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Construction and Analysis of the Immune Effects of a *Streptococcus agalactiae* Surface Protein ScpB Vaccine Encapsulated with Polylactic-Co-Glycolic Acid (PLGA)

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Abstract

In order to find an effective immune preparation to control tilapia streptococcus disease, the *Streptococcus agalactiae* surface protein serine protease C5a peptidase (ScpB) was cloned and the recombinant protein was encapsulated in poly (lactide-co-glycolic acid) (PLGA) microspheres, which were comprised of biodegradable materials. The ScpB-PLGA vaccine was then administered to the tilapia intraperitoneally at different concentrations, with PBS used as a control, and the relative percent survival (RPS) of each group was calculated. Serum lysozyme and superoxide dismutase (SOD) activity levels and antibody levels (OD_{450nm}) were tested weekly for the duration of the experiment. The results showed that the ScpB loading rate in the PLGA microspheres was 2.55% and the encapsulation efficiency reached 48.76%. The RPS ranged from 66.80% to 87.66%, with the highest RPS noted in group P1 (1 μg/g). The serum lysozyme, SOD and antibody (IgM) levels were significantly higher in the vaccinated fish relative to the control groups ($P < 0.01$). These results showed that PLGA could serve as an effective adjuvant for a ScpB vaccine and could provide relatively sustained immune protection.

Keywords

Tilapia, *Streptococcus agalactiae*, C5a Peptidase, Immunogenicity

Subject Areas: **Aquaculture, Fisheries & Fish Science**

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1. Introduction

In tilapia, streptococcal infection result in large economic losses annually for global tilapia farming markets. In China, the most prevalent streptococcus prior to 2008 was *Streptococcus iniae*, but now *Streptococcus agalactiae* dominates [1]. *S. agalactiae*, also called group B streptococcus (GBS), is a gram-positive diplococci that produces a polysaccharide capsule and contains 10 different serotypes [2]. Currently in China, the most dominant *S. agalactiae* serotype in tilapia is Ia [3], with no effective control methods, such as vaccination, currently available.

While the use of *S. agalactiae* bacterins, such as inactivated whole cells and extracellular products, has been shown to be effective when challenged with experimental *S. agalactiae* [4] [5], the use of these kinds of vaccines is seriously limited due to the large injection doses, single serotype coverage or the potential threat of virulence recovery. Additionally, a number of GBS surface proteins have been investigated as potential vaccine candidates. These proteins were shown to be highly conserved and can induce cross-protective immunity against different GBS seotypes [6]-[11]. One of the identified proteins, serine protease C5a peptidase (ScpB), was present on all of the tested GBS strains and serotypes and showed little or no antigenic variability [12]. It was capable of inducing antibodies that were opsonically active [10] [13] and was found to induce serotype-independent protection following immunization [6]. Interestingly, all human but only some bovine *S. agalactiae* isolates possess the scpB gene [14] [15].

While proteins and peptides have received extensive interest for their therapeutic applications in diverse clinical settings, they generally have short plasma half-lives, are unstable in the gastrointestinal tract and also have low bioavailabilities due to their relatively large molecular weight and high aqueous solubilities. These properties prevent them from being effectively used clinically. To overcome these obstacles, polymeric particulate carriers (micro- and nanospheres) have been developed as an effective way to control the release profile of the contained substance and to protect unstable biologically active molecules from degradation. Both natural and synthetic biodegradable polymers have been investigated for controlled drug release. Among these polymers, polylacticacid (PLA) and poly (lactic-co-glycolic acid) (PLGA) were ideal for drug delivery due to their excellent biocompatibility and biodegradability through natural pathways.

