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GroEL, a novel vaccine candidate of piscine *Streptococcus agalactiae* identified by immunoproteome

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ABSTRACT

Streptococcus agalactiae is the major etiological agent of streptococcosis, which is responsible for huge economic losses in fishery, particularly in tilapia (*Oreochromis niloticus*) aquaculture. A research priority to control streptococcosis is to develop vaccines, so we sought to figure out the immunogenic proteins of *S. agalactiae* and screen the vaccine candidates for streptococcosis in the present study. Immunoproteomics, a technique involving two-dimensional gel electrophoresis (2-DE) followed by immunoblotting and mass spectrometry (MS), was employed to investigate the immunogenic proteins of *S. agalactiae* THN0901. Whole-cell soluble proteins were separated using 2-DE, and the immunogenic proteins were detected by western blotting using rabbit anti-*S. agalactiae* sera. A total of 17 immunoreactive spots on the soluble protein profile, corresponding to 15 different proteins, were identified by MALDI-TOF/TOF MS. Among the immunogenic proteins, GroEL attracted our attention as it was demonstrated to be immunogenic and protective against other streptococci. Nevertheless, to date, there have been no published reports on the immunogenicity and protective efficacy of GroEL against piscine *S. agalactiae*. Therefore, recombinant GroEL (rGroEL) was expressed in *Escherichia coli* BL21 (DE3) and purified by affinity chromatography. Immunization of tilapia with rGroEL resulted in an increase in antibody titers and conferred protection against *S. agalactiae*, with the relative percentage survival of 68.61 ± 7.39%. The immunoproteome in the present study narrows the scope of vaccine candidates, and the evaluation of GroEL immunogenicity and protective efficacy shows that GroEL forms an ideal candidate molecule in subunit vaccine against *S. agalactiae*.

1. Introduction

Streptococcus agalactiae is known as a major causative agent of streptococcosis in tilapia (*Oreochromis niloticus*), which is one of the leading farmed species in the world with commercial production capacity of 3.67 million tons in 2014 [1]. Due to the high stocking densities in tilapia farms, the streptococcosis outbroke spread rapidly in major cultivation areas worldwide in the recent years, including China [2,3], Brazil [4], Thailand [5], and Ghanaian [6]. The streptococcosis caused the mortality rates of over 95% in some tilapia farms [2], which resulted in significant economic loss in the tilapia aquaculture.

The development of a vaccine for *S. agalactiae* is essential to reduce economic losses in the aquaculture industry. Researches on vaccines have been focused on the formalin-killed *S. agalactiae* vaccine [7] and live attenuated *S. agalactiae* vaccine [8] during the last 10 years. With the discovery of rarer piscine *S. agalactiae* serotypes, such as serotype Ib, II, III and IX [3,9], the formalin-killed vaccine and attenuated vaccine can't provide comprehensive protection against all the piscine *S. agalactiae* serotypes. Hence, the focus has shifted toward recombinant vaccines with wider serotype coverage spectra. It has been shown that several immunogenic proteins such as fibrinogen-binding protein, α-enolase [10], cell wall surface anchor family protein [11], phosphoglycerate kinase [12], truncated surface immunogenic protein (tSip) [13], immunogenic secreted protein [14], and elongation factor Tu [15] are able to provide some degree of protection against challenge with piscine *S. agalactiae*, with relative protection rates from 35.3% to 90%. In addition, a previous study proposed that combination of the recombinant proteins could be more efficient than a single recombinant protein, which provides a new strategy for development of subunit vaccines against *S. agalactiae* infection [12]. Therefore, further studies are still needed to find novel and more efficient immunoprotective antigens, and then combine the recombinant proteins to develop new

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vaccines against multi-serotype piscine *S. agalactiae*.

The immunoproteomics, which combines of two-dimensional electrophoresis (2-DE), immunoblotting and mass spectrometric analysis, is an effective approach for identification of immunogenic proteins that potentially suited for discovering novel antigens [16]. This approach has been proved to be useful for high-throughput identification of immunogenic proteins and screening of candidates for vaccines and diagnostic markers in many species of streptococci, such as *Streptococcus parauberis* [17], *Streptococcus pneumoniae* [18] and *Streptococcus suis* [19].

Therefore, the immunoproteomics analysis of *S. agalactiae* THN0901 isolated from infected tilapia (*Oreochromis niloticus*) was obtained in the present study. Based on the immunoproteome, an immunogenic protein was expressed and tested whether it is efficacy protective against piscine *S. agalactiae* infection. The immunoproteome of piscine *S. agalactiae* narrows the scope of screening protective antigens, which have potential as novel vaccine candidates. In addition, understanding the protective efficacy of the immunogenic proteins provides guidance in the development of multi-component vaccines against piscine *S. agalactiae*.

