precipitate the lectin out of solution nor inhibit its binding activity (Fig. 23).

The procedure used for the simultaneous precipitation of coho egg proteins including the lectin, and extraction of the lipids has also been found to be efficient for the purification of other materials such as immunoglobulins from chicken eggs (Bade and Stegemann 1984). The use of organic solvents such as isopropyl alcohol and acetone to precipitate the egg lectin, had no effect on the protein as well as its binding activity, and the lectin was readily dissolved in PBS together with other egg proteins.

The lectin was purified from coho eggs by affinity chromatography (Fig. 24). Following adsorption of the lectin onto D-galactose Sepharose 4B column, proteins other than lectin were washed off the column with PBS, and the lectin was then eluted with the competitive sugar (D-galactose) in a single peak (65 ug total protein). The eluted lectin

FIGURE 23

The effect of heat on the yolk extract and lectin of coho salmon eggs. Yolk extract (YE) was incubated at 100°C for 15 min and centrifuged. The resultant supernatant was then used in the adsorption test with *A. salmonicida*. (A) adsorbed; (B) unadsorbed lectin (arrow); and (C) YE. MM= BIO-RAD Low Molecular Weight Standards. Reduced (2-mercaptoethanol) samples were run on a 5-20% gradient SDS-polyacrylamide gel, and silver stained.

MM A B C

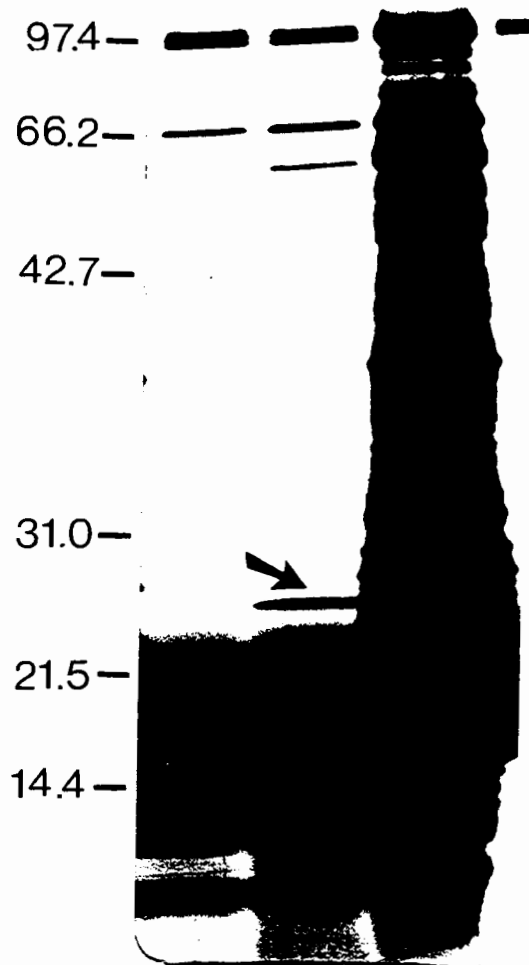
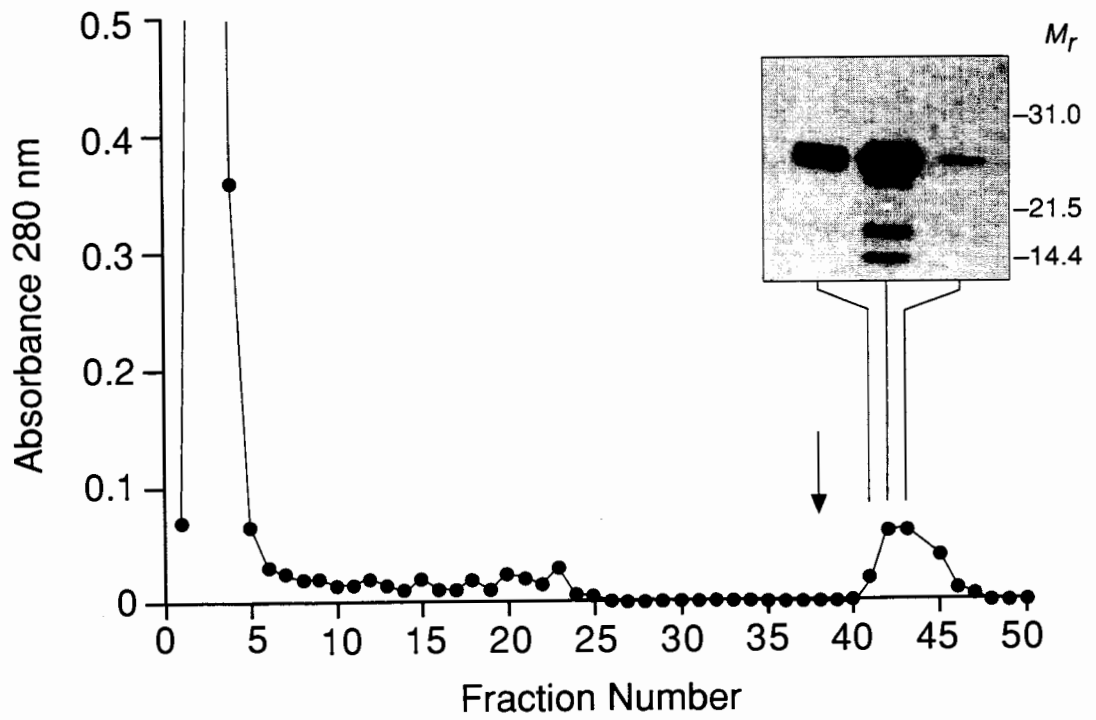


FIGURE 24

Affinity chromatography of coho salmon *O. kisutch* egg lectin on D-galactose-Sepharose 4B column (1.6 x 5.2 cm). Ten ml of yolk extract containing approximately 90 mg of protein was applied to the column. After washing the column with PBS, the lectin was eluted with 0.2M D-galactose in the buffer (indicated by an arrow). Fractions of 2 ml were analyzed for UV absorbance (• - •), and for proteins using SDS-PAGE (inset).



