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Application of immunoproteomics in developing a *Streptococcus iniae* vaccine for olive flounder (*Paralichthys olivaceus*)☆

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Abstract

Streptococcus iniae is the major etiological agent of streptococcosis, which is responsible for hemorrhagic septicemia in fish, particularly olive flounder (*Paralichthys olivaceus*). In the present study, we sought to understand the pathogenicity and immunogenicity of *S. iniae* in order to develop a vaccine for streptococcosis. Immunoproteomics, a technique involving two-dimensional gel electrophoresis (2-DE) followed by immunoblotting, was employed to investigate the pathogenicity and immunogenicity of two *S. iniae* isolates, Jeju-13 and Jeju-45, in olive flounder. The virulence of Jeju-13 was moderate whereas that of Jeju-45 was high. A vaccination trial with formalin-killed Jeju-45 demonstrated relatively low protection against the homologous isolate compared with the heterologous isolate. A significant difference in the secretion of extracellular products (ECPs) was noticed between the two *S. iniae* isolates. ECP antigens were highly immunogenic compared to those from whole cell lysates as determined by 2-DE immunoblot assay of Jeju-13 and Jeju-45 anti-sera collected from post-challenge survival fish. Furthermore, there were differences in the appearance of antigenic spots on 2-DE immunoblot profiles of ECPs of the respective sera. Interestingly, the mixture of killed-cells and concentrated ECPs from Jeju-45 led to significant protection against the homologous isolate of *S. iniae* in olive flounder. The present study demonstrates the usefulness of immunoproteomics in understanding the pathogenicity of *S. iniae* to aid the development of a vaccine for fish streptococcosis.

Keywords: *Streptococcus iniae*; Immunoproteome; Vaccine; Two-dimensional gel electrophoresis (2-DE); Immunoblotting; Extracellular products (ECPs)

1. Introduction

The gram-positive fish pathogen *Streptococcus iniae* is a causative agent of fish hemorrhagic septicemia accompanied with meningoencephalitis, exophthalmia and corneal opacity [1]. Since its first isolation from subcutaneous abscesses of freshwater dolphin [2], outbreaks due to the bacterium have been reported in several commercial fish species, such as olive flounder [3,4], yellowtail [5], tilapia [6], hybrid striped bass [6], rainbow trout [7] and barramundi [8]. The bacterium has also been isolated from a soft tissue infection of an old man engaged in handling fish and hence may be considered an emerging zoonotic agent [9–11]. *S. iniae* is known as a major causative agent of streptococcosis in olive flounder (*P. olivaceus*) [3,4], which is a major mariculture species in Korea (record production of 32,141 metric tonnes during the year 2004; Statistical Year Book of Maritime Affairs & Fisheries, 2005). In recent years the flounder farming industry has been adversely affected by streptococcosis outbreaks and this in having a significant impact on the economy of fish farmers in Korea [3,4].

The development of a vaccine for *S. iniae* is essential to reduce economic losses in the aquaculture industry and to protect people involved in fish and fisheries activities [6–8]. Only a few studies have sought to develop a vaccine for *S. iniae*, and

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their results highlight the problems associated with antigenic variation depending on strains and/or isolates [12–14]. Vaccines generated from formalin-killed *S. iniae* give significant protection against the homologous strain but not the heterologous strain of *S. iniae*. This led to the classification of the *S. iniae* strains into serotype I and II based on the polysaccharide components of the capsule envelope [7,12,13,15–17]. By comparison, vaccines generated from killed cells and concentrated extracellular products (ECPs) from a specific *S. iniae* isolate gave a high level of protection in tilapia against the heterologous compared to the homologous isolate [14].

Group A streptococci (GAS) have a variety of virulence factors, including surface structure proteins, capsular polysaccharide components, ECPs, and factors associated with invading, adhering, and escaping from defense mechanisms to establish within the host environment [18,19]. In *S. iniae*, the capsule functions to resist opsonophagocytosis in phagocytic cells and is involved in serum killing of the host [16]. The capsule also inhibits the humoral immune reaction of the host by masking the antigenic protein located on the cell surface [17]. ECPs from GAS contain a variety of exotoxins, including streptolysin O, streptolysin S, 4 types of DNases, hyaluronidase, streptokinase, streptococcal pyrogenic exotoxins and C5a peptidase [18,19]. The secretion of ECPs is controlled by several factors including the growth phase, nutrient level, pH, and temperature [20–22] and plays a role in antiphagocytosis, invasion and host systemic toxicity [18–22]. In addition, streptococcal ECPs include a high level of cell cytosolic and wall proteins associated with virulence and immunity to the host, in particular glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-biphosphate aldolase (FBA) and enolase [20–22].