2. Materials and methods

2.1. S. agalactiae strain

S. agalactiae strain THN0901 used in this study was originally isolated from infected tilapia during the outbreak of *S. agalactiae* in Hainan province of China [20]. *S. agalactiae* THN0901 was grown in Brain Heart Infusion (BHI) medium aerobically overnight at 28 °C, and the overnight cultured cells were collected and then diluted into 1:100. The bacterial were collected at early stationary (12 h) and washed by sterile phosphate-buffered saline (PBS) for 3 times.

2.2. Preparation of antisera against S. agalactiae

S. agalactiae THN0901 was grown in BHI medium for 12 h, and then the cultures were inactivated by 0.2% formaldehyde at 4 °C overnight. The bacteria were harvested and diluted with sterile PBS to 1×10^9 CFU/ml. Five milliliter bloods of a New Zealand rabbit were collected at the day before primary immunization for negative control. Then the rabbit was primary immunized with 1×10^8 CFU bacteria cells emulsified in complete Freund's adjuvant (Sigma, MO, USA) by subcutaneous injection. Two booster injections were followed using 1×10^8 CFU bacteria cells emulsified in incomplete Freund's adjuvant on the 14th and 28th day, respectively. Seven days after the last immunization, bloods of the rabbit were collected and the serum was prepared for western blotting.

2.3. Preparation of whole-cell soluble proteins

The preparation of cellular proteins was performed as previously described [20]. Briefly, the cultures were collected (12 h) by centrifugation and washed by sterile PBS for 3 times. Then the pellets were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS), and sonicated with the ultrasound on ice. The supernatant was collected and then 100% trichloroacetic acid was added to a final concentration of 10%. After keeping at 4 °C for 4 h, the pellets were collected and washed by ice-cold acetone. Finally, the pellets were dissolved in lysis buffer, and the concentration of the soluble proteins was determined by the Bradford method.

2.4. Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis was carried out according to a previously described study [21]. Briefly, 800 μg cellular proteins were diluted to a total volume of 350 μl with rehydration buffer, and then separated on 17 cm pH 4–7 NL ReadyStrips™ IPG Strips (Bio-Rad, CA, USA). First dimensional isoelectric focusing (IEF) was performed with a PROTEAN® IEF cell (Bio-Rad) using the program: 50 V active rehydration for 12 h; 150 V (rapid ramp), 150 Vh; 300 V (rapid ramp), 300 Vh; 500 V (rapid ramp), 500 Vh; 1000 V (rapid ramp), 1000 Vh; 5000 V (rapid ramp), 5000 Vh; 10,000 V (rapid ramp), 1 h; 10,000 V (line ramp), 55,000 Vh at 20 °C. After IEF, IPG strips were first soaked with equilibration buffer supplemented with 20 mg/ml DTT for 15 min, and then in another equilibration buffer supplemented with 25 mg/ml iodoacetamide for additional 15 min. Twin gels were loaded 17 cm IPG strips with soluble proteins, and the second dimensional electrophoresis was performed at 15 mA for 12 h, until the bromophenol blue front reached the edge of the gel. One of the twin gels was stained with Coomassie Brilliant Blue G-250, and then scanned by GSC-8000 (Bio-Rad) and analyzed by PDQuest™ v. 8.0 analysis software. The remaining gel was used for detection of immunogenic proteins.

2.5. Detection of immunogenic proteins

The remaining gel was transferred onto a nitrocellulose filter (Pall, MI, USA) in a gel sandwich using Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) at 200 mA for 3 h. The NC membrane was blocked with 5% skim milk in TBS overnight at 4 °C. The NC membrane subsequently incubated with primary antibodies (rabbit anti-*S. agalactiae* sera diluted at 1:500 in TBS) for 2 h, and then incubated with secondary antibodies (goat anti-rabbit IgG antibodies diluted at 1: 5000 in TBS) at room temperature for 1 h. The membrane was developed with DAB substrate (0.002% DAB, 0.03% H_2O_2), and the immunogenic spots were presented on the membrane.