Previously, *S. agalactiae* isolates were found to possess the scpB gene and the ScpB protein was found to confer immune protection against *S. agalactiae* infection when administered with Freud's adjuvant at a dose of 5 μg/g [16]. In the present study, Freud's adjuvant was replaced with PLGA to see if a higher efficacy could be achieved to protect tilapia from colonization by GBS.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

S. agalactiae ZP-N was isolated from tilapia (*Oreochromis niloticus*) and preserved in the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences. *Escherichia coli* DH5*α* and BL21 (DE3) were purchased from Takara, Dalian, China. The *S. agalactiae* ZP-N strain was cultured in brain heart infusion broth (BHI, Huankai Co Ltd., Guangzhou, China) at 28˚C, while the *E. coli* strains were cultured in Luria-Bertani broth (LB) medium at 37˚C.

2.2. Purification of Recombinant Protein

The Purification of recombinant protein ScpB was described as previously [16]. Briefly, *E. coli* BL21 (DE3) was transformed with pET32a (+)-scpB and the transformants were grown in LB medium to an optical density of 0.7 at 600 nm (OD_{600}), with recombinant protein expression induced using 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG). After an additional 6 h of growth, the cells were harvested. Recombinant scpB was purified from BL21 (DE3)/pET32a(+)-scpB under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) columns (GE Healthcare, USA) as according to the manufacturer's protocols. The purified protein were subsequently analyzed using sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE), followed by visualized with coomassie brilliant blue R-250.

2.3. Western Blotting Analysis

The obtained purified proteins were subjected to SDS-PAGE using a vertical gel apparatus containing a 5%

stacking gel and 10% separating gel. After electrophoresis, the proteins were transferred to a BioTrace™ PVDF Transfer Membrane (0.45 μm pore size, PALL, USA). Polypeptides from the gels were electroblotted onto 0.45 μm nitrocellulose membranes with a semidry blotter at 100 v for 90 min (Biorad, USA), following the supplier's instructions. For immunodetection, strips were cut from previously blotted membranes and blocked for 1 h with 5% skimmed milk in TBST. Membranes were then incubated with primary antibodies (rat anti-his-tag; Abmart Inc., Shanghai, China) diluted 1:5000 in TBS-T for 2 h at 37˚C. The strips were then washed three times with TBST and incubated for 1 - 2 h with horseradish peroxidase (HRP) labeled goat-anti-mouse second antibody (BOSTER, Wuhan, China). Finally, the strips were washed three times with TBST and visualized using aDAB-kit (BOSTER, Wuhan, China), with the reaction stopped by washing the strips with distilled H_2O.

2.4. PLGA Nanoparticle Preparation

Recombinant ScpB protein was encapsulated in PLGA (molar ratio of 85:15 for D,L-lactic to glycolic acid; MW: 50,000 - 75,000 kDa; Sigma, USA) by modification of the double emulsion (W1/O/W2) solvent evaporation method described elsewhere [17] [18]. Briefly, the oil (O) phase was prepared by dissolving 50 mg PLGA in 5 ml chloroform (5%, w/v). Ten milligrams scpB were added to the above PLGA solution and sonicated at 35% (100 w) for 20 s, followed by a 3 min vortex. The W1/O-emulsion was transferred to 250 ml 1% PVA (W2), sonicated at 35% (15 w) for 5 min on an ice bath (Sonics Vibra cell VC750, 3 mm tapered micro tip), vortexed for 30s, and agitated for 4 h on a magnetic stirrer(MS-H-Pro$^+$, SCILOGEX, America) with 800 rpm/min at room temperature. A pre-centrifugation step at 500 × g for 10 min was used to remove any aggregates at room temperature and the PLGA nanoparticles were washed three times in ddH_2O at 5000, 15,000 and 18,000 × g for 10 min to remove PVA-residues from the suspension at room temperature (Eppendorf AG5415R, Hamburg, Germany). The PLGA nanoparticles were then freeze-dried for 24 h at 0.001 hPa and −110˚C (LGJ-12, Songyuan Huaxing Technology Develop Co., Ltd, Beijing, China) and stored at 4˚C until use.