constituted less than 0.1% of the total protein loaded onto the column. SDS-PAGE (with 2-mercaptoethanol) of the peak fractions revealed one major band ($M_r = 24,500$ daltons) and three additional faint bands ($\sim M_r = 23,000$; $18,000$; $14,400$ daltons). Noteworthy however, a fourth faint band ($\sim M_r = 42,700$ daltons) was visible when the loading density of lectin was increased (Fig. 25A, lane 2). Lectins from the eggs of rainbow trout have been described as proteins in the form of monomers and noncovalently linked dimers (Krajhanzl and Kocourek 1986), and an estimated molecular weight of 25 kDa, based on Sephadex G-200 molecular sieving, was reported for a lectin from the eggs of the powan *Coregonus lavaretus maraena*, a fish of the *Salmonidae* family (Krajhanzl et al. 1978). The isolated lectin was tested for purity by western blot. A single band was obtained that corresponded to the major lectin band ($M_r = 24,500$) in the purified sample. The band $M_r = 18,000$, that happened to occur in trace quantities in the purified material, was concluded to represent contamination by a major antigenic protein present in coho eggs. The other faint bands are probably degradation fragments of the $24,500 M_r$ protein.

The 45-amino acid N-terminal sequence of the purified lectin has been established and is indicated in Table 4. The BLAST N-terminal sequence comparison between the 24.5-kDa lectin and protein sequences listed in the data bases did not reveal any other protein with a sequence similar to that of

FIGURE 25

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (A), and western blot (B) of reduced (2-mercaptoethanol) samples of coho salmon *O. kisutch* egg yolk extract (lane 1), purified lectin from galactose affinity chromatography (lane 2). The primary antibody was rabbit immune serum raised against lectin. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase. M_r standards are indicated (kDa).

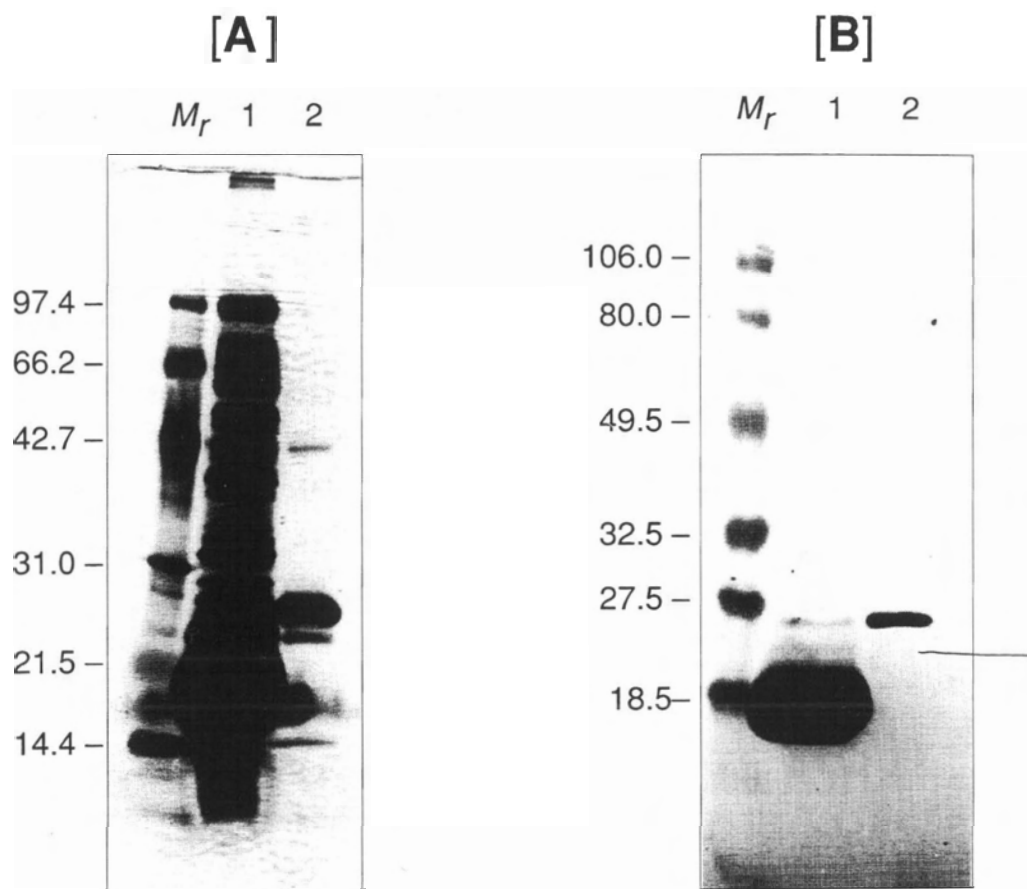


Table 4: N-terminal sequence of coho salmon *Oncorhynchus kisutch* egg lectin. Determinations were made using the major (24.5 kDa) subunit of the purified protein.

Cycle	Identified residue	Cycle	Identified residue
1	Ala	26	Asn
2	Ile	27	Tyr
3	Ser	28	Gly
4	Ile	29	Arg
5	Thr	30	Cys
6	Cys	31	Gln
7	Glu	32	His
8	Gly	33	Asp
9	Ser	34	Val
10	Asp	35	Cys
11	Ala	36	Ser
12	Leu	37	Ile
13	Leu	38	Gly
14	Gln	39	Cys
15	Cys	40	Pro
16	Asp	41	Asp
17	Gly	42	Asn
18	Gly	43	Gln
19	Lys	44	Leu
20	Ile	45	Thr
21	His		
22	Ile		
23	Lys		
24	Arg		
25	Ala		

the lectin. However, there were weak homologies, the most frequent being sequences like those of *kappa* chains of immunoglobulins and to a lesser extent ovoinhibitor from eggs. The analysis also revealed the presence of "gaps" in the sequence. The most likely residues in these gaps being cysteine and the homologies were based upon this. However, it is also possible that the gaps were glycosylated residues, the most common of which would have been serine and threonine in that order.

Amino acid composition of the lectin is given in Table 5. The lectin contained no detectable methionine, and was characterized by a relative abundance of hydrophobic amino acids, such as glycine, leucine and valine.