Despite advances ion the technologies available to study proteins, such as multidimensional protein identification, stable isotope labeling, and protein or antibody arrays, two-dimensional gel electrophoresis is the only technique that can be routinely used for quantitative expression profiling of complex mixtures of proteins according to p*I*, MW, solubility, and relative abundance [23]. For this reason, 2-DE has been used to study the mechanisms involved in pathogenicity [24,25], antibiotic resistance [26], environmental adaptation [21,22] and responses to host immune systems [20,27–33]. Immunoproteomics, a technique involving 2-DE followed by immunoblotting, has been widely used for studying the relationship between hosts and pathogenic bacteria, such as *S. pyogenes* [20,27], *S. pneumoniae* [28], *Staphylococcus aureous* [29], *Francisella tularensis* [30], *Helicobacter pylori* [31], *Borrelia burgdorferi* [32] and *Mycobacterium tuberculosis* [33].

The aim of the present study was to develop a vaccine against *S. iniae* in olive flounder employing immunoproteomics. This approach was used to investigate the low level of protectivity of whole cell bacteria by exploring the role of ECPs in pathogenicity and to compare the antigenicity of whole cell and ECPs of two virulent *S. iniae* isolates, Jeju-13 and Jeju-45. The efficacy of ECPs in protecting fish from infection was also investigated by developing a trial vaccine generated from a combination of ECPs and whole cells.

2. Materials and methods

2.1. Bacteria and vaccine preparation

Jeju-13 and Jeju-45 isolates were identified as *S. iniae* in a previous study [4]. The isolates were stored at −70 °C in tryptic soy broth (TSB) containing 10% glycerol until use. For amplification, the stored isolates were used to inoculate Todd–Hewitt broth (THB), incubated at 30 °C for 12 h, and then incubated at 30 °C for 24 h on blood agar. A single colony was subcultured to an $OD_{610\,nm}$ of 1.0 (4×10^8 CFU ml^{-1}) in THB for use in the challenge test and 2-DE. Jeju-45 cultured in THB was used for immunization studies in olive flounder. The cultured bacterium was centrifuged at 2000 × *g* for 30 min and the resulting pellet was treated with neutral buffered formalin at 10% for 12 h at 4 °C. The killed bacteria were washed three times and resuspended in PBS to an $OD_{610\,nm}$ of 1.0 (about 10^8 CFU ml^{-1}). Confirmation of bacterial death was confirmed by incubating a culture for 48 h at 30 °C on TSA.

2.2. Vaccination and challenge experiment

Healthy olive flounder with the average body weight of 150 g and an average length of 15 cm were obtained from a commercial fish farm in the Namhae County on the southern coast of Korea. Fish were divided into 8 groups of 15 each and were maintained in 200 l FRP tanks at 26–27 °C. The fish in 4 groups were anesthetized with AQUI-S® (5% Isoeugenol, New Zealand, 0.3 ml l^{-1}) and immunized intraperitoneally (IP) with 0.1 ml of formalin-killed Jeju-45 suspension (approximately 10^8 CFU ml^{-1}). At day 7 post-immunization, all fish from immunized and non-immunized groups were IP challenged with 0.1 ml of broth from the homologous (Jeju-45) and heterologous (Jeju-13) isolates containing approximately 10^8 CFU ml^{-1}. All fish were maintained under identical conditions and observed for clinical signs and cumulative mortality for 30 days following the challenge. Bacteria were isolated from dead fish and examined by hemolysis on blood agar, gram staining, catalase, oxidase and motility tests.

2.3. Preparation of whole cell lysates from the Jeju-45 isolate for 2-DE

Jeju-45 cultured in THB was harvested by centrifugation at 2000 × *g* for 30 min at 4 °C. The pellet was washed three times with phosphate buffered saline (PBS; 3 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4) then resuspended in 5 ml of PBS. The bacterial suspensions were transferred to new tubes (1 ml each) and centrifuged at 2000 × *g* for 10 min at 4 °C, and the resulting pellets were stored at −20 °C until use. Samples for 2-DE were prepared using chemical followed by mechanical extraction processes to obtain a high yield and optimum solubility of whole cell proteins [25]. For the chemical extraction process, the bacterial pellets were resuspended in 100 μl of lysis buffer-A (12 mM Tris, 5% glycerol, 0.4% SDS, and 200 mM dithiothreitol (DTT)). For mechanical extraction, the bacterial mixture was then placed on an ice

slurry and sonicated eight times (XL-2020, Misonix Inc. Farmingdale, NY, USA) at 5.5 W for 30 s. Bacterial cells were then disrupted by boiling the sonicated mixture for 10 min, cooling on ice, then lysing cells in 2-DE lysis buffer-B consisting of 2 M thiourea, 7 M urea, 40 mM Tris, 1% (w/v) DTT, 4% (w/v) 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) and 0.5% (v/v) IPG-buffer pH 4–7. Protein concentrations were estimated by the Bradford method [34] using bovine serum albumin (BSA) as the standard. The lysates were incubated on ice for 30 min, pelleted by centrifugation at 16,000 × *g* for 30 min at 4 °C, then stored at −70 °C until use for 2-DE.

