Interaction of coho salmon *Oncorhynchus kisutch* egg lectin with the fish pathogen *Aeromonas salmonicida*

A. N. Yousif¹, L. J. Albright¹, T. P. T. Evelyn²

¹ Institute for Aquaculture Research, Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6
² Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, British Columbia, Canada V9R 5K6

**ABSTRACT:** The yolk of coho salmon *Oncorhynchus kisutch* eggs contains a galactophilic lectin which proved capable of binding to *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids. Immunofluorescence staining of whole bacterial cells, and western blot analysis of cellular fractions from various phenotypes of *A. salmonicida*, revealed that the lipopolysaccharide's core region and other soluble proteins and/or glycoproteins were the bacterial lectin-binding sites. The protective role of the egg's lectin was investigated by testing the lectin for its ability to prevent growth of *A. salmonicida*. The lectin did not show antibacterial activities against any of the tested strains of *A. salmonicida*. It was concluded that the lectin's role lay in some direction other than protection against disease agents.

**KEY WORDS:** Lectin · Salmonids · Eggs · *Aeromonas salmonicida*

**INTRODUCTION**

Sugar-binding proteins or lectins are found in a wide variety of life forms. Although those from plants, particularly legumes, have been extensively studied, information has also been collected on lectins derived from vertebrates. In fish, the presence of lectins has been recorded in the ova of many species of the family Salmonidae (Uhlenbuck & 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 & Inoue 1986, 1989).

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 protection, of maternal origin, has been developed in fishes.

Recently, unfertilized eggs from coho salmon *Oncorhynchus kisutch* were found to contain a galactophilic lectin that specifically binds to bacterial cells of *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids (Yousif et al. 1994). Binding to bacterial cells could be important in the defense against bacterial infection in eggs and yolk sac fry.

We provide herein unequivocal evidence that coho egg lectin reacts with the carbohydrate-bearing components of *Aeromonas salmonicida*. The significance of the egg lectin was studied by investigating its antibacterial property — a reported characteristic of some lectins.

**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 (B.C.), 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 to 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 3 times with the alcohol and twice with precooled acetone. The final residue was collected in a Buchner funnel (filter paper no. 1, Whatman International Ltd, Maidstone, UK), washed with a small amount of acetone, and air dried. The dry powder was extracted with 75 ml of PBS by stirring for 1 h at room temperature. The supernatant (yolk extract, YE) was collected following centrifugation (12,000 x g) and stored at 4°C. Egg lectin was purified from YE following the method of Yousif et al. (1994). Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

**Bacterial strains.** The bacterial strains were obtained from the following sources: *Aeromonas salmonicida* [strain 76-30 A*, (virulent, A* LPS*)] was isolated from juvenile salmon at the Department of Fisheries and Oceans (DFO) Quinsam River Hatchery, Vancouver Island, B.C., in 1976. 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, USA) supplemented with 1% gelatin. Propagation of the cells was carried out using brain heart infusion (BHI, Difco) agar at 15°C.

**Treatment of the bacteria.** Bacterial cultures were aseptically scraped off agar plates, suspended and washed twice in sterile PBS by centrifugation (6000 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 µl of bacterial suspension of each strain) with 100 µl of YE (total protein: 650 µg) 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 (SDS-PAGE). Bacterial pellets were then washed in PBS (1.5 ml), and bound lectin was eluted by resuspending the pellet in 100 µl of 0.2 M D-galactose for 2 h at room temperature and then centrifuging. The supernatants were dialysed extensively (2 d) against distilled water and examined for lectin using SDS-PAGE.

**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 & Thorpe (1967). The samples 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, Hoeffer Scientific Instruments, San Francisco, CA, USA).

**Bacterial preparations:** *Aeromonas salmonicida* strains 76-30 A*, A450, A450-1, and A450-3 were grown statically in BHI broth for 48 h at 15°C. Bacteria were centrifuged for 15 min at 6000 x g and whole cell (WC) lysates were prepared in sample buffer as described by Laemmli (1970) using a concentration of 1.0 g (weight) of bacteria per 20 ml of sample buffer. A portion of the WC lysate was also treated with proteinase K (PK) according to the procedure of Hitchcock & Brown (1983). Membrane preparation (MP) of A450 was kindly provided by W. W. Kay.

