Research Article

SEROREACTIVITY ASSAY OF CRUDE AND FRACTIONATED OUTER MEMBRANE PROTEIN OF *Aeromonas sobria* ISOLATED FROM GOLDFISH (*Carassius auratus* Linnaeus, 1758)

Prasenjit Mali[1*], Koel Bhattacharya Sanyal[2], Debapriyo Mukherjee[3], Avijit Biswas[4], Biswadeep Dey[5], T. J. Abraham[6], Gadadhar Dash[7] and S.N. Joardar[8]

[1,4,5,6,7]Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, West Bengal, INDIA;

[2,3]National Surveillance Programme for Aquatic Animal Diseases, West Bengal University of Animal and Fishery Sciences, West Bengal, INDIA;

[8]Department of Veterinary Microbiology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, West Bengal, INDIA

**Corresponding author's E-mail: prasenjitmali78@gmail.com*

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ABSTRACT

***Aeromonas sobria* has frequently been reported as a causative agent of motile *Aeromonas* septicemia (MAS) along with *A. hydrophila* in fish and other aquatic organisms. Till now there is lack of precise tool for early diagnosis of this disease. The aim of the present study was to fractionate and characterize the outer membrane protein (OMP) antigen of *A. sobria* by serological techniques so as to identify immunoreactive molecules that might be useful in preparing immunodiagnostic 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_1 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_1 was assessed by using dip-stick ELISA. Therefore, fractionated antigen G_1 (molecular wt 42-67 kDa) should be further studied in immunodiagnostic tool preparations for *A. sobria* infection.**

INTRODUCTION

Like other ornamental fishes, gold fish also subjected to disease caused by infectious agent. Ulcer disease due to *Aeromonas* sp. is most common in gold fish. *Aeromonas sobria* (one of the important motile *Aeromonas* sp.) was frequently reported as a causative agent of ulcerative conditions in aquatic organisms including gold fish. But till now there is a scarcity of immunodiagnostic test to detect as well as to prevent *Aeromonas* infections of the aquarium fishes. *Aeromonas sobria*, along with *A. hydrophila*, has often been reported as a causative agent of Motile *Aeromonas* septicaemia or as MAS in fish and other aquatic animals (Rahaman *et al.*, 2002; Rehulka, 2002; Taylor, 2003; Wahli *et al*., 2005). Motile *Aeromonas* septicaemia causes considerable 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. Information related to the antigenicity of *A. hydrophila* is available for other fish species (Loghothetis and Austin, 1994, 1996ab; Shayo *et al.*, 2012). However, very rare published information on antigenic components of *A. sobria* isolated from goldfish (*Carassius auratus*) is available till now. Therefore, the identification of immune dominant antigens of *Aeromonas sobria* is urgently needed that can be used in immunodiagnostic purpose. In this background the present study was undertaken with the following objectives: To isolate and identify *Aeromonas sobria* from gold fish and to fractionate and characterize *Aeromonas sobria* outer membrane protein antigens using column chromatography and serological tests, respectively.

MATERIALS AND METHODS

Bacterial strain

Aeromonas sobria (GFVL20) strain was isolated from an ulcerative lesion of goldfish, *Carassius auratus* Linnaeus, 1758, as per Austin and Austin (1999), 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 (Le Chevallier *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 minus (-) 70°C in tryptose soya broth (TSB) containing 10% glycerol (Sharp and Secombes, 1993).

Preparation of the crude OMP antigen

Preparation of the outer membrane protein antigen (OMP) was done by the method of Chakraborty *et al.* (1982) with slight modifications (Dooley and Trust, 1988). In brief, 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 rpm 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). In brief, 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 Freunds' adjuvant (Sigma, USA) at intervals of 10 days. The first dose was given with Freunds' complete adjuvant (FCA) and the subsequent three doses with Freunds' 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 was followed as the standard 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 mm 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_1 and G_2) were observed, pooled, and dialyzed against distilled water at 4°C. The two peaks were separately subjected to DEAE-cellulose chromatography. The G_1 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_2 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). Dip-stick ELISA was used for rapid detection of antigen specificity following the procedure of Jiahao *et al.* (1997). The gel permeated fraction G_1 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_2O_2 and 0.025 g diaminobenzidine in 10 ml Tris-HCl (pH 7.5) was used.

RESULTS AND DISCUSSION

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_1 and G_2 (Fig. 1). Four peaks from G_1 (G_1C_1, G_1A_1, G_1A_2, G_1A_3) and two from G_2 (G_2C_1, G_2A_1) resulted from DEAE-cellulose elution (Fig. 2 & 3).