To obtain a qualitative assessment of the lectin-bacterium association, the cell surface composition of various *A. salmonicida* phenotypes was analysed by SDS-PAGE combined with protein, silver, and immunochemical staining (Fig. 26). Whole cell lysates (Fig. 26A), while naturally complex due to the large number of proteins represented, were dissimilar in several aspects. A major band at 49,000 daltons, which corresponds to the A-protein, was present in strains 76-30 A⁺ and A450 (Fig. 26A, lanes 1&2). These strains also produced their normal complement of liposaccharides (Fig. 26B, lanes 1&2), comprising the core region lipo-oligosaccharide (lower band) and the O antigen-containing complete LPS, therefore corresponding to the

Table 5: Total amino acid analysis of 24.5 kDa coho salmon *Oncorhynchus kisutch* egg lectin.

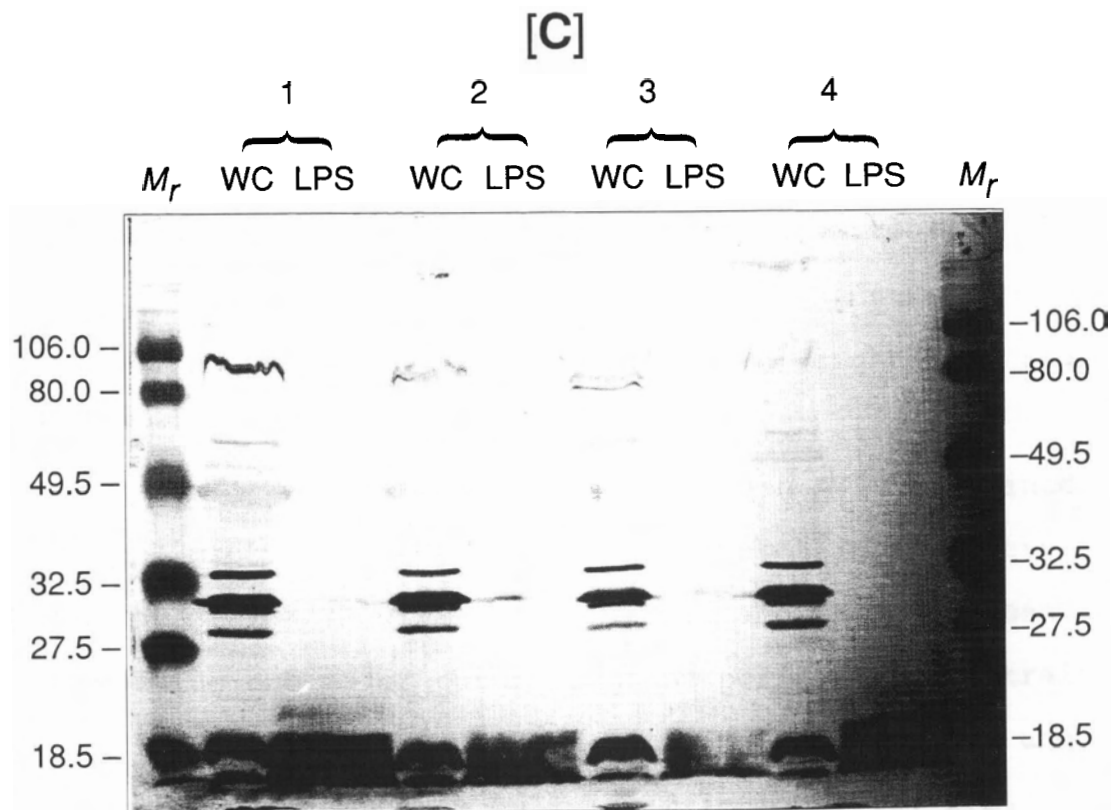
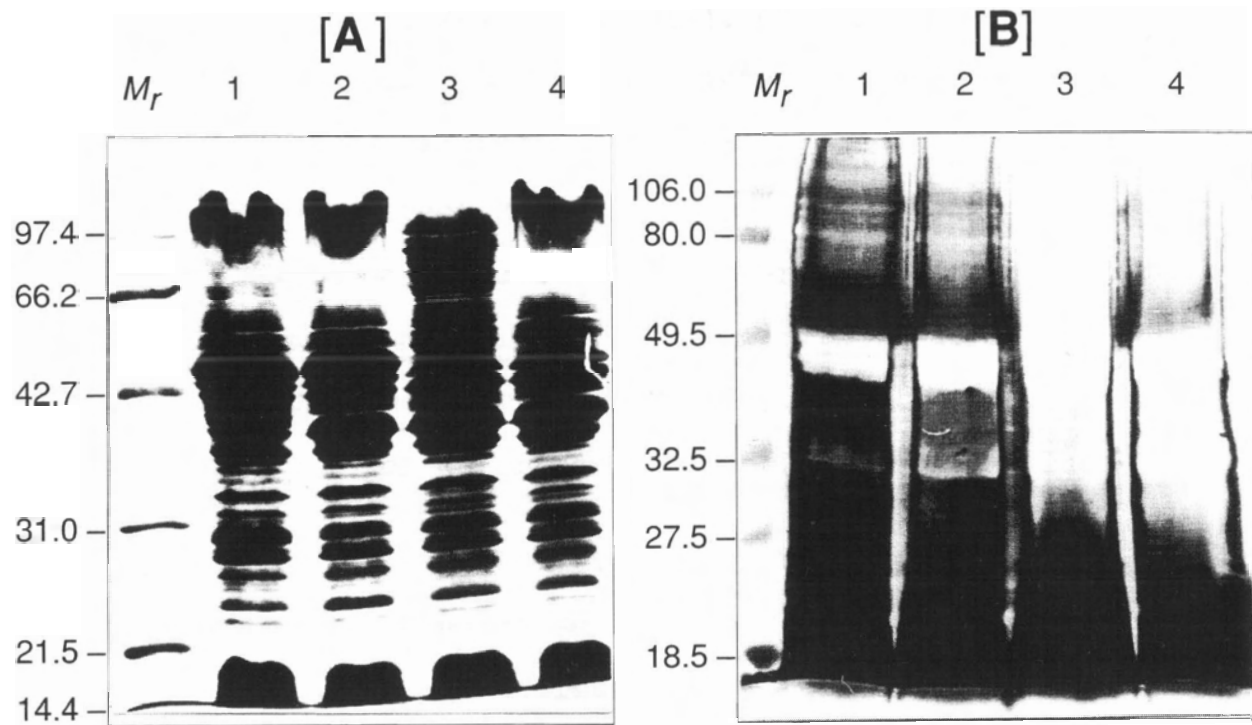
Amino acid residue	No. of residues per lectin subunit ^a
Asx	17
Glx	20
Ser	17
Gly	32
His	3
Arg	8
Thr	18
Ala	20
Pro	9
Tyr	4
Val	23
Cys	ND ^b
Met	0
Ile	23
Leu	26
Phe	6
Trp	ND
Lys	8
Total	234

^a The molar ratio of each amino acid was determined from the compositional analysis, and the alanine content was adjusted for a lectin molecular mass approximating 24.5 kDa, the apparent molecular mass of the lectin subunit as determined by SDS-PAGE.