2.6. Mass spectrometric analysis

Immunogenic spots corresponding to the Coomassie Brilliant Blue G-250 stained duplicate gel were excised. The gel pieces of spots were subjected to destaining with 50% methanol, and 50% acetonitrile (ACN), followed by desiccation under vacuum. The dried gel pieces were then rehydrated and incubated in 40 μl coverage solution (10% ACN, 50 mM NH_4HCO_3) containing 10 ng trypsin (Promega, Madison, USA) for 16 h at 37 °C. Next, the extracted peptides were dissolved in 1.5 μl of resolution solution (30% ACN and 0.1% trifluoroacetic acid (TFA)). The mass spectrometric analysis was performed on an ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, CA, USA), the UV laser was operated at a 200 Hz repetition rate with a wavelength of 355 nm and an accelerated voltage of 20 kV. Data were searched against the Mascot database, with error tolerances of 50 ppm and 50 ppm for MS and MS/MS, respectively. One missed cleavage was allowed; carbamidomethy and oxidation modifications were selected. The criteria for successfully identified proteins were one or more tryptic peptides match to the protein sequence and at least one peptide with $p < 0.05$.

2.7. Cloning and expression of recombinant GroEL (rGroEL)

Chromosomal DNA of *S. agalactiae* THN0901 was isolated using the Wizard Genomic DNA Purification Kit (Promega, WI, USA) according to the manufacturer's instructions. The nucleotide sequence of the *groEL* gene (1623 bp) was retrieved from GeneBank (GeneID: 1014885), and amplified with the forward primer 5′-GGAATTCATGGCAAAAGATATT AAATTTTC-3′ and reverse primer 5′-GGGTTCGAAGAAGCCACCCATC ATAGATGG-3′ with EcoRI and HindIII restriction sites. Then, the purified PCR product of *groEL* was linked into pMD-18T plasmid (TaKaRa, Dalian, China) and sequenced. After sequencing, the *groEL* gene fragment was cut by restriction enzymes and ligated into pET28a plasmid (Novagen, WI, USA), which was transformed into *Escherichia coli* (*E. coli*) BL21 (DE3). The transformats were selected on Luria-Bertani (LB) agar with 50 μg/ml kanamycin.

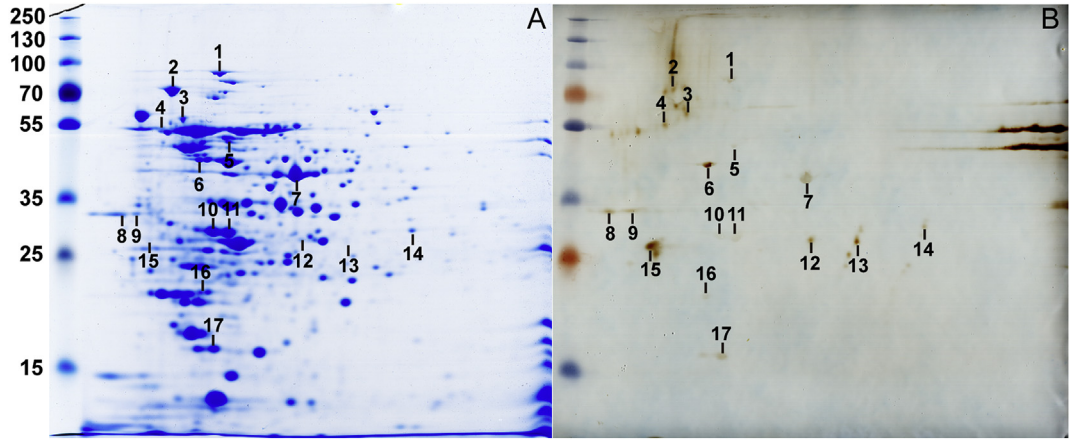

Fig. 1. Two-dimensional electrophoresis (2-DE) gel and Western blotting profile of whole-cell soluble proteins of *S. agalactiae* THN0901. (A) Whole-cell soluble proteins (800 μg) of *S. agalactiae* were separated by 2-DE, and visualized by Coomassie brilliant blue G-250. Protein spot numbers correspond to immunogenic proteins in Fig. 1B and were identified by MS. Molecular mass markers are indicated to the left of the gel. (B) Western blotting analysis of the whole-cell soluble proteins of *S. agalactiae* was performed using rabbit anti-*S. agalactiae* THN0901 serum. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.8. Protein synthesis and purification of recombinant GroEL