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 µl well⁻¹), PK digested lysates (30 µl well⁻¹), and MP (7.4 mg total protein well⁻¹) were separated on the mini-Protean II Slab Cell (BIO-RAD, Richmond, CA) 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 & Frasch (1982), to visualize LPS.

**Western blotting. Preparation of rabbit antiserum:** Antiserum against coho egg lectin was produced by injection of a New Zealand white female rabbit with purified coho egg lectin mixed with AdjuPrime immune modulator (Pierce, Rockford, IL, USA). AdjuPrime was ground in a mortar and pestle and suspended in the antigen solution to a final concentration of 1.0 g (wet weight) of bacteria per 20 ml of sample buffer. A portion of the WC lysate was also treated with proteinase K (PK) according to the procedure of Hitchcock & Brown (1983). Membrane preparation (MP) of A450 was kindly provided by W. W. Kay.

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**Molecular-weight protein standards (BIO-RAD)** were included in each run. All chemicals used in electrophoresis were purchased from Sigma (St. Louis, MO, USA).

**Western blotting. Preparation of rabbit antiserum:** Antiserum against coho egg lectin was produced by injection of a New Zealand white female rabbit with purified coho egg lectin mixed with AdjuPrime immune modulator (Pierce, Rockford, IL, USA). 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 µg of lectin was injected subcutaneously and the rabbit was allowed to rest for 2 wk after which it was boosted with 98 µg of the antigen. 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 by centrifugation and kept at -20°C. Normal rabbit serum was collected before immunization for use as a negative control.
**Blotting technique:** Discontinuous SDS-PAGE of samples was performed as described above. Pre-stained markers of low molecular mass (BIO-RAD) were included with each gel. Polypeptides were then transferred electrophoretically onto a 0.45 mm nitrocellulose (N/C) membrane (BIO-RAD), using a semidry transblot apparatus (LKB 2117-250 Novablot, Bromma, Sweden) at 0.8 mA cm⁻² for 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 1 h 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-l-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 mg 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.

**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 diameter) were drawn on the smear using a Tek pen (Manostat, New York, NY, USA). The area within the circle was then treated with coho egg lectin (170 µg ml⁻¹) for 1 h. This was followed by 1:20 dilution of rabbit anti-coho egg lectin antiserum for 30 min and by 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 3 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.

**Growth inhibition.** 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 µg 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**

Fig. 1 demonstrates the ability of Aeromonas salmonicida cells to adsorb the lectin from yolk materials. The resulting linkage between bacterial cells and the lectin that took place during the adsorption was strong enough to allow rigorous washing, with PBS, to remove the unbound egg proteins. The lectin was then eluted from the surface of bacterial cells in a semipurified form by the addition of D-galactose (Fig. 1, lanes F, G, H, I). The upper protein bands (M1 = 97400 and 66000) that occurred in sugar eluates were considered contamination by egg proteins because PBS washes also contained these proteins (Yousif et al. 1994).

To obtain a qualitative assessment of the lectin-bacterium association, the cell surface composition of various Aeromonas salmonicida phenotypes was analysed by SDS-PAGE combined with protein, silver, and immunochemical staining. Whole cell lysates (Fig. 2A), while naturally complex due to the large number of proteins present, were dissimilar in several

![Electrophoretic patterns of the yolk extract (YE) used in the adsorption test. Adsorbed YE with Aeromonas salmonicida: (A) strain 76-30 A⁺, (B) strain A450, (C) strain A450-1, (D) strain A450-3, (E) unadsorbed. (F) to (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. M₁ standards are indicated (kDa)](image-url)
Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of whole cell lysates (WC), and proteinase K digested-WC (LPS) from Aeromonas salmonicida. Lanes: 1, strain 76-30 A'; 2, strain A450; 3, strain A450-1; 4, strain A450-3. (A) Coo massie-blue-stained WC. (B) Silver-stained LPS. (C) Immunoblot of WC and LPS from the 4 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, standards are indicated (kDa).

Respects. A major band at 49,000 daltons, that corresponded to the A-protein, was present in strains 76-30 A' and A450 (Fig. 2A, lanes 1 & 2). These strains also produced their normal complement of liposaccharides (Fig. 2B, lanes 1 & 2), comprising the core region lipooligosaccharide (lower band) and the O antigen-containing complete LPS, therefore corresponding to the phenotype A' LPS'. The remaining 2 strains did not possess A-protein (Fig. 2A, lanes 3 & 4). Of these 2 A strains, 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 in 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 \( M_r = 37,000 \) daltons that likely represents a major outer membrane porin (Fig. 2A, lane 1).