2.5. Characterization of PLGA-ScpB Nanoparticles

PLGA-ScpB particles were viewed via scanning electron microscope (Quanta 200), with the loading and encapsulation efficiencies measured directly from the particles using a BCA assay. For antigen calculations, 30mg of PLGA-ScpB particles were dissolved in 3 ml 1 M NaOH (0.5% SDS; pH = 10) with shaking at room temperature for 24 h. After completely dissolving the particle suspension, the pH was adjusted to neutral (pH = 7) and proteins quantified using a BCA assay (Biocolors, Shanghai, China). Antigen loading and encapsulation efficiencies were calculated as the amount of antigen entrapped in the particles in relation to the amount of antigen that was added to the W1-phase during vaccine formulation.

2.6. Antigen Release/*In Vitro* Stability Study

Ten milligrams of PLGA-ScpB particles were suspended in 2 mL PBS (pH 7.4) and incubated at 37˚C on a rotator (HZ-9211K, Jiangsu, China). At sampling, a portion of the particle suspensions was collected and centrifuged at 20,000 × g for 5 min, with 1 mL supernatant collected and stored at −20˚C. The particle pellet was re-suspended in 1 mL PBS and transferred back to the original particle containers. Three independent samples were obtained at days 1, 2, 3, 4, 5, 6, 7, 12, 17, 22, 27, and 32 and released antigen was quantified using a BCA assay.

2.7. Fish

Healthy tilapia (*Oreochromis niloticus*, 25 ± 0.5 g) were purchased from a local fish farm (Panyu, Guangzhou, PR China) and acclimatized in the laboratory for 2 weeks. Fish were maintained at 28˚C ± 2˚C in aerated freshwater and fed daily with commercial dry pellets. Fish were anaesthetized with tricaine methanesulfonate (Sigma, USA) prior to experimentation involving injection, blood collection or sacrifice. Prior to experimentation, fish were randomly sampled for relevant bacteria, with no bacteria detected.

2.8. Vaccination and Bacterial Challenge

All vaccination experiments were performed in triplicate with a group size of 40. Throughout the time of vaccination and mortality observation, each group of fish was maintained separately in 12 tanks containing 500 L of

aerated freshwater that was maintained at 28˚C ± 2˚C For vaccination, PLGA-ScpB particles were diluted to 1 μg/g (ScpB weight /fish body weight; P1), 3 μg/g (P3) and 5 μg/g (P5). For each concentration, three groups of fish were injected intraperitoneally (i.p.) with 100 μL each of the appropriate concentration, with another three groups offish sham boosted with 100 μL PBS as a control (C). At the 28th day post-boost, the fish were challenged with GBS ZP-N that had been cultured in BHI medium (Huankai Co Ltd., Guangzhou, China) to logarithmic phase, washed, and resuspended in PBS.

When vaccinating with live bacteria, the cells were cultured in BHI medium to an OD_{600} of ~0.8 and resuspended in PBS to 8.4×10^7 CFU/ml. All groups of fish were injected i.p. with 100μL of the live bacteria, with mortality monitored for 20 days post-challenge and the relative percent of survival (RPS) calculated according to the following formula: RPS = (1 − vaccinated fish mortality/control fish mortality) × 100% [19]. All of the experiments were approved by the Institutional Animal Care and Use Committee at the research facility.

2.9. Sera Collection and Enzyme Activity Analysis

Blood was collected from the caudal veins every 7 days until the end of the experiment and allowed to clot overnight at 4˚C. The serum was then obtained by centrifugation at 3500 × g for 10 min and stored at −20˚C. Serum lysozyme activity was tested using alysozyme activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. Superoxide dismutase (SOD) activity was also assayed using a WST-1 assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing).

