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## Antigenic Characterization of Outer Membrane Protein of *Aeromonas sobria* Isolated from Goldfish (*Carassius auratus* L.)

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### Abstract

*Aeromonas sobria*, along with *A. hydrophila*, has frequently been reported as a causative agent of motile aeromonas septicemia (MAS) in fish and other aquatic organisms. Currently, there are no precise tools to diagnose or vaccinate against this disease. The aim of the present study was to fractionate and characterize the outer membrane protein (OMP) antigen of *A. sobria* by serological and cellular techniques so as to identify immunoreactive molecules that might be useful in preparing immunodiagnostic and/or immunoprophylactic tools against *A. sobria* infection in goldfish. Eight fractions were isolated from the crude OMP antigen using sephacryl S-200 and DEAE-cellulose chromatography. The highest seroreactivity was observed in the gel-permeated protein G<sub>1</sub> which had an optical density (OD) of 0.72 nm, higher even than that of the crude OMP antigen, 0.63 nm. The serodiagnostic potential of G<sub>1</sub> was assessed by using dip-stick ELISA. The *in vitro* goldfish lympho-proliferation ability of the fractionated antigen, G<sub>1</sub>A<sub>3</sub>, was higher than of all the other fractionated antigens and the crude OMP. Therefore, fractionated antigen G<sub>1</sub> (molecular wt 42-67 kDa) and G<sub>1</sub>A<sub>3</sub> (molecular wt 45-47 kDa) should be further studied in immunodiagnostic and/or immunoprophylactic preparations for *A. sobria* infection.

### Introduction

*Aeromonas sobria*, together with *A. hydrophila*, has frequently been reported as a causative agent of motile aeromonas septicemia (MAS) in fish and other aquatic animals (Rehulka, 2002; Taylor, 2003; Wahli et al., 2005). MAS causes significant losses in the ornamental fish

industry and *A. sobria* can produce extracellular substances such as proteases, amylase, chitinase, lipase, and nuclease as virulence factors (Janda, 1991). One of the major drawbacks in goldfish farming is the lack of a precise diagnostic tool and vaccine against MAS.

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Information related to the antigenicity of *A. hydrophila* is available for other fish species (Loghothetis and Austin, 1994, 1996ab) and, in earlier work, we detected immunoreactive antigens of the outer membrane protein (OMP) of *A. hydrophila* isolated from goldfish (Maji et al., 2006). However, we found no published information on antigenic components of *A. sobria* isolated from goldfish (*Carassius auratus*).

With this background, the present study was designed to fractionate and characterize the OMP antigen of *A. sobria* by serological and cellular techniques to identify immunoreactive molecules that might be useful in preparing immunodiagnostic and/or immunoprophylactic tools for *A. sobria* infection in goldfish.

#### Materials and Methods

**Bacterial strain.** *Aeromonas sobria* (GFVL20) was obtained from the Department of Fishery Pathology and Microbiology of the Faculty of Fishery Sciences at West Bengal University of Animal and Fishery Sciences in West Bengal, India. The strain was isolated in 2003 from an ulcerative lesion of goldfish, *Carassius auratus* L., as per Austin and Austin (1999). The strain was isolated using tryptose soya agar (TSA), nutrient agar (NA), and starch ampicillin agar (SAA). The isolate was identified to the genus level using cultural, morphological, and biochemical characteristics according to the scheme for identifying gram-negative bacteria (LeChevallier et al., 1980) and to the species level using the microbial identification system, BIOLOG-GN (BIOLOG Inc., Hayward, CA, USA) with a similarity index of 80%. The strain was stored in 1-ml aliquots at -70°C in tryptose soya broth (TSB) containing 10% glycerol (Sharp and Secombes, 1993).

**Preparation of the crude OMP antigen.** The outer membrane protein antigen (OMP) was prepared by the method of Chakraborty et al. (1982) with slight modifications (Dooley and Trust, 1988). Briefly, the somatic antigen was prepared by disintegration in an ultrasonic homogenizer (Labsonic U, Biotech Int., UK) using a titanium probe operated at 150 W and 0°C for 20 cycles of 2 min each at intervals of 1 min. After centrifugation at 10,000 x g for 20 min at 4°C, the pellet was put into 20 ml of a

normal saline solution and treated with 2% sodium dodecyl sulphate and 2% mercaptoethanol for 45 min at 60°C for solubilization. The solubilized extract was centrifuged at 10,000 x g for 20 min and the supernatant (the OMP) was stored at -20°C. The protein content of the crude OMP antigen was estimated using the method of Lowry et al. (1951).