ELISA

Reactivity to rabbit hyperimmunized sera (1:200 dilution) of the crude and fractionated antigens were expressed in OD values at 492 nm (Fig. 4). The seroreactivity of the G_1 fraction was higher (OD 0.72) than that of the crude OMP antigen and all other fractions. Therefore, the G_1 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_1 fraction for diagnostic purposes.

The crude OMP antigen of *A. sobria* (GFVL20) was fractioned by gel permeation and ion-exchange chromatography to identify the principal 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. Whereas, 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 fraction(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.

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.

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

From the present study, we conclude that the fractionated antigen G_1 should be further studied as a serodiagnostic of pathogenic *A. sobria* infection in fish. At the same time, the cross-reactivity of G_1 should be tested with related bacterial species. The fractionated antigen might prove important in immunodiagnostic preparations for *A. sobria* infection.

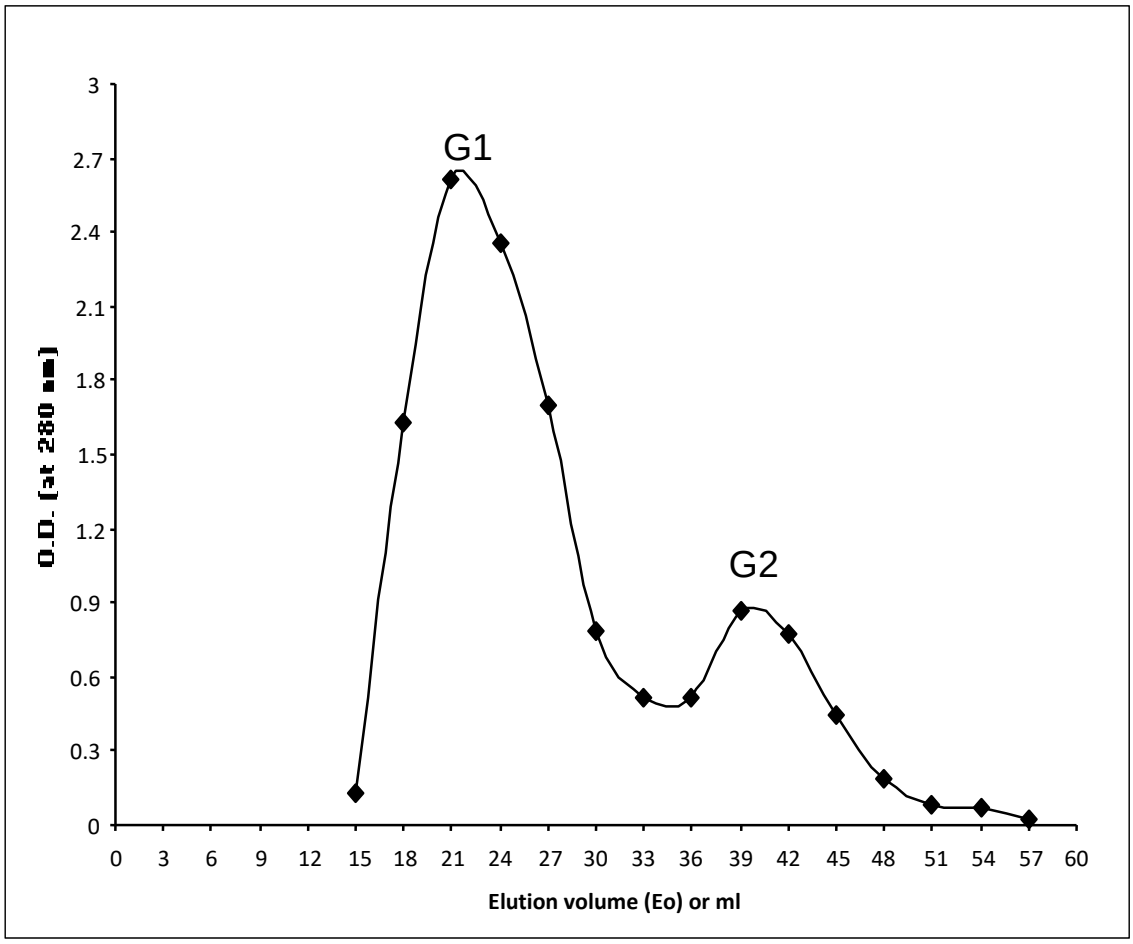

Fig. 1. Elution profile of molecular exclusion chromatography of *A. sobria* GFVL20 OMP antigens