E. coli BL21 (DE3) were cultured in 200 ml of LB medium containing 50 μg/ml kanamycin. IPTG was then added to the culture to a final concentration of 1 mM during mid-log phase. After IPTG treatment at 16 °C for 10 h, *E. coli* cultures were collected and resuspended with phosphate buffered saline (PBS). The cultures were homogenized by ultrasonication, and the supernatant was collected and incubated with nickel-nitrilotriacetic acid (Ni-NTA) agarose. The mixture in the supernatant was washed with wash buffer (20 mM and 100 mM imidazole), and the rGroEL protein was eluted with 200 mM imidazole. The elution supernatant was concentrated and ultrafiltered through a 3 kDa ultrafiltration to remove imidazole. The concentration of purified rGroEL was determined by the Bradford Assay and then adjusted to 1 mg/ml using 0.1 M PBS. The purified rGroEL was emulsified with complete Freund's oil adjuvant or incomplete Freund's oil adjuvant at a ratio of 1:1.

2.9. Vaccination and challenge

Nile tilapia were purchased from Guangzhou Ashare Aquatech Co., Ltd (Guangdong, China), with weights of 55 ± 5 g. They were acclimated for two weeks (28 ± 0.5 °C) and divided into three groups (Ctrl, adjuvant and rGroEL). All tilapia received two subcutaneous doses (100 μl) on day 0 and 14. Each tilapia of the rGroEL group was intraperitoneally injected with a suspension, which contained 50 μg of rGroEL mixed with an equal volume of Freund's complete adjuvant. Tilapia in the adjuvant group was injected with 50 μl PBS emulsified with an equal volume of Freund's complete adjuvant. Tilapia of the ctrl group received 100 μl PBS, which was used as experimental ctrl for immunization. All groups received booster injections on day 14 with the same dose of the corresponding antigen, which were emulsified with Freund's incomplete adjuvant. Blood samples were collected via the caudal peduncular vein on day 14 and 28 after primary immunization, while under light anesthesia with eugenol. Then, each group was divided equally into three tanks, and each tank contained 14 fish. Each tilapia was challenged with 5×10^4 CFU *S. agalactiae* per fish. Tilapia were monitored daily for clinical signs of disease and mortality for 14 days post-challenge. The efficacy of the vaccine was calculated as relative percent survival (RPS) [22]. Approval was obtained from the Animal Ethics Committee of the school of life sciences of Sun Yat-sen University prior to using the animals for research.

2.10. Enzyme-linked immunosorbent assay (ELISA)

To determine the IgM antibody level against *S. agalactiae*, ELISAs were performed according to the instructions of the anti-tilapia (*O. niloticus*) IgM monoclonal antibodies (Aquatic Diagnostics, Stirling, UK). Ninety-six-well ELISA plates were coated with 100 μl/well of rGroEL (20 μg/ml) and incubated overnight at 4 °C. Plates were washed three times with low salt wash buffer (0.2 M Trisma base, 3.8 M NaCl, 2.5 mM Merthiolate, 0.5% Tween 20), and then blocked with coating buffer containing 1% (w/v) bovine serum albumin in coating buffer for 2 h at 25 °C. Following blocking, the plates were washed three times with low salt wash buffer. Two-fold dilutions of tilapia serum from non-vaccinated fish or vaccinated fish were prepared in PBS starting with a 1:2 dilution, which were added to each well and incubated for 3 h at 25 °C. A mouse anti-tilapia (*O. niloticus*) IgM monoclonal antibodies were diluted 1:33 in PBS, and then 100 μl of the dilution was added to each well for 3 h at 25 °C. The plates were washed five times with high salt wash buffer (0.2 M Trisma base, 5 M NaCl, 2.5 mM Merthiolate, 1% Tween 20), and 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution) was then added to each well and incubated at 25 °C for 1 h. After washing five times with high salt wash buffer, 100 μl of tetramethylbiphenyl substrate solution (Sangon Biotech, Shanghai, China) was added. Ten minutes later, the reactions were terminated by adding 50 μl of 2 M H_2SO_4, and the absorbance was read at 450 nm using a microplate reader (Bio-Rad).

2.11. Statistical analysis

Each group in the present experiment was performed in triplicate. All the experimental data were independent and normally distributed, and analyzed using ANOVA and Ducan's test by GraphPad Prism software version 7 (GraphPad, CA, USA). Data were expressed as means ± SEM, and $p < 0.05$ was considered to be significant.

3. Results

3.1. Soluble protein profile and immunoblotting

Two-dimensional electrophoresis gel of *S. agalactiae* whole-cell soluble proteins was stained by Coomassie brilliant blue (CBB) G250 and was shown in Fig. 1A. This gel was a representative example of the three independent repeats, approximately 400 soluble protein spots were visualized. A twin gel was used for electroblotting onto NC

Table 1
Immunogenic spots in the whole-cell soluble proteins profile of *S. agalactiae* THN0901.

