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Immunostimulation by phospholipopeptide biosurfactant from *Staphylococcus hominis* in *Oreochromis mossambicus*

Veluchamy Rajeswari, Sekaran Kalaivani Priyadarshini, Viswanathan Saranya, Ponnusamy Suguna, Rajaiah Shenbagarathai*

Postgraduate and Research Department of Zoology and Biotechnology, Lady Doak College, Madurai 625002, Tamil Nadu, India

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ABSTRACT

The immunostimulatory effect of phospholipopeptide biosurfactant from *Staphylococcus hominis* (GenBank Accession No: KJ564272) was assessed with *Oreochromis mossambicus*. The non-specific (serum lysozyme activity, serum antiprotease activity, serum peroxidase activity and serum bactericidal activity), specific (bacterial agglutination assay) immune responses and disease resistance activity against *Aeromonas hydrophila* were examined. Fish were intraperitonially injected with water soluble secondary metabolite (biosurfactant) of *S. hominis* at a dose of 2 mg, 20 mg and 200 mg kg^{-1} body weight. Commercial surfactant surfactin (sigma) at 20 mg kg^{-1} was used as standard and saline as negative control. All the doses of water soluble biosurfactant tested, significantly enhanced the specific, nonspecific immunity and disease resistance from the day of post administration of phospholipopeptide biosurfactant till the tail of the experimental period. These results clearly indicated that the secondary metabolite isolated from *S. hominis* stimulates the immunity of finfish thereby could enhance aquaculture production.

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1. Introduction

Aquaculture is a rapidly growing economic area that contributes nearly half (47.3%) of the world's fish food consumption. Nevertheless, one of the main threats to aquaculture is the infectious outbreaks leading to mass mortality in fish [1,2]. *A. hydrophila*, the motile aeromonads affects a wide variety of freshwater fish species and occasionally marine fish [3]. Improving fish performance in terms of immunity and disease resistance are the major challenges faced by the fish culturists. Though, many measures including routine use of antibiotics have been tried, the excessive and inappropriate use of antibiotics has resulted in cross resistance against pathogens and accumulation of residues in tissues [4]. The use of organic, inorganic and synthetic compounds such as levamisole [5,6], glucan [7,8], vitamin C and E [9–11], chitin [12], FK-565 (Lactoyl tetrapeptide) [13], FCA (*Freund's Complete Adjuvant*) [14,15] as immunostimulant has been increased in recent years. Generally, many microbial products with low molar mass and the group of naturally occurring polymers are surveyed to have an immunoregulatory activity [16]. The components of bacterial cell wall, such as MDP (Muramyl dipeptide), peptidoglucan and LPS (Lipopolysaccharide), attributed to the immunostimulatory effects of aquatic animals [17–19]. However, their role as immunostimulants is controversial for commercial application due to their adverse health effects [20,21]. Microbial secondary metabolites, a protective subcellular component has received more attention in disease control. Newaj-Fyzul et al. [22], has demonstrated that feeding rainbow trout with cell-free supernatant of *Bacillus subtilis* AB1 significantly reduced cumulative mortalities after challenge with Aeromonas sp. Arijo et al. [23], also reported that subcellular components of *Vibrio harveyi* were successful for the stimulation of immunity and the prevention of *V. harveyi* infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Hence, screening of new immunostimulants from the secondary metabolites (biosurfactants) of microorganisms could be advantageous to strengthen fish immune system and to reduce the quantity of antibiotics required to control infectious diseases.

Many Staphylococcus strains have been reported to produce a number of antimicrobial peptides [24–27]. *Staphylococcus hominis* MBBL 2–9 produced an antimicrobial peptide, called hominicin which exhibited a high bactericidal activity against methicillin-

* Corresponding author.
E-mail addresses: shenbagarathai@gmail.com, shenbagarathai@rediffmail.com (R. Shenbagarathai).

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resistant *Staphylococcus aureus* (MRSA) ATCC 11435 and vancomycin-intermediate *S. aureus* (VISA) CCARM 3501 but also showcased heat-tolerance and pH stability. The main advantages of biosurfactants are their low toxicity, high biodegradability and high stability at extreme conditions, such as high levels of temperatures, salinity and pH [28,29]. In some cases, stability is higher than that of chemical based surfactants [30]. Also, biosurfactant molecules are able to form stable emulsions in solutions with high ionic force, which is especially relevant in aquacultural applications, since most fish farms are located in high salinity environments, making the stability of biosurfactants at high ionic strength is essential. For example, biosurfactants from Cobetia sp. MM1IDA2H-1 [31] exhibited high stability at wide ranges of pH, temperature and strength force (3–20% w/v of sodium chloride). Researchers across the globe have evaluated the use of biosurfactants in a wide variety of potential applications such as environmental bioremediation, biomedical applications, cosmetics, personal care products, perfume and fragrance industry [31]. Similarly, WH1fungin (WF), a lipopeptide surfactin, was verified to have an immunoadjuvant property in mice groups when WF plus Hepatitis B surface antigen (HbsAg) was immunized intranasally or parenterally suggesting its potential for development of more efficient HBV vaccines in the future [32]. Also, a synthetic lipopeptide derived from bacterial lipoprotein P_3CSK_4, has shown to be an effective immunoadjuvant in parenteral, nasal and oral immunization [33]. Synthetic lipopeptide P_3CSK_4 constitutes a potent macrophage/monocyte activator resulting in the induction of lymphokine production, phagocytosis, activation for tumour cytotoxicity, tumour necrosis factor- α (TNF-α) production, and release of reactive oxygen and nitrogen intermediates [34,35]. However, limited research is available regarding its immunomodulatory activity. Hence the current study focus on the evaluation of immunostimulatory property of extracellular secondary metabolite phospholipopeptide biosurfactant isolated from *S. hominis* to fish (*Oreochromis mossambicus*), but the ideas are also applicable to other groups of organisms relevant to aquaculture.