^b ND, not determined.

FIGURE 26

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of whole cell lysates (WC), and proteinase K digested-WC (LPS) from *A. salmonicida*. Lanes:1, strain 76-30 A⁺;2, strain A450;3, strain A450-1;4, strain A450-3. (A) Coomassie blue stained WC. (B) Silver stained LPS. (C) Immunoblot of WC and LPS from the four strains, after treatment with lectin or yolk extract and antisera as described in the Materials and Methods. The primary antibody was rabbit immune serum raised against lectin. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase. M_r standards are indicated (kDa)



phenotype A⁺ LPS⁺. The remaining two strains did not possess an A-protein (Fig. 26A, lanes 3&4). Of these two A⁻ isolates, strain A450-3 possessed LPS (A⁻ LPS⁺ phenotype) while strain A450-1 was of the A⁻ LPS⁻ phenotype since no high molecular weight LPS was detected on this isolate. In addition to these differences in major surface characteristics (presence or absence of the A-protein and LPS), strain 76-30 A⁺ did not possess a principal protein ($\sim M_r = 37,000$ daltons) that likely represents a major outer membrane porin (Fig. 26A, lane 1).

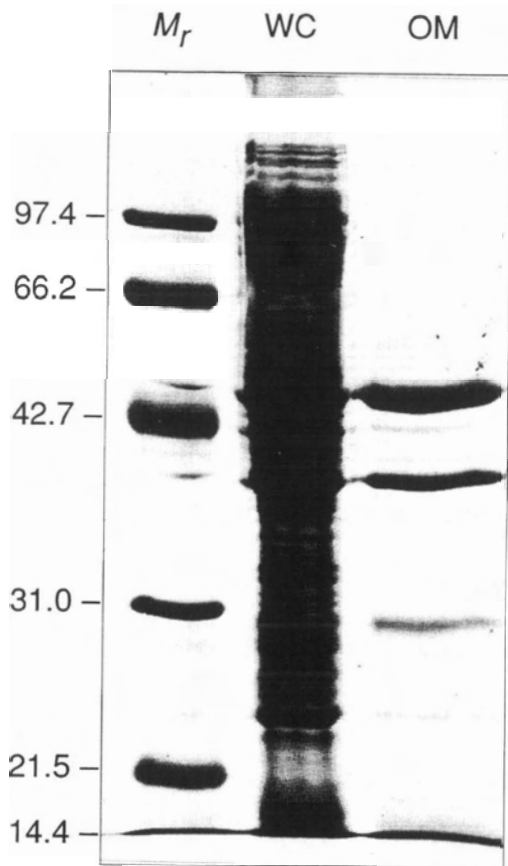
To determine the bacterial lectin-binding site(s), blots of whole cell lysates as well as LPS (Fig 26C) were first probed with the purified lectin or yolk extract followed by antilectin serum. At least five cellular proteins or glycoproteins from whole cell lysates of the four strains reacted strongly with the lectin and antisera. According to the molecular-mass markers used in the experiment, these cellular components had $\sim M_r = 32,500-18,500$ daltons. Similarly, LPS of the four strains tested was strongly reactive. However, only the low molecular weight core region was visible.

The outer membrane fraction of strain A450 contained two principal proteins of this fraction, A-protein, the main component of the 2D crystalline array, and the lower band representing the major outer membrane porin of this strain (Fig. 27A). Once again, western blotting, with lectin and antilectin serum, of this cellular fraction (Fig. 27B)

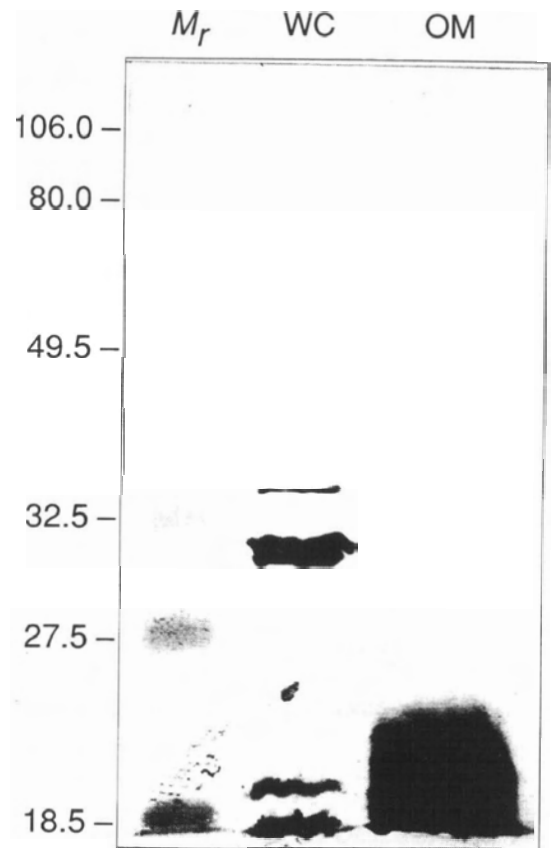
FIGURE 27

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cellular fractions from *A. salmonicida* strain A450. (A) Coomassie blue stained whole cell lysate (WC) and outer membrane (OM). (B) Immunoblot of WC and OM from the same strain, after treatment with lectin or yolk extract and antisera as described in the Materials and Methods. The primary antibody was rabbit immune serum raised against lectin. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase. M_r standards are indicated (kDa)

[A]



[B]



resulted in similar reactivity (compared to those obtained in Figure 26C), with LPS's core region being the major and only binding site. Furthermore, a key experiment that demonstrated lectin receptor sites and localized them on the bacterial surface was performed by first labeling the cells (strain 76-30 A⁺) with lectin followed by antilectin serum and the fluorescent dye fluorescein isothiocyanate (FITC) conjugated to the secondary antibodies as described in Materials and Methods. In the present strain the FITC-labeled lectin receptors were uniformly distributed at the cell periphery, indicating a specific receptor, presumably involved in the adsorption of egg lectin onto cells of *A. salmonicida* (Fig. 28).