Spot number[a]	Protein name	GI number[b]	Theoretical M_W/pI[c]	Score[d]	Function[e]
Carbohydrate metabolism					
3	Phosphoglucosamine mutase	gi|84029258	48681.7/4.64	255	Catalyzes the conversion of glucosamine-6-phosphate to glucosamine-1-phosphate
4	S-adenosylmethionine synthase	gi|29611809	43308.2/4.86	411	Catalyzes the formation of S-adenosylmethionine from methionine and ATP
6	Glyceraldehyde 3-phosphate dehydrogenase	gi|81744478	36097.4/5.17	165	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
7	2,5-diketo-D-gluconic acid reductase	gi|597809249	31294.9/5.22	212	Oxidoreductase activity
8, 9	Acetoin reductase	gi|597808608	26860.5/4.79	337	Acetoin catabolic process acetoin dehydrogenase activity
Translation					
1	Elongation factor G	gi|34395600	76579.5/4.79	413	Catalyzes the GTP-dependent ribosomal translocation step during translation elongation
5	Elongation factor Ts	gi|109828059	37593.2/4.76	276	Translation elongation factor activity; associates with the EF-Tu; GDP complex and induces the exchange of GDP to GTP
10, 11	30S ribosomal protein S2	gi|61215772	28596.9/5.22	343	Structural constituent of ribosome
15	50S ribosomal protein L3	gi|109893560	22412.1/10.13	267	Structural constituent of ribosome
Cellular process					
12	PcsB protein	gi|406649424	45412.2/9.12	130	A signal peptide; a peptidase C51 Domain
16	Superoxide dismutase [Mn/Fe]	gi|61246080	22607.2/4.73	136	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems
Nucleotide metabolism					
13	Exodeoxyribonuclease III	gi|76563474	31308.9/5.63	260	Bidirectionally degrades single-stranded DNA into large acid-insoluble oligonucleotides
14	GntR family transcriptional regulator	gi|597809021	23683.3/5.55	185	DNA binding; transcription factor activity, sequence-specific DNA binding
Folding, sorting and degradation					
2	GroEL protein	gi|119366272	57295.9/4.69	656	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions
Unknown function					
17	Conserved hypothetical protein	gi|76562877	14925.2/4.66	276	Unknown

[a] Refers to the proteins labeled in Fig. 1.
[b] Accession ID of each protein is the GenInfo number in the NCBI protein database.
[c] Molecular weights and pI values represent reported values for the full-length protein.
[d] Mascot score.
[e] As given in the SWissprot database for *S. agalactiae*.

membrane, and the membrane was probed with a rabbit anti-*S. agalactiae* THN0901 serum. The pattern of immunogenic proteins is shown on the membrane in Fig. 1B. This membrane is a representative of the three membranes from the immunoblotting repeats. Seventeen protein spots were detected by western blotting, and were selected for MS analysis. After destaining and in-gel trypsin digestion, a total of 17 spots representing 15 proteins were successfully identified by MALDI-TOF/TOF MS (Table 1). As expected, most of the proteins originated from the cytoplasm, and the proteins were assigned into 6 classes of cellular function, with more than half proteins being assigned to carbohydrate metabolism (33.33%) and translation (26.67%), followed by groups of proteins involved in cellular process (13.33%), nucleotide metabolism (13.33%), folding, sorting and degradation (6.67%), and unknown function (6.67%).

3.2. Cloning, expression, and purification of rGroEL of S. agalactiae

The gene *groEL* was amplified from the *S. agalactiae* THN0901 using specific primers, with an expected size of around 1600 bp. The PCR products were examined by agarose gel, and the position of the band was consistent with the expected size (Fig. 2A). After sequencing, the amplified sequence of *groEL* is 1623 bp in length and encodes 540 amino acids according to sequence analysis (Fig. S1). Recombinant protein GroEL was expressed in the *E. coli* BL21 (DE3), the purification profile (Fig. 2B) shows the rGroEL of the approximately 70 kDa fusion protein, which contains two histidine tags and a T7 tag from the expression vector pET28a. In addition, the rGroEL was predominantly expressed in the soluble fraction and purified by Ni^{2+}-NTA affinity chromatography under native conditions, which confirms the successful expression of rGroEL in the prokaryotic system.