**Preparation of antiserum.** Rabbit antisera to *A. sobria* was produced following the method mentioned by Mishra and Sekhar (1997). Briefly, two healthy NZW male rabbits were maintained in the laboratory. One was injected intramuscularly with four doses of *A. sobria* OMP antigen mixed with an equal volume of Freund's adjuvant (Sigma, USA) at intervals of 10 days. The first dose was given with Freund's complete adjuvant (FCA) and the subsequent three doses with Freund's incomplete adjuvant (FIA). Boostering was done with an increasing dose of proteins. Five days after the last injection, blood from the rabbit was collected and the serum was separated and stored at -20°C. Serum from the uninjected rabbit was used as a control.

**Fractionation of the crude OMP antigen.** Fractionation of the crude OMP antigen followed the procedure of Joardar and Ram (1999). At first, the crude OMP was fractionated by molecular exclusion chromatography on a 50 x 1.1 cm column bed of sephacryl S-200. Two ml of crude OMP with 9.4 mg protein/ml was eluted at 20 ml/h with Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl. Two peaks (G<sub>1</sub> and G<sub>2</sub>) were observed, pooled, and dialyzed against distilled water at 4°C. The two peaks were separately subjected to DEAE-cellulose chromatography. The G<sub>1</sub> protein was dialyzed and 11.5 mg was applied to a 12 x 3 cm column bed of DEAE-cellulose, equilibrated with 25 mM Tris-HCl buffer (pH 8) containing 3M urea and a linear gradient of NaCl from 0.15 to 0.3 mM. Fractions under each region were pooled and dialyzed against distilled water at 4°C. Proteins were concentrated by sucrose, sterilized through a membrane filter (0.22 µ), and stored at -20°C. The G<sub>2</sub> protein was dialyzed and 6.6 mg was applied to DEAE-cellulose as above; it was fractionated and stored at -20°C.

**Enzyme linked immunosorbent assay (ELISA).** Seroreactivity of the OMP crude antigen and its fractionated components was assessed by ELISA as per Mishra and Sekhar (1997) with slight modifications (Maji et al., 2006). Dip-stick ELISA was used for rapid detection of antigen specificity following the procedure of Jiahao et al. (1997). The gel permeated fraction G<sub>1</sub> had the highest seroreactivity and was used as a coating antigen. The steps were almost the same as plate ELISA, except that the procedure was performed on a nitrocellulose membrane strip and a substrate solution of 40 µl H<sub>2</sub>O<sub>2</sub> and 0.025 g diaminobenzidine in 10 ml Tris-HCl (pH 7.5) was used.

**Tetrazolium based colorimetric assay of goldfish leucocytes.** To assess cellular reactivity of the OMP crude and fractionated antigens, tetrazolium based colorimetric assay of goldfish leucocytes was performed. Goldfish (3-4 cm) were purchased from the local market, maintained at ambient temperature, and fed a commercial dry pellet feed daily. The fish were acclimatized for three weeks before the experiment. Leukocytes were isolated from the head kidneys of the goldfish and purified in a cell suspension layered on Histopaque® (Sigma, USA) at a ratio of 1:3 and centrifuged at 400 x g for 3 min (Chung and Secombes, 1988). The cells at the interface were transferred to sterile tubes and washed three times by centrifugation at 300 x g for 10 min, resulting in a cell population consisting of 90% lymphocytes.

Viability and enumeration of the cells were estimated by the trypan blue dye exclusion method. The number of viable cells was adjusted to 2 x 10<sup>6</sup> cells per ml by dilution with RPMI-1640 growth medium, supplemented with 10.41 g/l media powder, 2 mM L-glutamine, 25 mM sodium bicarbonate, 24 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% v/v FCS. The cell-containing medium (100 ml) was dispensed into a 96-well tissue culture plate (Nunc, Denmark). A stock solution of Concanavalin A (Con A) was prepared at a concentration of 20 µg/ml of the proliferation medium (RPMI-1640 growth medium) and stored at -20°C. One hundred ml

of this stock solution at a final concentration of 10 µg/ml served as the positive control. Stock solutions of the crude OMP antigen and its fractionated components were prepared at concentrations of 40 µg/ml of the medium and stored at -20°C until needed. The volumes of the wells were made up to 200 µl with different stock solutions of fractionated antigens along with crude OMP and Con A. The plate was incubated at 28°C for five days in a humid atmosphere containing 5% CO<sub>2</sub>. Then 20 µl of MTT (3-[4,5-dimethyl thiazol-2-yl]2,5-diphenyl-tetrazolium bromide) with a concentration of 5 mg/ml in PBS was added to each well. The plate was incubated for an additional 4 h at 28°C after which formazan production was determined by the method of Plumb et al. (1989). Next, 150 µl of the cell suspension in each well was replaced by an equal volume of DMSO and the optical density (OD) of each well was measured at 595 nm ten minutes later (Daly et al., 1995) using an ELISA reader (ECIL, India) to assess lymphoproliferation. The stimulation index (SI) was calculated as SI = (mean OD of lymphocyte wells with Con A or antigen/mean OD of control lymphocyte wells) - 1.