2. Materials and methods

2.1. Fish and their maintenance

Wild strain of *O. mossambicus* (Mossambique tilapia), a cichlid fish was used as the model organism for this study. About 260 male fish weighing 25 ± 5 g were collected from a local fish farm and were acclimatized to laboratory conditions in 21 fibre reinforced plastic tanks (vol. 150 L) consisting of 10 fish each for a period of 4 weeks. They were kept at an ambient temperature of 28 ± 2 °C under natural photoperiod (between March and June). Water was changed on alternate days to avoid ammonia accumulation. Fish were fed at a rate of 3% body weight once in a day with a balanced fish diet consisting of 42% dried fish meal, 20% groundnut oil cake, 15% tapioca flour, 15% wheat flour, 5% blood meal and 3% mineral-vitamin mixture [36] prepared in the lab. Its nutrient composition consisted of 39% protein, 24% carbohydrate, 11% lipid and 9% ash [37]. A total of 26 tanks with 10 fish per tank were used for the experiment.

2.2. Microbial identification

Bacterial isolate were obtained from petrol/diesel contaminated soil and screened for biosurfactant producing ability. The potent strain was subjected to 16 S rDNA based molecular characterization. The genomic DNA of the isolate was amplified using Eu-bacterial primer (5′- AGA GTT TGA TCC TGG CTC AG -3′; 5′- ACG GCT ACC TTG TTA CGA CTT -3′) following the method described by Relman [38] and sequenced (Eurofins India Ltd.,). The resulted nucleotide sequence was analysed using NCBI BLAST and were submitted to NCBI and accession number was obtained.

2.3. Biosurfactant screening, production, extraction and characterization

Different biosurfactant screening methods were performed to select the potential biosurfactant producing microorganism. The methods adopted were (a) Hemolytic activity in 5% blood agar plate [39]; (b) Oil spreading technique [39]; (c) Parafilm M test [40] and (d) Emulsification activity by adding kerosene and equal volume of cell free supernatant [41].

Bacterial strain was grown in a minimal salt (MS) medium containing (g/l): yeast extract – 1; KH_2PO_4 – 1.4; K_2HPO_4 – 2.2; $MgSO_4$ 7. H_2O – 0.6; was used throughout the study. The basal minimal medium was supplemented with 2 ml of trace element solution and palmolein oil 2% (v/v) as the sole carbon source. The composition of trace element solution involved (g/l): Ortho phosphoric acid – 0.6; Cobaltous chloride – 0.4; Zinc sulphate – 0.2; Magnesium chloride – 0.06; Sodium molybdate – 0.06; Copper sulphate – 0.02. The trace element solution was added after the production media were autoclaved, prior to inoculation by filtering it through 0.2 μm membrane filters, sterilized by filtration (Millipore Corp., Bedford, MA, USA). The cultures were centrifuged after incubation at 30 °C for 3 days, and the cell-free filtrates were used in the experiments (Modified method of Gudiña et al., [42]).

Biosurfactant was precipitated by adjusting the pH to 2.0 in the cell-free filtrate using 6 N HCl and kept at 4 °C overnight. Pellet thus precipitated was collected by centrifugation (8000 rpm for 15 min at 20 °C) and dissolved in distilled water. Then the pH was adjusted to 8.0 with 1 N NaOH, and the extract was lyophilized [43]. Partial purification of water soluble biosurfactant was performed by dialysis.

Preliminary identification of partially purified phospholipopeptide biosurfactant was detected using biuret test [44], phosphate test [45] FTIR (Perkin–Elmer Spectrum RX1, Shelton, Connecticut), spectrum in the range of 450–4000 cm^{-1} at a resolution of 4 cm^{-1} (Madurai Kamaraj University, Madurai) [46], GC – MS spectra coupled to mass detector turbo mass gold – perkin Elmer (Indian Institute of Crop Processing Technology, Thanjavur) [47] and Agilent 1100 HP – HPLC (Sankara Nethralaya, Chennai) were used to confirm the phospholipopeptide nature of the extracted biosurfactant [48].

2.4. Assessment of nonspecific immunity

To analyze the effect of phospholipopeptide biosurfactant on nonspecific immune mechanisms, five groups/tanks each containing 10 fish each were administered, ip, with 0.2 ml water soluble fraction of biosurfactant at doses of 2, 20 and 200 mg/kg body weight and 20 mg/kg concentration of surfactin. A preliminary test was performed to determine the LC_{50} dose of the biosurfactant [49] and the sub-lethal concentrations were derived for test substance. Then fish were bled at regular intervals of 5 days till day 20 post-immunization. To detect pre-treatment levels of immune mechanisms, fish were also bled 2 days prior to the start of the experiment. The blood was collected in serological tubes and stored overnight at 4 °C in a refrigerator. The clot was then spun down at 400 × g for 10 min to separate the serum and stored at −20 °C until further use.

2.4.1. Lysozyme activity

Lysozyme activity was measured by the method of Parry et al. [50], in combination with the microplate adaptation of Hutchinson

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