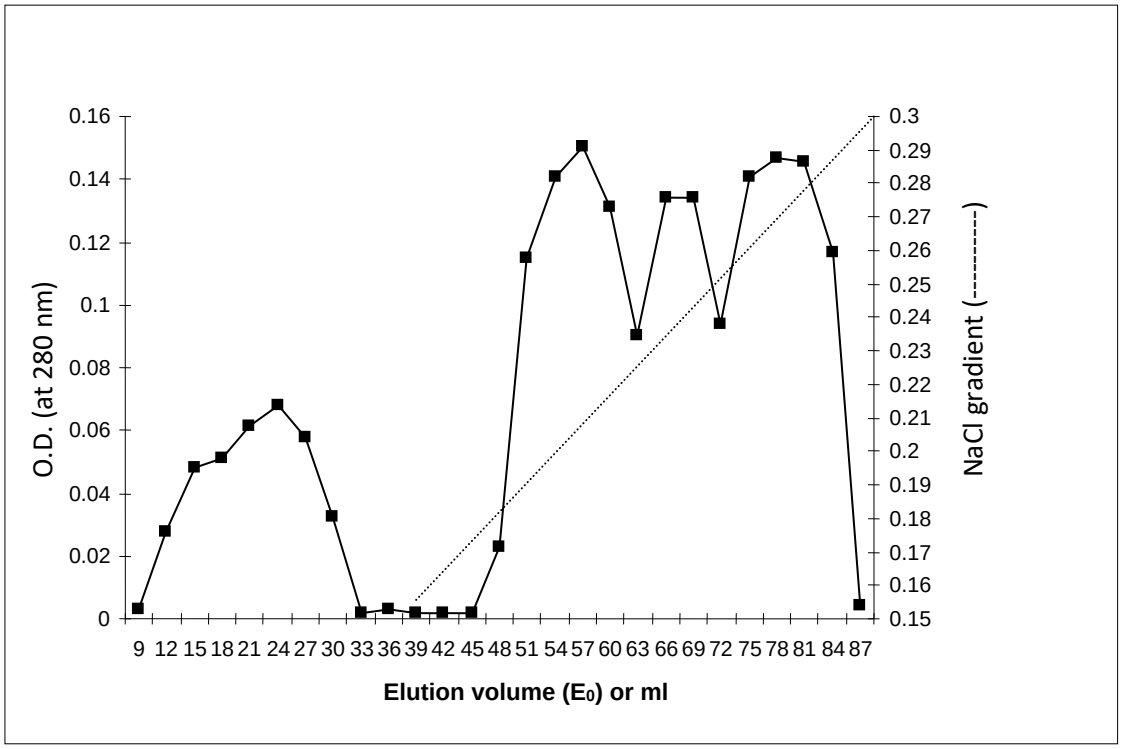

Fig. 2. Elution profile of Ion-exchange chromatography of gel permeated fraction-I (G1) of *A. sobria* GFVL20 OMP antigens

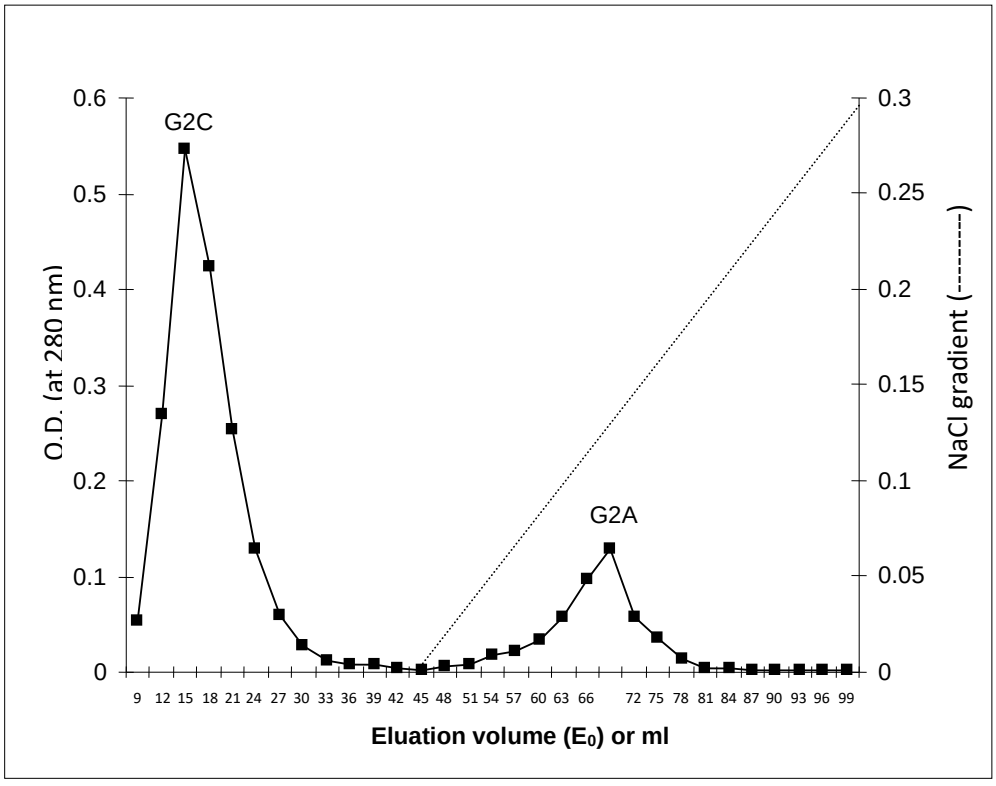

Fig. 3. Elution profile of Ion-exchange chromatography of gel permeated fraction II (G2) of *A. sobria* GFVL20 OMP antigens