2.10. Enzyme-Linked Immunosorbent Assay (ELISA)

Sera were collected from vaccinated fish at various times post-vaccination and were diluted 20-fold in PBST (0.1% Tween-20 in PBS) containing 2% bovine serum albumin. Briefly, 96-well ELISA plates were coated with ScpB protein (2 μg/μL), with 100 μL of sample added per well, and incubated overnight at 4˚C. After wash three times with PBST, blocking buffer was added and the plates were incubated at 37˚C for 2 h. Diluted sera from vaccinated fish from groups C, P1, P3 and P5 were added in triplicate to the wells of the plates and incubated at 37˚C for 1 h. Wells were then washed with PBST and HRP conjugated rabbit anti-tilapia IgM antibodies (1:4000; Zoonbio Biotechnology Co. Ltd.) were added. The plates were then incubated and washed as described above. Samples were visualized using a TMB Kit (Bios, Beijing, PR China) and read at 450 nm with a microplate reader (Molecular Devices, Canada).

2.11. Statistical Analysis

All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., USA) and Excel 2003. Differences in antibody titers and nonspecific immunity were analyzed using a Student's t-test, with data displayed as a mean ± SD. Differences in groups of nonspecific immunity were determined using a one-way ANOVA. In all cases, $P < 0.01$ was deemed significant. The release curve was calculated using GraphPad Prism 5.0.

3. Results

3.1. Characterization of PLGA Nanoparticles

A scanning electron micrograph showed a spherical shape, smooth surface, lack of porosity and relatively uniform size of the microparticles containing ScpB (**Figure 1**). All particle formulations had an average size between 300 and 400 nm, with more than 90% of the particles in each batch being less than 500 nm. ScpB encapsulation in PLGA particles showed an efficiency of 48.76%, while antigen loading was found to be 25.5 μg antigen/mg PLGA in vaccine formulation.

Nanoparticle stability was assessed by quantifying antigen release from the particles over a period of 32 days, with high initial release noted in antigen loaded formulations within the first seven days of incubation (**Figure 2**). Release amounts during this period were 0.117 mg and reached 47.8% for PLGA-ScpB formulations. Ten days later, there was a moderate continuous cumulative release (**Table 1**), with a cumulative antigen release of 72.2% noted on the 32nd day.

3.2. Vaccine Efficacy

The mean percent mortality and RPS of PLGA nanoparticles vaccinated and non-vaccinated tilapia following *S.*

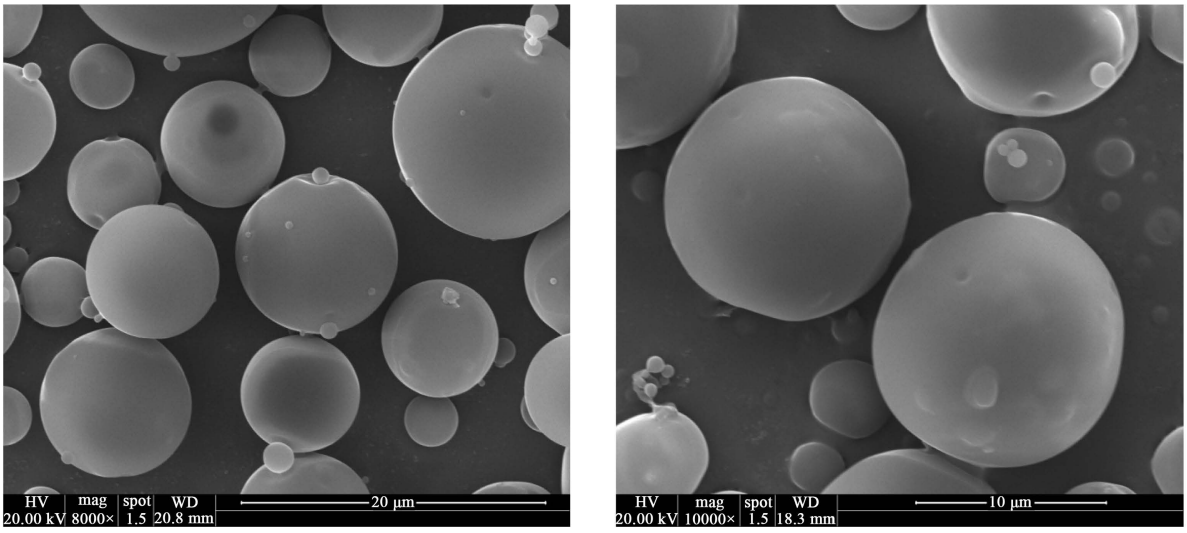

Figure 1. Scanning electron micrographs of PLGA microspheres incorporating ScpB.