## Results

**Fractionation of OMP antigens.** Eight fractions were generated from the crude OMP antigen. The elution profile, obtained from sephacryl S-200, had two prominent peaks, G<sub>1</sub> and G<sub>2</sub> (Fig. 1). Four peaks from G<sub>1</sub> (G<sub>1</sub>C<sub>1</sub>, G<sub>1</sub>A<sub>1</sub>, G<sub>1</sub>A<sub>2</sub>, G<sub>1</sub>A<sub>3</sub>) and two from G<sub>2</sub> (G<sub>2</sub>C<sub>1</sub>, G<sub>2</sub>A<sub>1</sub>) resulted from DEAE-cellulose elution (Fig. 2).

**ELISA.** Reactivity to rabbit hyperimmunized sera (1:200 dilution) of the crude and fractionated antigens was expressed in OD values at 492 nm (Fig. 3). The seroreactivity of the G<sub>1</sub> fraction was higher (OD 0.72) than that of the crude OMP antigen and all other fractions. Therefore, the G<sub>1</sub> fraction was further tested using dip-stick ELISA where a difference in color after exposure to hyperimmune (test) or normal (control) serum indicated the potential of using the G<sub>1</sub> fraction for diagnostic purposes (Fig. 4).

**MTT assay.** The proliferation of goldfish head kidney leukocytes caused by the crude

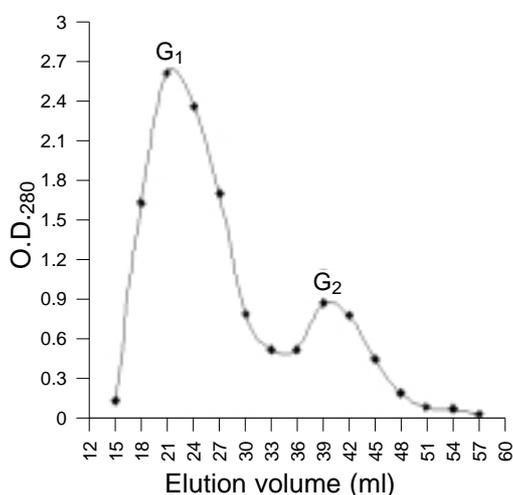


Fig. 1. The sephacryl S-200 elution profile of the outer membrane protein (OMP) antigen of *Aeromonas sobria* (GFVL20).

and fractionated antigens was determined. The fraction  $G_1A_3$  had the highest stimulation index (SI) after stimulation by a non-specific stimulator (mitogen), i.e., Con A (Fig. 5).

### Discussion

The crude OMP antigen of *A. sobria* (GFVL20) was fractionated by gel permeation and ion-exchange chromatography to identify the principle components (polypeptides) of the antigen responsible for serological and cellular (lymphoproliferation) reactivity. Eight fractions were generated from the crude OMP antigen using sephacryl S-200 and DEAE-cellulose chromatography with two (high and low molecular weight) protein peaks. In contrast, Smirnov et al. (2000) generated six fractions from water-soluble proteins of *A. sobria* by gel filtration chromatography on sephadex G-100 and Agarwal et al. (2000) obtained one peak in *A. sobria* toxin subjected to gel filtration chromatography on sephadex G-75.

In the second step of our experiment, ELISA was performed on the crude OMP and its fractionated components to detect frac-

tion(s) with high seroreactivity. There was clear variation among the fractions with the highest seroreactivity obtained in gel-permeated  $G_1$  protein (OD 0.72), higher than in the crude OMP antigen (OD 0.63). Fractionated  $G_2$  and  $G_1A_3$  also had high OD values, 0.495 and 0.479, respectively. The higher seroreactivity of these fractionated antigens might be due to the presence of semi-purified immunodominant (seroreactive) polypeptides in the antigen preparations. There is little published information on the seroreactivity of fractionated antigens of *Aeromonas* spp. with which to compare.

The potential of using the  $G_1$  fraction for field based diagnosis of *Aeromonas* infection was assessed using dip-stick ELISA. Results indicate the specificity of fractionated  $G_1$  with hyperimmune serum and, hence, the potential of using  $G_1$  for rapid diagnosis of *A. sobria* infections in fish.