The M ϕ monolayers of rainbow trout (Fig. 29) were composed of a morphologically homogeneous cell population. After 3 days in culture the majority of the cells were adherent and had spread to varying degrees. While some were very large and elongated others were still round or only slightly spread out. The nuclei contained dense chromatin, and the cytoplasm of some cells contained fine granules, most likely melanin.

The possible existence of a specific lectin receptor interaction between sensitized bacterial cells and the M ϕ surface was examined by immunochemical staining of M ϕ monolayers with lectin followed by antilectin serum and FITC conjugated antiserum. Figure 30 depicts a view of the stained

FIGURE 28

Binding of coho salmon *O. kisutch* egg lectin to cells of *A. salmonicida*. Heat-killed bacterial cells # 76-30 A⁺ were treated with lectin and antisera as described in Materials and Methods, and examined under UV. light. (Epifluorescence micrograph, x1000).

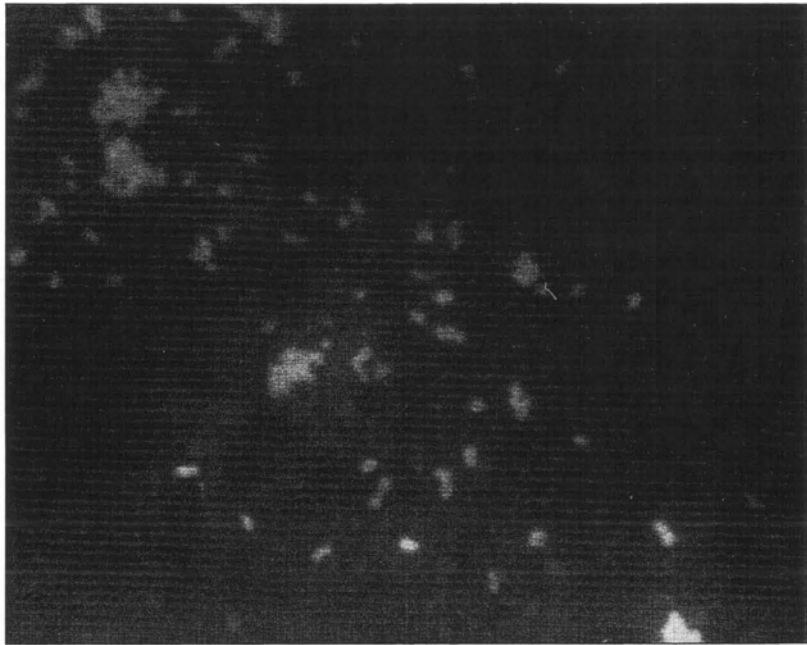


FIGURE 29

Light micrograph of macrophage culture of rainbow trout *O. mykiss*. 0.5×10^6 cells seeded, washed after 2 h, and fixed after 3 days in culture. Giemsa stain (x1000).

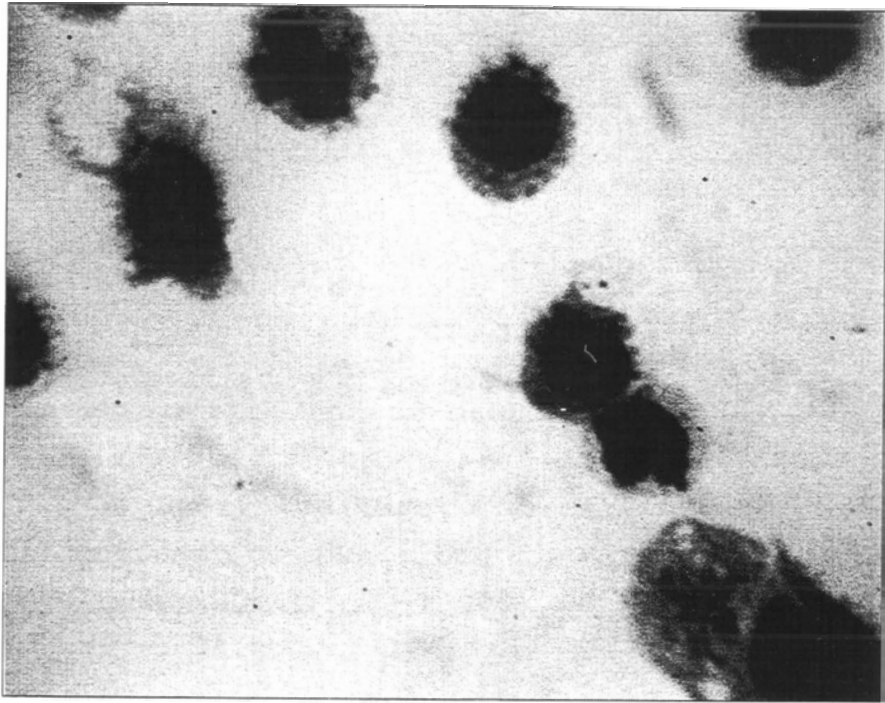
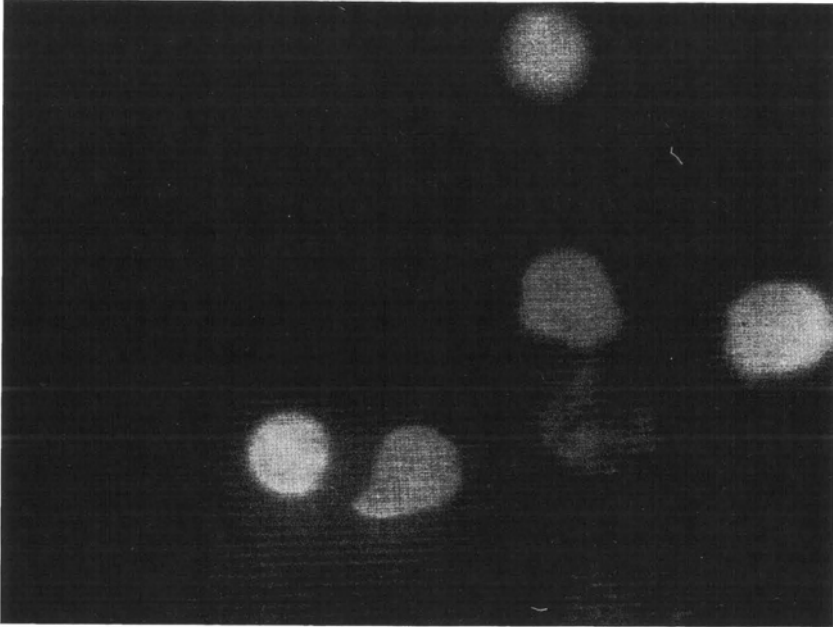