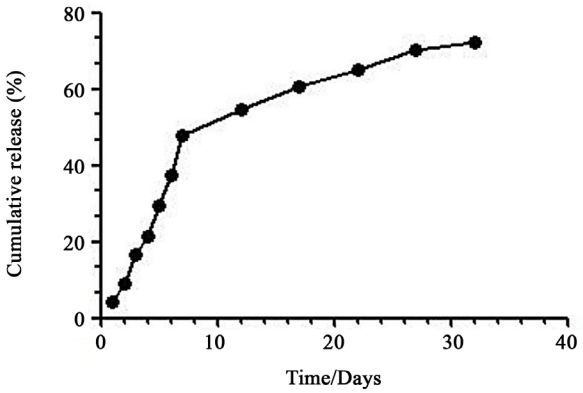

Figure 2. Cumulative release profiles of ScpB from PLGA microspheres.

Table 1. Quantification of antigen release from the particles over a period of 32 days.

Days	Cumulative release amount (mg)	Cumulative percentage (%)
1	0.011	4.3
2	0.023	8.9
3	0.043	16.7
4	0.054	21.3
5	0.075	29.6
6	0.095	37.4
7	0.117	47.8
12	0.126	54.5
17	0.147	60.7
22	0.166	65.1
27	0.181	70.4
32	0.184	72.2

agalactiae challenge are shown in **Table 2**. The first mortalities showed no classical symptoms and were observed on day 2 post-challenge, with *S. agalactiae* successfully isolated from the brain and liver. Three days later, most infected fish showed behavioral changes such as swimming in circles in lateral recumbence, with other clinical signs including opacity of the eyes (unilateral or bilateral) and exophthalmia. The control group (vaccinated with PBS) had the highest cumulative mortality (up to 55.96%). Group P1 (1 μg/g) had the lowest mortality (12.5%) and the highest RPS (87.66%), while the RPS of groups P3 (3 μg/g) and P5 (5 μg/g) reached 66.80% and 68.01%.

3.3. Lysozyme Activity

The lysozyme activities from groups P1, P3 and P5 were significant higher than that of control ($P < 0.01$; **Table 3**), with relatively higher levels noted 2 weeks post-vaccination followed by a steady decline in subsequent weeks through the fourth week. However, after challenge in the fourth week, the lysozyme activity of groups P1, P3 and P5 rapidly increased to peak in the fifth week. Of these groups, the highest activity levels were noted in group P1 (481.03 ± 0.26; $P < 0.01$) relative to the other vaccinated groups. In the ninth week, the activity levels for the vaccinated groups were still significantly higher than that of control groups ($P < 0.01$).

3.4. Superoxide Dismutase Activity

Prior to reaching nine weeks post-vaccination and GBS challenge, SOD activity was examined and found to be significantly higher in the P1, P3, and P5 groups relative to the control groups ($P < 0.01$; **Table 4**). In the ninth week, SOD activity was still significantly elevated in groups P1 and P3 relative to the control groups, but not in group P5. The overall trend of SOD activity showed an initial increase followed by a slow decline. In the fourth week post-challenge, the activity initially rose sharply and peaked in the sixth week, with the activity of group P1 being higher than the other groups.

3.5. Enzyme-Linked Immunosorbent Assay (ELISA)

After vaccination, the IgM titer levels of group P1, P3 and P5 were significantly elevated relative to the control groups (**Table 5**). In the third week post-vaccination, the IgM antibody levels for group P1 reached an OD of

Table 2. Immunoprotection of the ScpB vaccine against tilapia Streptococcicosis.