A colorimetric assay (MTT dye assay) was used to characterize *in vitro* cellular reactivity of crude and fractionated OMP antigens of *A. sobria* GFVL20 using goldfish head kidney leukocytes. The stimulation index (SI) of  $G_1A_3$  was higher than that of the other fractions and the crude antigen. Teleosts possess both humoral and cell mediated immune responses (Graham and Secombes, 1990). Hence, it might be possible to use  $G_1A_3$  to protect goldfish by stimulating cell-mediated immunity. Yin et al. (1997), also using MTT dye assay, showed that the proliferation of head kidney leukocytes from catfish was significantly enhanced four weeks after immunization with *A. hydrophila*. Again using MTT assay, Daly et al. (1995) obtained almost the same OD and SI values in mitogen (PHA) and *A. salmonicida* antigens for kidney lymphocytes, corroborating results of the present study.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) of the  $G_1$  and  $G_1A_3$  fractions, performed according to Laemmli (1970), revealed five bands ranging 42-67 kDa in molecular weight and three bands ranging 45-47 kDa, respectively. The 42-67 kDa polypeptides were predominant in inducing B lymphocytes and a humoral immune response (assessed by ELISA) while the 45-

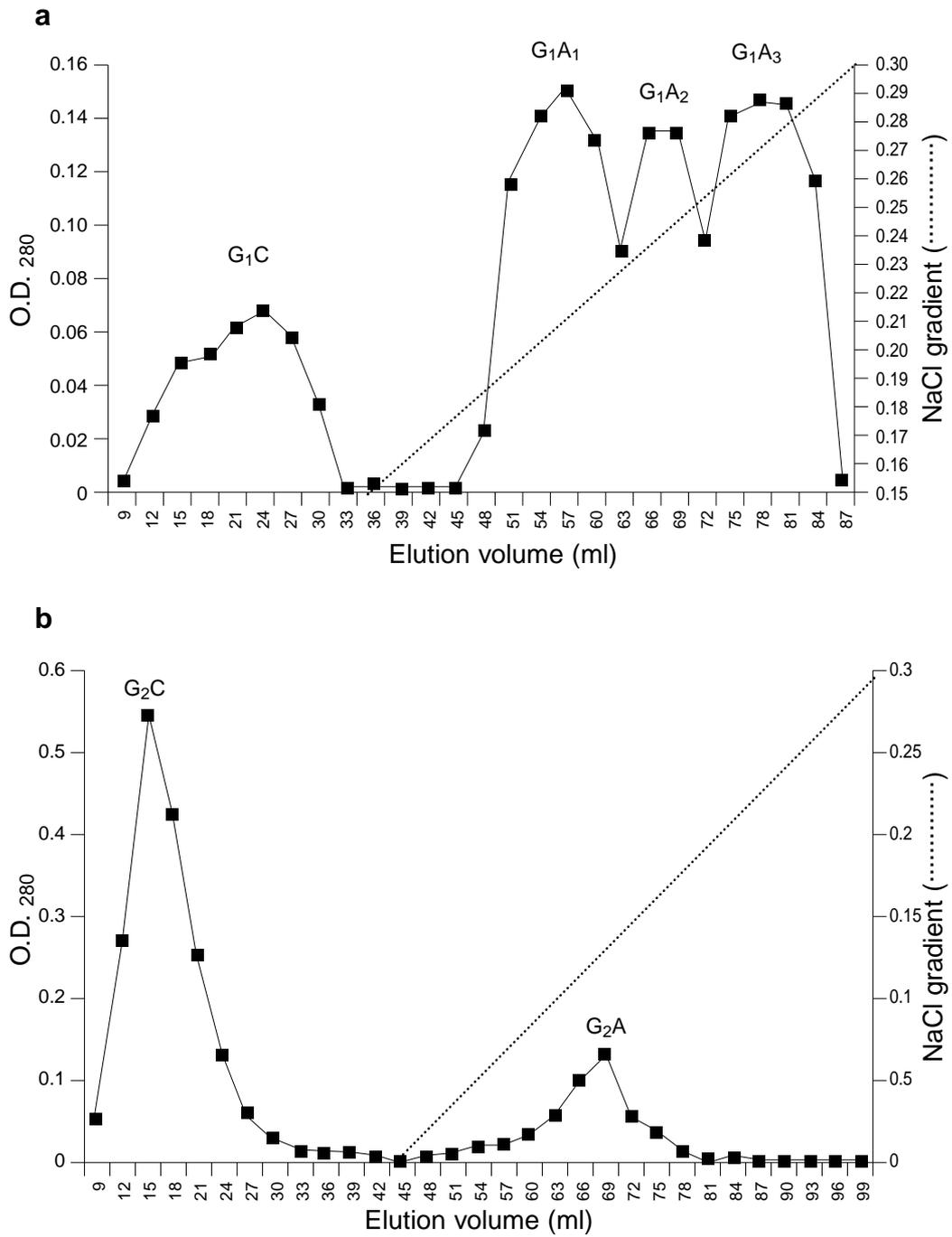


Fig. 2. Elution profiles obtained with ion-exchange chromatography of gel-permeated fractions of (a) G<sub>1</sub> and (b) G<sub>2</sub> of the outer membrane protein (OMP) antigen of *Aeromonas sobria* (GFVL20).

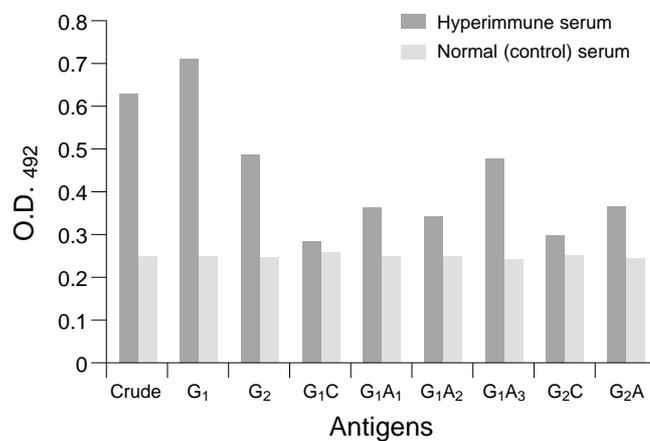