3.3. Protective efficacy of rGroEL

To assess the protective efficacy of rGroEL, tilapia of three groups were respectively immunized intraperitoneally with PBS, adjuvant and rGroEL on day 0 and day 14, and then challenged intraperitoneally with 5×10^4 CFU *S. agalactiae* per tilapia on day 28. The death of tilapia broke out in the first 12–24 h without any clinical sign, and the tilapia succumbed infection and death on the second to fifth days, exhibiting typical clinical signs of disease including lethargy, ataxia and corneal opacity. After 14 days of observation, the rGroEL group showed a significantly higher survival ($P < 0.001$) rate than the ctrl and adjuvant groups (Fig. 3). The RPSs of the rGroEL and adjuvant groups, compared to the ctrl group, were 68.61 ± 7.39% and 16.39 ± 2.17% respectively.

3.4. Enzyme-linked immunosorbent assay (ELISA)

rGroEL was able to induce an immune response in tilapia. Fig. 4 shows the antibody response against rGroEL in immunized tilapia, which was determined by ELISA. Four weeks after the primary immunization, the specific antibody titer of tilapia immunized with rGroEL was significantly higher ($P < 0.001$) than that of ctrl and adjuvant groups, while the specific antibody titer of rGroEL group didn't show significant change at two weeks after the primary immunization.

4. Discussions

Immunoproteomics, a combination of 2-DE, immunoblotting and MALDI-TOF/TOF MS analysis, is an effective approach for identification of immunoreactive proteins potentially suited for screening of vaccine candidates. The most straightforward way to find immunogenic

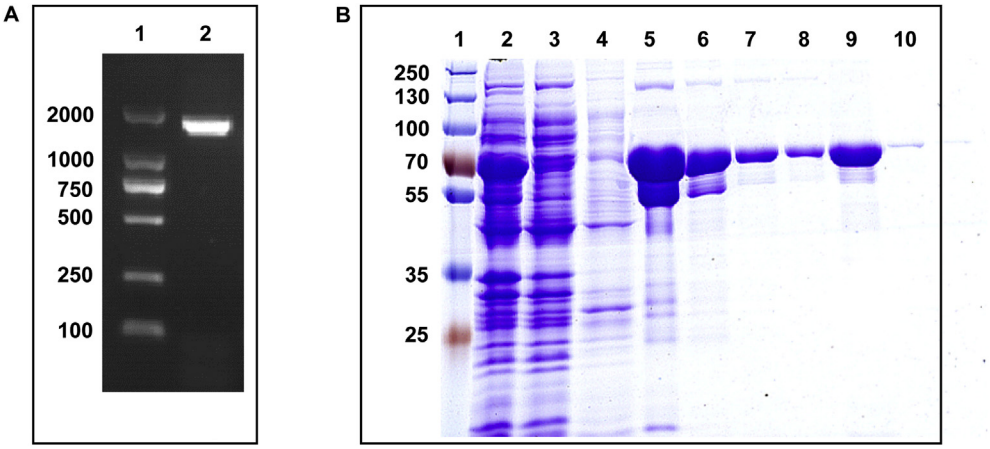

Fig. 2. Cloning, expression and purification of GroEL of *S. agalactiae*. (A) The PCR products of *groEL* were examined on agarose gel. Lane 1, DNA ladder marker; 2, PCR products of *groEL*. (B) Expression and purification of recombinant GroEL protein in *E. coli* BL21 (DE3). Purification was carried out using concentration of imidazole (20 mM, 100 mM and 200 mM). Lane 1, MW marker; 2, induced; 3, uninduced; 4, wash with PBS; 5 and 6, wash with 20 mM imidazole; 7 and 8, wash with 100 mM imidazole; 9 and 10, elution with 200 mM imidazole.

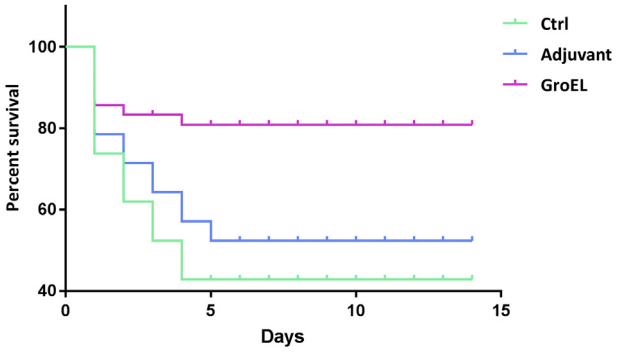

Fig. 3. Survival rate of group crtl, adjuvant and GroEL after challenge. The morbidity and death of tilapia were recorded daily for 14 days post intraperitoneal challenge with *S. agalactiae* THN0901 (5×10^4 CFU/fish). Tilapia mortalities occurred in large quantities in first 5 days, and the relative percent survival of the rGroEL group and adjuvant group, compared to the ctrl group, were 68.61 ± 7.39% and 16.39 ± 2.17%, respectively.