Group	Immunizing dose	Nos. of fish	Nos. of dead fish	Mortality (%)	RPS (%)
P1	1 μg/g	96	12	12.50	87.66
P3	3 μg/g	113	21	18.58	66.80
P5	5 μg/g	106	19	17.90	68.01
C	Control	109	61	55.96	—

Table 3. Kinetics of lysozyme activities in different vaccinated approaches.

Group weeks	C	P1	P3	P5
0	217.47 ± 0.95	217.47 ± 0.95	217.47 ± 0.95	217.47 ± 0.95
1	245.01 ± 0.77^{d}	337.23 ± 1.78^{b}	343.79 ± 1.25^{a}	325.30 ± 1.15^{c}
2	248.99 ± 0.35^{c}	458.53 ± 0.48^{a}	438.70 ± 1.75^{b}	434.14 ± 1.42^{b}
3	252.45 ± 0.48^{c}	361.34 ± 0.51^{a}	354.00 ± 1.71^{a}	326.51 ± 8.01^{b}
4	254.18 ± 4.29^{b}	319.82 ± 1.06^{a}	233.37 ± 0.81^{d}	247.96 ± 0.90^{c}
5	378.14 ± 1.76^{d}	481.03 ± 0.26^{a}	463.71 ± 1.34^{c}	470.54 ± 1.56^{b}
6	348.75 ± 1.20^{c}	477.30 ± 1.40^{a}	457.34 ± 1.93^{b}	462.95 ± 1.80^{b}
7	315.02 ± 1.27^{d}	462.16 ± 0.86^{a}	445.12 ± 1.60^{b}	452.31 ± 1.19^{c}
9	272.90 ± 1.06^{c}	443.27 ± 0.34^{a}	426.26 ± 1.35^{b}	430.78 ± 2.08^{b}

Notes: The different superscript show extremely significant difference in the same line ($P < 0.01$), and the same superscript demonstrate insignificant difference ($P > 0.05$).

Table 4. Kinetics of SOD activitieses in different vaccinated approaches.

Group weeks	C	P1	P3	P5
1	29.90 ± 0.11^{d}	61.38 ± 1.39^{a}	57.13 ± 0.20^{b}	54.86 ± 0.70^{c}
2	30.96 ± 0.31^{d}	71.83 ± 0.1^{a}	59.99 ± 0.04^{b}	58.68 ± 0.29^{c}
3	34.70 ± 0.27^{c}	74.82 ± 0.26^{a}	63.88 ± 0.14^{b}	63.38 ± 0.32^{b}
4	36.98 ± 0.09^{d}	64.68 ± 0.39^{a}	55.98 ± 0.41^{b}	54.86 ± 0.49^{c}
5	54.98 ± 0.15^{d}	83.62 ± 0.33^{a}	71.49 ± 0.00^{b}	68.96 ± 0.47^{c}
6	58.29 ± 0.70^{d}	98.16 ± 0.85^{a}	87.56 ± 0.49^{b}	81.60 ± 1.31^{c}
7	46.00 ± 0.33^{c}	63.92 ± 0.08^{a}	58.34 ± 0.31^{b}	59.04 ± 0.65^{b}
9	36.93 ± 0.06^{c}	45.86 ± 0.40^{a}	37.63 ± 0.99^{b}	35.21 ± 0.71^{c}

Notes: The different superscript show extremely significant difference in the same line ($P < 0.01$), and the same superscript demonstrate insignificant difference ($P > 0.05$).

Table 5. Kinetics of serum antibody titers in different vaccinated approaches.