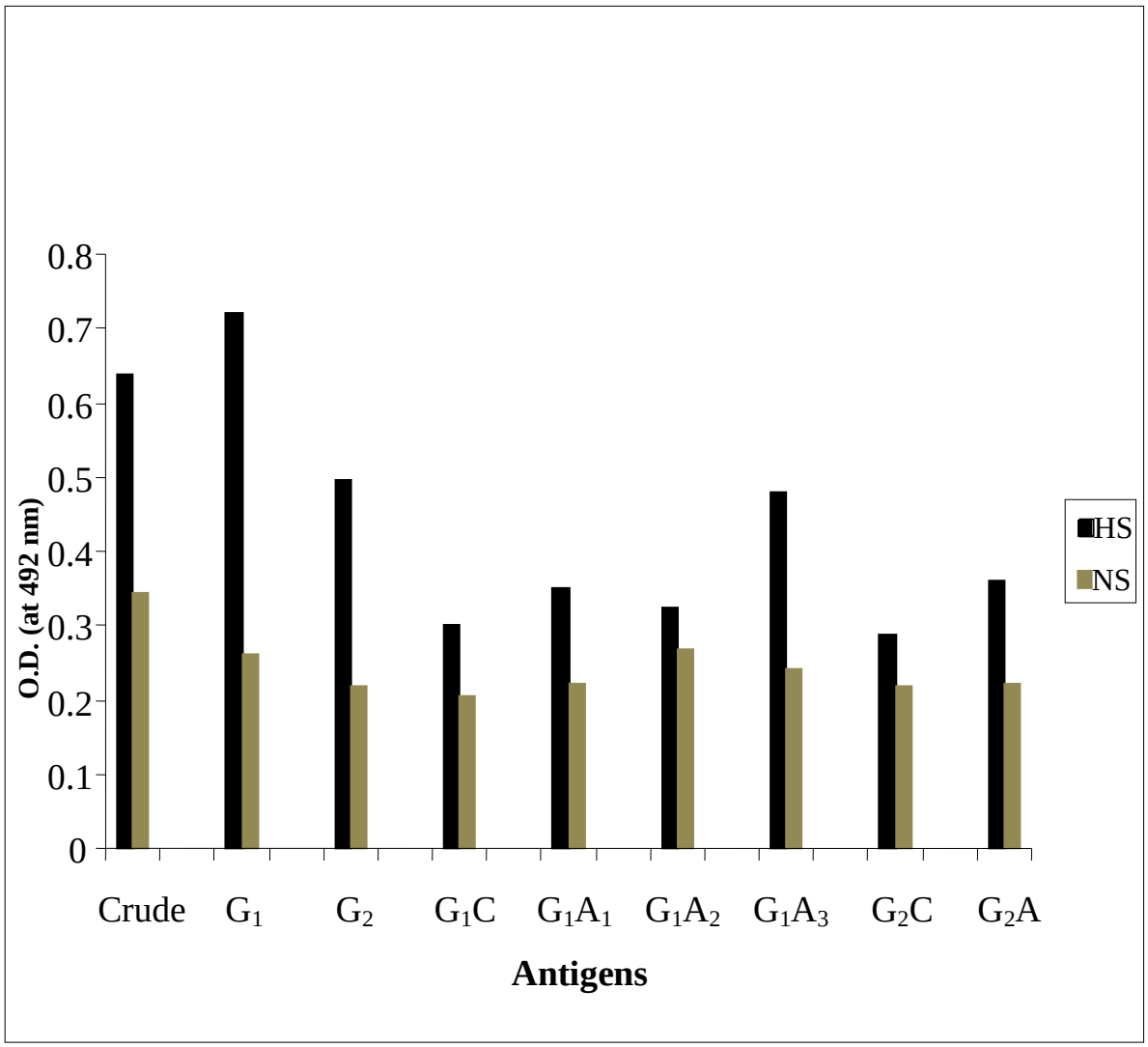

Fig. 4. Seroreactivity of *A. sobria* GFFVL20 crude and fractionated OMP antigens with hyper immune and control rabbit serum (1:200 dilution) as assessed by ELISA

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