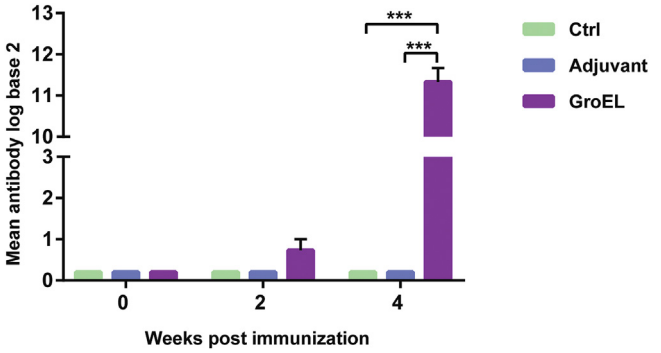

Fig. 4. Specific antibody titers post immunization with rGroEL. After two weeks of the first immunization, there was no difference in antibody titers between the three groups. While the antibody titers increased significantly in the group GroEL two weeks post the second immunization. *** represents significant difference ($P < 0.001$) between the antibody titers of group GroEL and other groups.

proteins is to perform immunoblotting with tilapia anti-*S. agalactiae* serum. However, the purchased secondary antibodies (anti-tilapia (*O. niloticus*) IgM monoclonal antibodies) titer is not enough to be used for Western blotting, and can only be used for ELISA. Therefore, we prepared rabbit anti-*S. agalactiae* serum instead of tilapia anti-*S. agalactiae* serum to perform the western blotting, like some previous studies [23,24]. A total of 17 immunoreactive spots, corresponding to 15 different proteins, were identified by immunoblotting in the present study. Two spots (spot # 8 and spot # 9, spot # 10 and spot # 11) were identified to be different fragments of one single protein, maybe because they have various charges, which has been observed in immunoproteomic analysis of other pathogens [19].

The majority of these proteins were cytoplasmic protein linked to housekeeping function such as energy metabolism, including phosphoglucosamine mutase (spot # 3), S-adenosylmethionine synthase (spot # 4), glyceraldehyde 3-phosphate dehydrogenase (spot # 6), 2,5-diketo-D-gluconic acid reductase (spot # 7) and acetoin reductase (spots # 8–9). Among them, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-biphosphoglycerate. GAPDH was also found in the outer surface proteins of *S. agalactiae* [25], which contributed to adherence in pathogenic *S. agalactiae*. In addition, a previous study demonstrated that the purified recombinant GAPDH could elicit a significant humoral antibody response in mice and conferred significant protection against challenge with a lethal dose of *Streptococcus zooepidemicus* [26]. As for the remaining four proteins, there are no reports on their immunogenicity, which may become new target antigens.

Elongation factor G (EF-G, spot # 1) and elongation factor Ts (EF-Ts, spot # 5) belong to translation elongation factors. EF-G is responsible for the translocation of the peptidyl-tRNA from the A-site to the P-site (peptidyl-tRNA site) of the ribosome. EF-Ts is a nucleotide exchange factor that is required to regenerate EF-Tu from its inactive form (EF-Tu-GDP) to its active form (EF-Tu-GTP) (https://www.ebi.ac.uk/interpro/entry/IPR001816?q=elongationfactor). EF-Ts is also found to be a surface-associated protein in other streptococci, such as *Streptococcus oralis* [27], *S. pyogenes* [28], and *S. suis* [29]. In addition, Martinez [30] reported that EF-Ts expressed by the reference strains of all *S. suis* serotypes are antigenically similar. In addition, EF-G has been also described as immunogenic protein in *S. suis*, *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis* [31].