Fig. 3. Seroreactivity to hyperimmune and control rabbit sera (1:200 dilution) of *Aeromonas sobria* (GFVL20) crude and fractionated OMP antigens as assessed by ELISA.

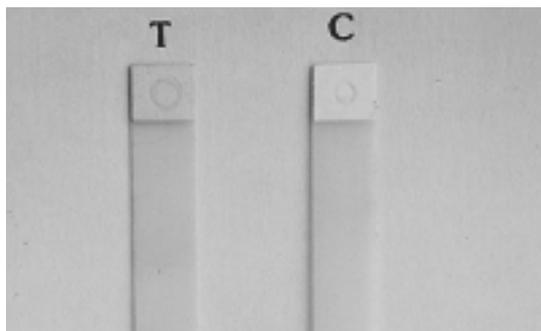


Fig. 4. Assessment of the diagnostic potential of G<sub>1</sub> by dip-stick ELISA using hyperimmune and normal sera. T = test, C = control.

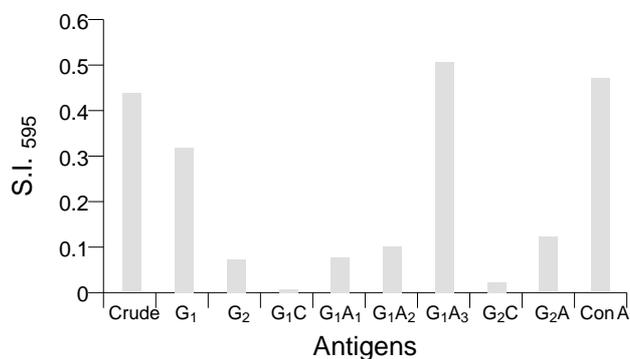


Fig. 5. *In vitro* proliferation of goldfish kidney lymphocytes after exposure to crude and fractionated OMP antigens of *A. sobria* stimulated by Concanavalin A, expressed as stimulation indices (SI).

47 kDa polypeptides induced T lymphocytes (assessed by lymphoproliferation assay). We earlier reported on two fractionated OMP antigens of *A. hydrophila*, GPID2 (primarily a 57 kDa polypeptide) and GPIID2 (primarily a 23 kDa polypeptide), which dominated induction of B and T lymphocytes, respectively (Maji et al., 2006).

From the present study, we conclude that the fractionated antigen G<sub>1</sub> should be further studied as a serodiagnostic of pathogenic *A. sobria* infection in fish. At the same time, the cross-reactivity of G<sub>1</sub> should be tested with related bacterial species. The fractionated antigen G<sub>1A3</sub>, having the highest cellular reactivity, might prove important in cellular immunodiagnostic and/or immunoprophylactic preparations for *A. sobria* infection. Challenge studies should be conducted to assess the potential of this fraction as an immunoprophylactic agent.

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