FIGURE 30

Epifluorescence micrograph of macrophage culture of rainbow trout *O. mykiss*. 0.5×10^6 cells seeded, washed after 2 h, and fixed after 3 days in culture. The cells were treated with lectin and antisera as described in Materials and Methods, and examined under UV. light (x1000).

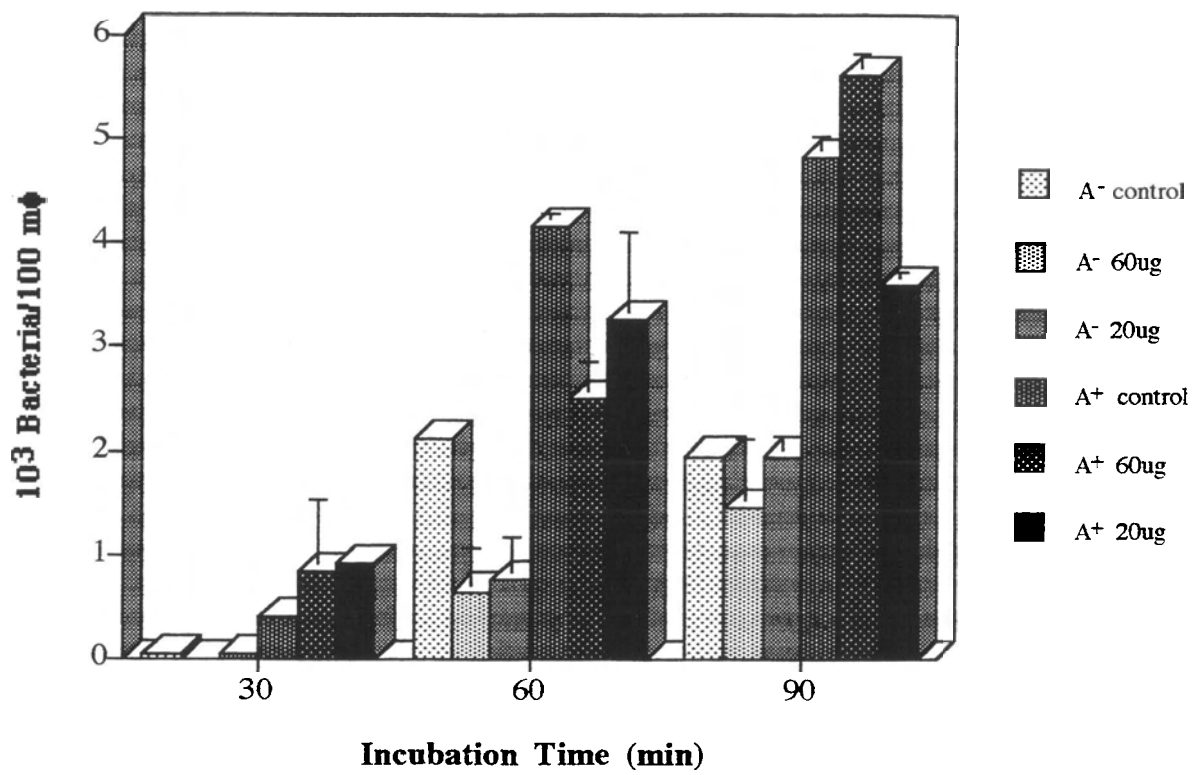


cells with FITC-labeled receptors that seemed to be surface localized. Control monolayers (PBS was substituted for lectin in the staining protocol) however, did not show any fluorescence (results not shown).

The interaction of *A. salmonicida* and M ϕ s mediated by coho egg lectin was studied using rainbow trout kidney M ϕ s and the virulent A⁺ LPS⁺ phenotype (strain A450) as well as an avirulent A⁻ LPS⁻ phenotype (strain A450-3). At all incubation times, the association of *A. salmonicida* with M ϕ s was much greater for A⁺ than A⁻ strains (Fig. 31), and in contrast with A⁻ cells the phagocytic index of A⁺ cells increased markedly with time. The effect of lectin on the process of adherence and internalization was unambiguous after 1 h incubation. Opsonization of A⁻ bacterial cells with lectin did not enhance phagocytosis; instead, the phagocytic index was markedly depressed when these cells were coated with as low as 20 ug of lectin. At longer incubation periods, however, these coated cells reached levels of association with the macrophages similar to those achieved by controls. In contrast, opsonization of A⁺ bacteria with lectin had no consistent effect on phagocytosis, and the process was highly variable throughout the experiment. This is most likely because of the presence of the A-layer and its effect on the accessibility of the lectin to those receptor sites located at the LPS.

FIGURE 31

Association of A⁺ (A450) and A⁻ (A450-3) *A. salmonicida* with rainbow trout Mφs as determined by direct microscopy counts in Mφ monolayers. Association assays were done in 24-well plates in which plain unsensitized (control) or sensitized, with 20 or 60 ug lectin, live bacterial cells were incubated with the Mφ monolayer. Each column represents the average of two counts from a single experiment with duplicate samples.



Despite its adsorption by different phenotypes of *A. salmonicida*, the purified lectin did not show antibacterial activity against these bacteria. This is in agreement with the results of Kamiya and Shimizu (1980), and Kamiya et al. (1990) who observed that the lectin-like proteins, purified from the mucus of the windowpane flounder *Lophopsetta maculata*, and the eggs of chum salmon *O. keta* which agglutinated marine yeasts and *V. anguillarum*, respectively, but did not inhibit their growth .