Another immunogenic protein PcsB (Spot # 12) was found in the surfome and secretome in our previous study [14]. PcsB is required for the ordered cell separation of *S. agalactiae*, and insertional inactivation of the *pcsB* gene exhibited a significant influence on cell septum formation [32]. In addition, PcsB of *S. agalactiae* was shown to be immunoreactive with sera from infected human [25]. Moreover, PcsB of *S. pneumoniae* was found to be exceptionally conserved among clinical isolates and cross-protective against four different serotypes in lethal sepsis and pneumonia models [33]. Spot 16 matched the superoxide dismutase. Superoxide dismutase has been identified as a virulence factor located in the periplasm of *S. agalactiae* [34] and *S. pneumonia* [35]. It has also provided protection in mice against infection with *Brucella abortus* [36]. Two ribosomal proteins were also identified in the present proteome, including 30S ribosomal protein S2 (spots # 10–11) and 50S ribosomal protein L3 (spots # 15). Ribosomal proteins were

present in most immunoproteome of bacteria, which may due to the large amounts of ribosomal proteins. In addition, some ribosomal proteins have been reported to catalyze posttranslational modifications involving acetylation and methylation of amino acids [37].

Among the immunogenic proteins, GroEL (spots # 2) attracted our attention. GroEL is one of the heat shock protein (Hsp) family members, which are ubiquitous and highly conserved molecular chaperons expressed in both eukaryotic and prokaryotic organisms. GroEL is an essential chaperone present in diverse bacteria that forms nanocages and provide central compartment to prevent aggregation of unfolded proteins [38]. GroEL is also characterized as cell surface protein in gram-positive bacteria that overexpresses during biofilm formation, indicating its potential role in interaction with the immune system of host [39]. GroEL may represent as a promising target for the prevention and treatment of piscine *S. agalactiae* infections. In addition, GroEL of *Riemerella anatipestifer* [40], *Streptococcus pyogenes* [41] and *Streptococcus* equi ssp. *Zooepidemicus* [42] have been shown to be highly immunogenic, which can elicit high antibody titers, promote lymphocyte proliferation. Furthermore, GroELs of *Streptococcus pneumonia* [43], *Bacillus anthracis* [44] and *Salmonella enterica serovar* Typhi [45] provide varying degrees of protection against infection in mice models. The best effect is that immunization with GroEL conferred 100% protection to mice against *B. anthracis* infection [44]. Thus, it could be speculated that GroEL would be a promising candidate antigen for the development of a universal vaccine against *S. agalactiae*.

To date, there has been no published report on the immunogenicity and protective efficacy of GroEL against piscine *S. agalactiae* infection. In the present study, GroEL was demonstrated to be immunogenic by the immunoproteome. However, being immunogenic does not necessarily mean that an antigen is capable of producing a protective immune response, with as little as 2.5% of antigens displaying protective properties [46]. It is necessary for us to demonstrate the protective efficacy of the immunogenic antigens. Therefore, rGroEL of strain *S. agalactiae* THN0901 was successfully expressed, and Coomassie-blue stained gels indicated that rGroEL was strongly expressed in the soluble form. A previous study indicated that there is a close relationship between the antigen dosage and protective effect of the vaccine for both intraperitoneal injection and cohabitation challenge models [47], and suitable antigen dosage can maximize the protection efficacy [48]. The suitable antigen dosages range from 0.50 to 1.50 μg/g fish [49–51], so antigen dosage was chosen to be 0.90 μg/g fish in the present study.

Previous studies have indicated that specific antibodies play a highly significant role in protection against *S. agalactiae* [52]. The results of ELISA suggest that a booster injection with rGroEL is needed to achieve the best protective effect, because the specific antibody titer of tilapia immunized with rGroEL was significantly higher ($P < 0.001$) than that of ctrl and adjuvant groups at 2 weeks after a booster injection. However, the specific antibody titer of serum in the GroEL group didn't show significant change at 2 weeks after the primary immunization.

In the present study, whole-cell soluble proteins of *S. agalactiae* THN0901 were separated using 2-DE, and the immunogenic proteins were detected by western blotting. A total of 17 immunoreactive spots on the soluble protein profile, corresponding to 15 different immunogenic proteins, were identified by MALDI-TOF/TOF MS. Based on the immunoproteome, rGroEL was expressed in *Escherichia coli* BL21 (DE3) and purified by affinity chromatography. Immunization of tilapia with rGroEL resulted in an increase in antibody titer and conferred protection against *S. agalactiae*, with the relative percentage survival of 68.61 ± 7.39%. Therefore, the immunoproteome in the present study narrows the scope of vaccine candidates. In addition, the evaluation of rGroEL immunogenicity and protective efficacy shows that GroEL forms an ideal candidate molecule in subunit vaccine. Our future studies will shed light on the protective efficacy of more immunogenic proteins against *S. agalactiae* challenge in tilapia models, which will accelerate the vaccine development against multi-serotype piscine *S. agalactiae*.

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Appendix A. Supplementary data

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