Separation of Secreted Immunosuppresssive Proteins of the Fish Pathogen, *Renibacterium Salmoninarum*, from Culture Medium and Infected Fish Tissues

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Introduction

Renibacterium salmoninarum, causative agent of bacterial kidney disease of salmonid fish, is a serious problem in cultured salmon and trout around the world.[1] The organism secretes a 57–58 kD doublet protein, described as a hemaglutinin[2] or as Antigen F,[3] which has been associated with *in vitro* immunosuppression of salmonid lymphocytes.[4] The present report describes use of the Rotofor® preparative isoelectric focusing apparatus for separation of this protein and its breakdown products from culture medium and kidney tissue of infected hosts.

A single 4 h Rotofor run purified Antigen F to homogeneity from kidney tissue preparations of infected salmonid hosts, as shown in Figure 1.

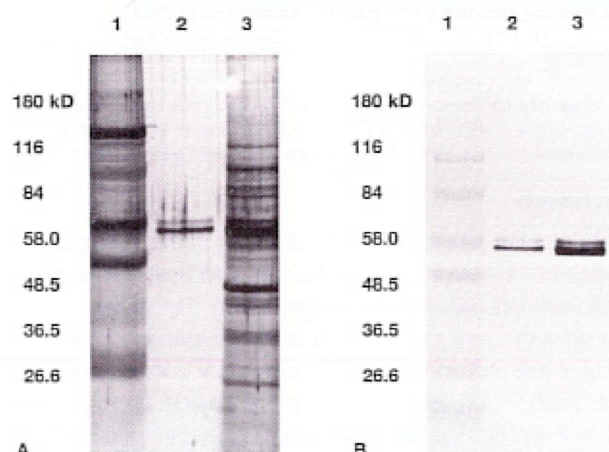

Fig. 1. Western blot analysis comparing crude infected kidney tissue preparation to Rotofor fraction 5. A. Total protein stain of the blot. Lane 1: prestained standards; 2: fraction 5; 3: crude prep. Fraction 5 contained the majority of the Antigen F. B. Immunodetection assay performed on the same material as above with MAbs against the 57 kD protein as primary antibody.

Methods

PREPARATION OF ANTIGEN F FROM KIDNEY TISSUE SAMPLES AND CULTURE SUPERNATANTS FOR ISOELECTRIC FOCUSING

Juvenile Coho salmon (*Oncorhynchus kisutch*) were infected by intraperitoneal injection of *R. salmoninarum*. Samples were prepared by harvesting kidney tissue from moribund coho salmon, mixing the tissue with an equal volume of PBS (phosphate buffered saline), and vortexing. The sample was centrifuged, and 1.0 ml of the supernatant (4.0 mg protein) was mixed with 40 ml water and 2.0 ml Bio-Lyte® ampholytes (pH range 3–10; 40% w/v). The mixture was loaded into the Rotofor cell and focused without further treatment.

R. salmoninarum (ATCC 33209) was cultured in Kidney Disease Medium II plus 10% calf serum[5] for 14 days at 17 °C. Bacterial cells were pelleted by centrifugation and the supernatant was concentrated with 50% saturated ammonium sulfate (SAS). The concentrate was dialyzed into PBS, pH 7.2, 10 mM PO_4. A total of 0.75 g material in 1.0 ml was added to 40 ml water and 2.0 ml Bio-Lyte ampholytes (pH range 3–10, 40% w/v). The mixture was loaded into the Rotofor cell and focused without further treatment.

RUNNING CONDITIONS

Focusing in the Rotofor cell required 4 h at 12 W constant power. The apparatus was cooled by recirculating water through an ice water bath. The initial conditions were ~300 V and 40 mA. At equilibrium the values were ~1,000 V and 12 mA.

SAMPLE ANALYSIS

Twenty fractions were harvested and their pH values measured. Fractions were analyzed on 12% SDS-PAGE gels. The presence of antigen in the harvested Rotofor fractions was determined by enzyme linked immunosorbent assay (ELISA). Detection was facilitated by monoclonal antibodies to the Antigen F.[6] The antibodies served as primary antibodies for both the ELISA assays and western blots.

Results

SEPARATION OF THE ANTIGEN F FROM INFECTED FISH KIDNEY

The usefulness of the Rotofor cell for the isolation of the hemaglutinin protein from infected fish tissues was examined. ELISA data shown in Figure 2 indicates that the protein was localized in Rotofor fractions 3 through 6, covering a pH range of 3.3–4.3, with fraction 5 containing the majority of the protein. Aliquots from the same fractions were also run on SDS-PAGE mini-gels. Proteins from these gels were visualized on western blots by total protein staining and immunodetection. The western blots revealed that the majority of the tissue proteins were localized in the high pH range (Figure 3). Fraction 5 of this separation was concentrated by ammonium sulfate precipitation and electrophoresed adjacent to the initial sample. This demonstrated the purification achieved using the Rotofor cell, shown in Figure 1.