DISCUSSION

Lectins specifically bind sugars, and therefore interact with the sugar-bearing surfaces of cells (Liener 1976, Lis and Sharon 1977, Goldstein and Hayes 1978). Studies on the interaction of lectins with bacterial cells have dealt with the following themes: (1) Lectins have been used to characterize bacterial surface components, thereby facilitating bacterial typing and identification (Kohler et al. 1973, Gilboa-Garber et al. 1977b, Curtis and Slack 1981, Garber et al. 1981, Doyle et al. 1984) (2) Lectins have been shown to function in establishing symbioses between bacteria and legume plants (Brill 1977, Muller et al. 1984) and (3) The role of lectins in protecting plants and animals, or their reproductive elements (seeds and eggs) against bacterial or fungal infections has been investigated (Mirelman et al. 1975, Sequeira and Graham 1977, Shimizu et al. 1977, Vasta and Marchalonis 1983).

The occurrence of lectins and haemagglutinins in the eggs of members of Pacific salmon *Oncorhynchus* spp. is well documented. These lectins have common D-galactosyl and L-rhamnosyl binding specificities (Voss et al. 1978, Ozaki et al. 1983, Kamiya et al. 1990). Similarly, the present study demonstrated the presence of a D-galactosyl specific lectin in the eggs of coho salmon as well as the presence of galactose-bearing lectin receptors on the surface of *A. salmonicida* cells.

The Gram-negative bacterium *A. salmonicida* subsp. *salmonicida* is the etiological agent of septicemic furunculosis in salmonids. This pathogenic bacterium possesses an additional cell envelope layer, the A-layer, located outside its outer membrane (Udey and Fryer 1978, Trust et al. 1980, Evenberg and Lugtenberg 1982a). The A-layer has been implicated in adhesion, enhanced virulence and autoagglutination (Udey and Fryer 1978, Trust et al. 1980, Evenberg and Lugtenberg 1982a). It is a 49 kDa protein (A protein) and covers most of the surface of virulent cells, although some lipopolysaccharide (LPS) may be exposed (Kay et al. 1981).

Among the sugars tested, D-galactose, L-rhamnose and D-galactosamine markedly inhibited the lectin. This indicates that the lectin is a member of a group of similar anti-B-like lectins found in salmonid eggs, which share the sugar binding specificity (Uhlenbuck and Prokop 1967, Anstee et al. 1973, Voak et al. 1974, Krajhanzl et al. 1978, Voss et al. 1978, Ozaki et al. 1983, Bildfell et al. 1992) and insensitivity to 2-mercaptoethanol (Anstee et al. 1973, Ozaki et al. 1983). These monosaccharide inhibitors are characterized by having a common structure at one of the carbon atoms in their pyranose ring. Those sugars, whose pyranose rings share the same configuration as to C₄ (i.e. hydroxyl group -OH) also possess similar orientation with regard to hydroxyl group. Some other sugars, such as N-acetyl D-galactosamine have similar (compared to L-rhamnosyl type sugars) configuration at C₄,

yet they were unable to inhibit the lectin, perhaps because of their bulky size exemplified by the presence of acetamido group ($\text{CH}_3\text{-CO-NH}$) at C_2 of their pyranose ring. This indicates that the stereochemical configuration of the hydroxyl group alone may not predict inhibition activity for the sugar. The lack of inhibition by other sugars also tested could be attributed to differences in configuration of the pyranose ring as well as orientation of the vicinal hydroxyl group on C_4 .

The sugar specificity of the lectin indicated that this lectin is different from those reported earlier (see Chapter II: Immunoglobulin-like protein). Also, it permitted the design of a suitable purification procedure. In this study, D-galactose coupled to Sepharose served as a matrix in affinity columns to immobilize the lectin. Proteins other than lectin were eluted with PBS, whereas the D-galactose binding lectin could only be eluted by a competitive reaction.

The anomer specificity of the lectin purified from coho eggs was not determined in this study. However, previously published works indicated that lectins found in salmonid eggs are specific for α anomer (Voak et al. 1973, Ozaki et al. 1983). This was primarily based on inhibition of haemagglutination with the α -galactosyl sugars melibiose and raffinose, but not with lactose (a β -galactosyl).

The adsorption of the lectin onto boiled cells indicated the relative resistance of the sugar-bearing residues of *A. salmonicida* cells to protein denaturing agents and facilitates the usage of fixed bacteria for studies of their interactions with the lectins. Furthermore, boiling the yolk material for 15 min did not affect the binding property of the lectin, but it removed most of the high molecular weight proteins present. The binding activity in the yolk was due to one, not multiple lectins because: (1) D-galactose/L-rhamnose elution of YE-*A. salmonicida* complex followed by L-rhamnose/D-galactose yielded no additional protein bands in SDS-PAGE (2) sugar inhibition profiles of rhamnose-, or galactose-eluted lectin were identical and (3) both have identical mobility in SDS-PAGE.

Cellular fractions from various *A. salmonicida* phenotypes reacted equally well with the lectin and showed that besides LPS's core region other cellular proteins and/or glycoproteins may also act as ligands for coho egg lectin. On the other hand, the A-layer appeared to have no significant role in the adsorption reaction. This appears to be the case because amino acid analysis of A protein revealed it lacks carbohydrates and amino sugars (Kay et al. 1981, Evenberg and Lugtenberg 1982b). It seems likely that binding of coho egg lectin to *A. salmonicida* was achieved via the carbohydrate moieties of those cellular proteins reactive with lectin (Fig. 26), and most likely by the bacterium's LPS. Further evidence for the involvement of these receptors in the

adsorption reaction was provided by the indirect fluorescent antibody staining (IFAT) of whole bacterial cells. The ability of the lectin and antilectin polyclonal antiserum to react by IFAT with *A. salmonicida* cells suggested that some of these receptors penetrated to the exterior surface of the A-layer. In addition, western blot analysis of isolated outer membrane constituents of an A⁺ LPS⁺ phenotype clearly indicates that only one of the two constituents of the LPS was reactive with the lectin - the core polysaccharide. This tendency of the lectin to react only with galactosyl/rhamnosyl moieties in the LPS's core of *A. salmonicida* strongly suggests that 2-keto-3-deoxyoctonate (KDO) acts as the immunodeterminant group in the core region of *A. salmonicida*. KDO was initially shown to be a component of the lipopolysaccharide of *Escherichia coli* (Heath et al. 1963) and *Salmonella typhimurium* (Osborn 1963). KDO occupies the innermost sugar residue position in the core polysaccharide and provides the point of attachment to the lipid A moiety which is embedded in the outer membrane of Gram-negative bacteria. Lipid A is composed of a glucosamine-phosphate backbone to which are attached ester- and amide-linked fatty acids (Galanos et al. 1977).