Group weeks	C	P1	P3	P5
1	0.116 ± 0.004^{d}	0.429 ± 0.004^{a}	0.227 ± 0.005^{b}	0.207 ± 0.003^{c}
2	0.148 ± 0.111^{c}	0.630 ± 0.010^{a}	0.591 ± 0.003^{b}	0.602 ± 0.002^{b}
3	0.153 ± 0.002^{c}	1.011 ± 0.127^{a}	0.968 ± 0.009^{b}	0.977 ± 0.002^{b}
4	0.158 ± 0.003^{d}	0.993 ± 0.004^{a}	0.895 ± 0.002^{b}	0.912 ± 0.000^{c}
5	0.435 ± 0.005^{d}	1.844 ± 0.002^{a}	1.489 ± 0.002^{b}	1.447 ± 0.004^{c}
6	0.442 ± 0.001^{c}	1.753 ± 0.001^{a}	1.408 ± 0.002^{b}	1.402 ± 0.018^{b}
7	0.519 ± 0.001^{c}	1.629 ± 0.002^{a}	1.216 ± 0.058^{b}	1.255 ± 0.005^{b}
9	0.466 ± 0.005^{d}	1.395 ± 0.002^{a}	0.965 ± 0.000^{b}	0.932 ± 0.002^{c}

Notes: The different superscript show extremely significant difference in the same line ($P < 0.01$), and the same superscript demonstrate insignificant difference ($P > 0.05$).

1.011 ± 0.127, which was significant higher than the other groups ($P < 0.05$). In the fourth week, the IgM antibody OD values showed a small decline in all groups. However, in the fifth week post-challenge, the IgM antibody OD values sharply increased to reach 1.844 ± 0.002. In the next weeks, the values slowly declined, but groups P1, P3 and P5 remained elevated relative to the control groups and group P1 always had a significantly higher value than the other groups.

4. Discussion

ScpB is a surface associated serine protease that plays an important role in the virulence of *S. agalactia* [10]. It cleaves the chemotactic complement component C5a [20], binds to fibronectin and contributes to cellular invasion [21]. While genetic polymorphisms alter the functional activity of C5a peptidase [22], they do not affect its ability to bind fibronectin [23]. ScpB exhibits a conserved nucleotide sequence in several hemolytic streptococcal species, is ubiquitously expressed and is localized to the surface, thus making it a good vaccine candidate for *S. agalactiae* and *S. pyogenes* infections [21] [24].

In this study, the *scpB* gene was cloned from tilapia *S. agalactiae* isolates and successfully expressed *in vivo*. ScpB is likely to be membrane-associated or released into extracellular space and thus can serve as a protective immunogen. The obtained subcellular location prediction results were consistent with C5a peptidases located in other sources [12] [25].

Studies in mammals have indicated that when compared to other types of vaccines, subunit vaccines are less immunogenic, especially those that are highly purified, and mainly induce humoral immunity. Since *S. agalac-*

tiae is an intracellular pathogen that can survive in phagocytes by subverting bactericidal responses, subunit vaccines should in theory be ineffective gainst a *S. agalactiae* infection. However, we and other investigators [16] [26] [27] have in several studies observed that some subunit vaccines, when in the form of recombinant proteins purified from *E. coli* under native conditions and formulated with certain adjuvants, are highly protective against experimental *S. agalactiae* challenge, with RPS rates around 70% or higher achievable.

PLGA is a degradable polymer microsphere which has been approved by the Food and Drug Administration (FDA), studied extensively for the controlled delivery of therapeutic proteins [28] and has a high potential for gene or protein delivery. While these microspheres are not readily internalized by cells, they are retained in the tissue, thus providing prolonged DNA release [28]. Therefore, the released DNA can transfect the cells at the delivery site with the protein product acting locally or distributed systematically. In this study, recombinant ScpB protein was encapsulated in PLGA and shown to possess good physical performance and sustained release *in vitro*. The release amount during the first seven days of incubation was 0.117 mg and reach 47.8% for NP/ScpB formulations. Ten days later, a moderate continuous cumulative release was noted (**Table 1**), with a cumulative antigen release of 72.2% reached on the 32nd day. This kind of sustained release character for PLGA was also confirmed by other reports [29].