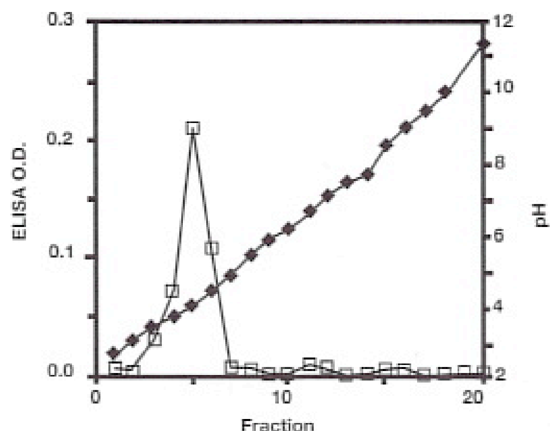

Fig. 2. ELISA (□) and pH (■) measurements from Rotofor fractions following focusing of proteins from infected coho salmon kidneys.

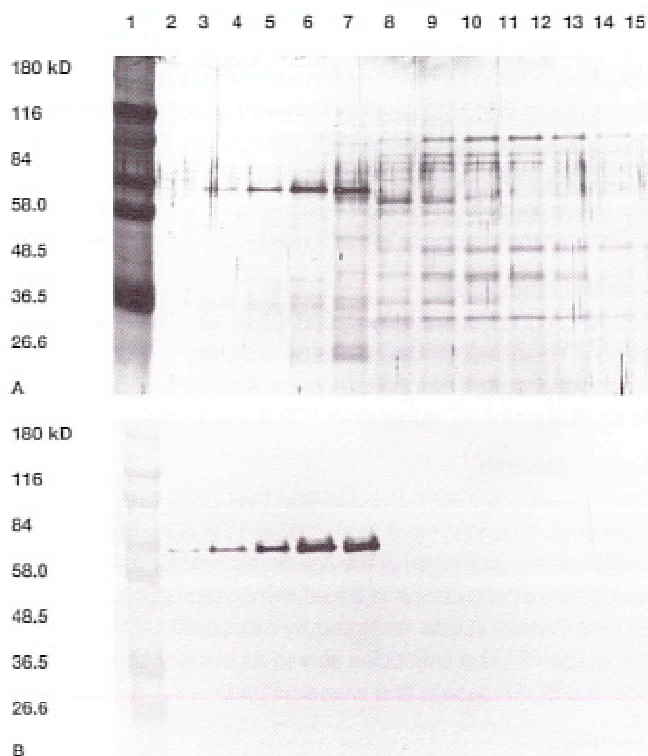

Fig. 3. Western blot of Rotofor fractions 2–15, proteins from infected fish kidneys. A. Total protein stain. Lane 1: MW standards followed by Rotofor fractions 2–15. B. Immunodetection assay of the same developed with MAbs against the 57 kD protein.

SEPARATION OF THE ANTIGEN F FROM *IN VITRO* CULTURE MEDIUM

To determine whether the protein isolated from diseased fish was the same as that produced *in vitro*, the Rotofor cell was used to isolate Antigen F from precipitated culture supernatant fluid. This material contains large quantities of calf or horse serum used in the culture medium. The Rotofor cell isolated the major constituents of the medium to neutral or high pH, while Anitgen F and its breakdown products were present in fractions from pH 3–4.5. Antigen F from culture medium was isolated in the same pH range as that obtained from infected fish tissues (Figure 4).

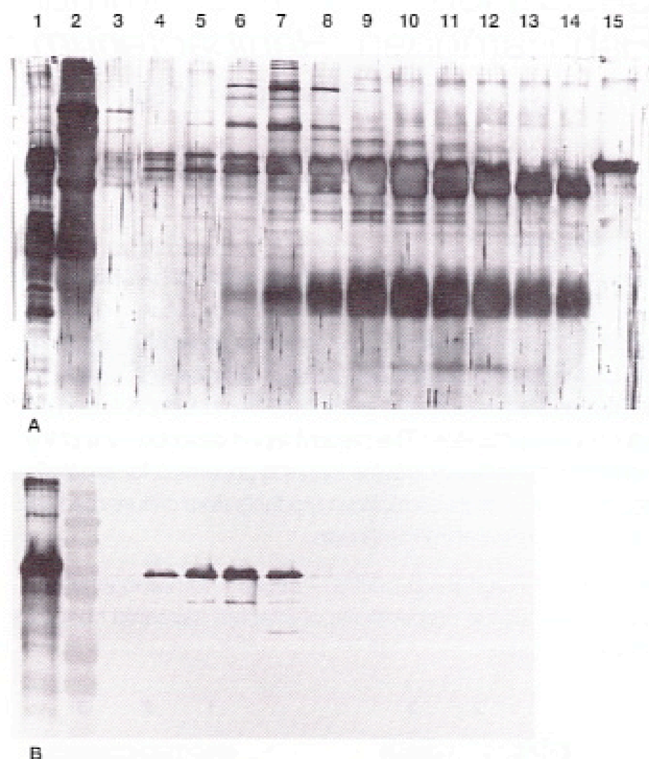

Fig. 4. Western blot of Rotofor fractions 2–15 (A) from cell culture supernatant. A. Total protein stain. Lane 1: crude supernatant fluid; 2: MW standards, 3–15: fractions 2–14. B. Immunodetection assay of the same developed with MAbs against Antigen F.

Conclusion

The Rotofor cell has facilitated the analysis of the interaction between *R. salmoninarum* and the salmonid host, especially in the experiments with infected kidney tissue. The purity obtained with these methods has allowed comparison of activity of this protein produced in laboratory culture and in infected fish. Future experiments on protein sequencing, probe synthesis and cloning are dependent on the data confirming that Antigen F isolated from infected tissues is the same as that secreted by the bacterium into cell culture medium.

References

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