No doubt owing to the tremendous variation that exists in the polysaccharide portion of LPS, many different lectins have been shown to react with one or more forms of LPS (Pistole 1981- a review). Rostam-Abadi and Pistole (1982) have shown that a lectin (limulin) from the serum of *Limulus*

polyphemus reacts specifically with LPS of *Salmonella minnesota* and KDO was proven to be the lectin-binding site on the LPS. This is also true for the KDO of *A. salmonicida* LPS which possesses three accessible galactose residues (W.W. Kay-personal communication). It is thus not unexpected that coho egg lectin was reactive only with the core region lipooligosaccharide and not the O-polysaccharide chain of LPS.

To gain further insight into the possible role of egg lectins in defense reactions, the lectin identified in the eggs of coho salmon was studied in detail using phagocytic cells from a closely related species, the macrophages of rainbow trout. The macrophage is the most important cell type for cellular reaction against infectious and noninfectious foreign agents. As is well known, mammalian macrophages phagocytize foreign particles via the Fc and C3 receptors with the aid of antibody and complement (Mantovani et al. 1972, Griffin et al. 1975). For non-mammalian vertebrates, however, little is still known about the specific receptors involved in phagocytosis by macrophages. It seemed possible that a lectin-dependent phagocytosis might occur. Such a phenomenon has been shown to occur with mouse macrophages sensitized with concanavalin A (Goldman and Cooper 1975, Bar-Shavit and Goldman 1976). Although this study demonstrated the existence of lectin receptors on the macrophage surface (likely galactosyl moiety-containing glycoconjugates similar to those of *A. salmonicida* LPS), direct evidence for the involvement of these receptor sites in lectin-mediated

phagocytosis was not established. This is in contrast to the results of workers who showed that fish macrophages carry receptors for homologous lectins as well as heterologous ones (Ozaki et al. 1983, Castagnaro et al. 1991), and subsequently proposed an opsonic role for these lectins in fish.

Trust et al. (1983) showed that A-layer provided *A. salmonicida* with an enhanced ability to associate with phagocytic monocytes, and suggested that bacteria displaying increased surface hydrophobicity are more likely to be readily phagocytized. This enhanced ability, in the absence of lectin, to associate with macrophages was confirmed in this study where strain A450 ($A^+ LPS^+$) was phagocytized more readily than the other less hydrophobic phenotype ($A^- LPS^-$). Nevertheless, the egg lectin exhibited an interesting biological activity - it blocked the attachment and subsequent uptake by macrophages of A^- cells at a concentration as low as 20 $\mu\text{g protein ml}^{-1}$. This ability to block the uptake of a bacterium represents a potentially significant finding, and suggests a macrophage protecting role for the egg lectin in coho embryos and yolk sac fry. This is important especially in view of the finding that avirulent $A^- LPS^-$ cells exhibit substantial cytotoxicity against salmonid macrophages (Olivier et al. 1992). Alternatively, the egg lectin may be implicated in carbohydrate metabolism and transport of glycoconjugates in developing eggs and the adult fish (Krajhanzl et al. 1985).

The mechanism by which the lectin may act in blocking the uptake of these cells is unclear. However, since this protein binds galactose on either cell, and does not actually link the two cells (as judged from absence of enhanced phagocytosis) there is a possibility that this protein is a monovalent lectin which by binding to a sugar terminal moiety like galactose on the surface of *A. salmonicida* simultaneously blocks and/or modifies those terminals via which association with macrophages takes place, subsequently resulting in reduced phagocytosis. It is expected that lectin will block phagocytosis for longer periods even with A⁺ cells, if a higher density of lectin was established on the surface of the bacterial cell. It is noteworthy that this particular property of coho egg lectin resembled the *in vitro* behavior of oyster haemagglutinin in similar experiments by Tripp and Kent (1967) where phagocytosis of red blood cells was depressed in the presence of half-strength oyster hemolymph. Similarly, the lectin from the gastropod mollusk *Otala lactea* albumin gland depressed phagocytosis of sheep red blood cells (Anderson and Good 1976).

The source of the lectin present in coho salmon eggs was not investigated. However, cytochemical data indicated that sugar binding proteins (lectins) in fish ova are present in the yolk vesicles of small oocytes and in cortical alveoli of full-grown oocytes and eggs (Nosek et al. 1983, Nosek 1984).

Although the existence of these egg proteins has been known for decades, few of them have been purified and biochemically characterized. In the present study, the amino acid composition and N- terminal amino acid sequence of coho egg lectin were established. However, the BLAST computer search of N- terminal sequence data bases did not reveal sequences homologous to those of the purified lectin. Of interest, however, was the weak homology that occurred with the variable region of *kappa* light chains of mammalian immunoglobulins (Igs). These variable regions are known to form antigen-binding domains with their heavy chain counterparts. This similarity to an Ig-like structure may therefore classify the lectin as a member of an ancient superfamily of recognition molecules such as antibodies, T cell receptors, major histocompatibility complex glycoproteins and lymphocyte Fc receptors. Studies using DNA techniques have shown that all of these molecules that mediate cell-cell recognition or antigen recognition in the immune system contain related structural elements (Ig-like domains) suggesting that the genes that encode them have a common evolutionary history (Williams and Barclay 1988).

In addition to *kappa* chains, there was weak homology to ovoinhibitor of eggs. Ovoinhibitor is a protein that belongs to a major group of proteinase inhibitors present in egg white. It is characterized by inhibiting serine proteinases such as trypsin, α -chymotrypsin, subtilisin and alkaline proteinases. Although the function of this protein is not

known for certain, it is believed it may have a protective role against bacterial proteinases (Stevens 1991).

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