To determine the bacterial lectin-binding site(s), blots of whole cell lysates as well as LPS (Fig. 2C) were first probed with the purified lectin or yolk extract followed by antilectin serum. At least 5 cellular proteins, probably glycoproteins, from whole cell lysates of the 4 strains reacted strongly with the lectin and antisera. According to the molecular-mass markers used in the experiment, these cellular components had \( M_r = 32,500 \) to 18,500 daltons. Similarly, LPS of the 4 strains tested was strongly reactive. However, only the low-molecular-weight core region was reactive.

The outer membrane fraction of strain A450 contained 2 principal proteins: A-protein, the main component of the 2D crystalline array, and the lower band representing the major outer membrane porin of this strain (Fig. 3A). Once again, western blotting, with lectin and antilectin serum, of this cellular fraction (Fig. 3B) resulted in similar reactivity (compared to that obtained in Fig. 2C), with the LPS core region being the 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 antibody. In this strain the FITC-labeled lectin receptors appeared to be uniformly distributed on the cell surface (Fig. 4).

Despite its adsorption by different phenotypes of *Aeromonas salmonicida*, the purified lectin did not show antibacterial activity against these bacteria.

**DISCUSSION**

Cellular fractions from various *Aeromonas salmonicida* phenotypes reacted equally well with the lectin and showed that in addition to the LPS core region other cellular proteins (probably glycoproteins) may...
also act as ligands for the lectin. On the other hand, the A-layer appeared to have no significant role in the adsorption reaction. Lack of reactivity with the A-protein may have reflected the fact that analysis of A-protein revealed an absence of any carbohydrates and amino sugars (Kay et al. 1981, Evenberg & Lugtenberg 1982). 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, and most likely via the bacterium’s LPS. Further evidence for the involvement of these receptors in the adsorption reaction was provided by the indirect fluorescent antibody staining technique (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 external surface of the A-layer. In addition, western blot analysis of isolated outer membrane constituents of the A’ LPS’ A. salmonicida phenotype clearly indicated that only 1 of the 2 constituents of the LPS was reactive with the lectin—the core polysaccharide. This tendency of the lectin to react only with galactosyl/hmannosyl moieties in the LPS core of A. salmonicida strongly suggested that 2-keto-3-deoxyoctonate (KDO) was the reactive group in the core region of A. salmonicida because this group is known to contain 3 accessible galactose residues (W. W. Kay pers. comm.). KDO was initially shown to be a component of the LPS of Escherichia coli (Heath et al. 1963) and Salmonella typhimurium (Osborn 1963) that 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 (see review in Pistole 1981). Reactivity of coho egg lectin with the core (KDO) region of the Aeromonas salmonicida LPS was not surprising. Rostam-Abadi & Pistole (1982) have shown that a lectin (limulin) from the serum of Limulus polyphemus reacts specifically with LPS of Salmonella minnesota and KDO was shown to be the lectin-binding site in the LPS.

Lack of antibacterial activity in this lectin was not unique because such lectins have been reported before from other fishes. Kamiya & Shimizu (1980) and Kamiya et al. (1990) observed that lectin-like proteins isolated from the mucus of the windowpane flounder Lophopsetta maculata and from the eggs of chum salmon Oncorhynchus keta agglutinated marine yeasts and Vibrio anguillarum, respectively, but did not inhibit the growth of these microorganisms.

The main function of the coho salmon egg lectin is uncertain. The present study suggests that protection against disease agents may not be its role. Instead, as suggested by Krajhanzl et al. (1985), the lectin’s main function may be in carbohydrate metabolism where it may be involved in transport of glycoconjugates in developing eggs and adult fish.

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Fig. 4. Binding of coho salmon Oncorhynchus kisutch egg lectin to cells of Aeromonas salmonicida. Heat-killed bacterial cells 76-30 A’ were treated with lectin and antiserum as described in the 'Materials and methods', and examined under ultraviolet light. (Epi ﬂuorescence micrograph, ×1000)
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