Moreover, ScpB formulated with PLGA afforded a RPS of more than 87% over a period of two months following vaccination. Previously, our laboratory showed that Freund's adjuvant had a strong boosting effect on the specific immunity induced by an *S. agalactiae* subunit vaccine. However, in order to achieve more than 87% protection when using Freund's adjuvant, 5 μg/g of recombinant ScpB was necessary. In the present study, only 1 μg/g of recombinant ScpB provided the same level of protection. These findings suggest that PLGA was more effective in providing an improved recombinant ScpB uptake and thus may serve as a more robust adjuvant.

In general, microspheres are too large to enter cells by endocytosis, so instead they can be preferentially taken up by phagocytic cells such as macrophages due to size exclusion [28]. This makes microspheres an ideal DNA carrier for vaccinations or the induction of a cytotoxic T cell response. Furthermore, some microspheres possess bioadhesive properties, making them suitable for mucosal immunizations or oral delivery.

The innate immune defense system is the first line of defense against invading pathogens [30], with lysozymes and SODs being an important part of this system. Lysozymes are catalytic enzymes able to hydrolyze 1, 4-beta-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid, which are contained within peptidogly can heteropolymers of gram positive bacterial cell walls, and with complement components pertaining to gram negative bacteria also possibly affected [31]-[33]. Lysozymes are abundant in the blood and a number of secretions such as tears, saliva, human milk and mucus. They are also present in cytoplasmic granules of macrophages and polymorphonuclear neutrophils (PMNs) [34]. Following the use of a PLGA encapsulated vaccine to inoculate kelp grouper (*Epinephelus bruneus*), the vaccinated fish sera lysozyme activity was up to 12,000 U/mL, which was significantly higher than levels noted in non-vaccinated or inactivated antigen vaccinated individuals [35]. In the present study, the sera lysozyme activities from the ScpB-PLGA vaccinated groups were significantly higher than those of control groups. These findings suggest that ScpB-PLGA vaccinations can increase lysozyme activity and thus antimicrobial activity, and can enhance the ability of tilapia to ward off pathogen infections.

SODs are enzymes that alternately catalyze the dismutation of superoxide (O^{2-}) radicals into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage [36] [37]. Thus, SODs are an important antioxidant defense in nearly all living cells exposed to oxygen and serve as one of the key antioxidant enzymes in fish [38]. While SODs can remove excess free radicals, a dynamic equilibrium may exist between antioxidant levels and the rate of free radical formation. One study found that the SOD activity was closely correlated to immune levels in the body [39] and that it plays an important role in enhancing defense capabilities and immune functions in phagocytic cells. In a study that vaccinate grouper (*Epinephelus bruneus*) with OND-PLGA, serum SOD activity was maintained a high level in 2 - 4 weeks and infections were held at bay [40]. In this study, tilapia vaccinated with ScpB-PLGA showed an increase in serum SOD activity, followed by a later decrease and stabilization at the 9th week.

Santillan *et al*. [24] immunized mice intramuscularly and intranasally with ScpB-PLGA, which induce specific serum IgG antibodies and high IgA levels were detected in the vaginal secretions. Furthermore, the high levels of antibodies could still be detected 40 weeks post-immunization and were significantly higher than the controls [2]. In this study, the vaccinated tilapia serum IgM levels remained relatively high even at 9 weeks

post-immunization, which may correlate to a slow-release as is characteristics of PLGA. The ScpB-PLGA vaccination provided a 87% survival rate, with a survival rate of only 9% achieved when using ScpB only.

5. Conclusion

In conclusion, the recombinant ScpB encapsulated in PLGA had good immunogenicity against *S. agalactiae*. These results suggest that PLGA is a more effective than Freund's adjuvant and enabled a decreased ScpB dosage for immunization.

Acknowledgements

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Conflict of Interest

There is no conflict of interest.

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