2.4. Extracellular products from S. iniae isolates

The cellophane plate technique described by Liu [35] was used with slight modifications to obtain ECPs from the *S. iniae* isolates. Briefly, isolates were cultured in THB at 30 °C for 17 h without shaking. A 0.5 ml aliquot of cultured suspension was then spread with a sterilized scraper on TSA plates (700 ml) overlaid with a cellophane sheet sterilized by EO gas. Plates were then incubated for 48 h or 60 h at 30 °C. Bacteria and ECPs were harvested by washing the cellophane sheet with 50 ml of PBS then centrifuged at 2000 × *g* for 30 min at 4 °C to separate pellets and supernatant. The supernatants were filtered with a 0.45 μm disposable filter then concentrated with a Mini-Plus concentrator (amicorn). Protein concentrations were estimated by the Bradford method using bovine serum albumin as the standard. The concentrated supernatants were diluted with sterile PBS for immunization of olive flounder and cold ethanol precipitated for 2-DE. Prepared samples of ECPs were stored at −70 °C until use.

2.5. Two-dimensional gel electrophoresis and immunoblot assays

2.5.1. Isoelectric focusing (IEF) and SDS-PAGE

IEF was performed using the IPGphor^{TM} system (Amersham Bioscience, Uppsala, Sweden) with IPG strips (Immobiline DryStrip^{TM}, pH 4–7, 13 cm; Amersham Bioscience), according to the previously reported method [36]. The protein loading volume for whole cell lysates and ECPs was adjusted to 0.6 mg ml^{-1} and 0.2 mg ml^{-1} with rehydration buffer (9 M urea, 2% CHAPS, 0.4% DTT, 0.5% IPG-buffer, and 0.002% bromophenol blue), respectively. Prepared samples were loaded onto the IPG strips and focused at 86.1 kV h at 20 °C using an automated system as follows: rehydration for 14 h (7 h at 30 V followed by 7 h at 60 V), and focusing for 17 h (2 h at 200 V, 1 h at 500 V, 1 h 1000 V, 1 h at 2000 V, 2 h at 4000 V and 10 h at 8000 V). After IEF, the IPG strips were equilibrated with 10 mg ml^{-1} of DTT in equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 0.002% bromophenol blue, and 50 mM Tris–HCl, pH 8.8) for 15 min, and then with 40 mg ml^{-1} of iodoacetamide in the same buffer for another 15 min. Equilibrated IPG strips were placed onto 12.5% SDS-polyacrylamide gels (18 cm × 16 cm × 0.1 cm), sealed with 0.5%, w/v agarose, then electrophoresed at 10 mA gel^{-1} for 15 min followed by application of 20 mA gel^{-1} until the dye reached the bottom of the gel.

2.5.2. Protein visualization and image analysis

Electrophoresed gels were silver stained as previously described [36]. Briefly, each 2-DE gel was fixed with solution A (50% methanol, 12% acetic acid, and 0.05% (v/v) of 37% formaldehyde) for 1 h, washed twice with 50% ethanol then sensitized with sensitizing solution (0.01% sodium thiosulfate) for 1 min. The sensitized gels were rinsed with three changes of double distilled water (dDW) and then incubated with solution B (0.1% silver nitrate and 0.1% of 37% formaldehyde) for 30 min. Gels were then washed twice with dDW for 30 s then treated with developing solution (12% sodium carbonate and 0.05% of 37% formaldehyde) until the desired level of staining was obtained. The developing solution was then removed and the reaction was stopped by the addition of solution A without formaldehyde. The stained gels were stored in 50% methanol at 4 °C until analysis. Images of the gels were digitalized with an Agfa Arcus 1200^{TM} image scanner (Agfa-Gevaert, Mortsel, Belgium) then analyzed using Phoretix 2D software (Ver. 5.01, NonLinear Dynamics, Newcastle, UK).

2.5.3. 2-DE immunoblot assays

2-DE immunoblot assays of whole cell lysates and ECPs were performed by comparing antigens recognized by sera of surviving olive flounder exposed to approximately 10^5 CFU ml^{-1} of Jeju-13 or Jeju-45 as described by Kang et al. [37]. Briefly, 2-DE was performed as described above, and gels were either stained with silver nitrate for visualization of the protein spots, or transferred to a PVDF membrane (0.45 μm; Millipore, USA) for the detection of antigens to whole cell lysates and ECPs. The membranes were blocked with 5% skim milk in PBS-T for 1 h at room temperature (RT) and then washed three times with PBS-T (PBS including 0.05% (v/v) Tween-20). The membranes were incubated with anti-isolate Jeju-13 or Jeju-45 sera for 4 h at RT. The membranes were washed three times with PBS-T and then incubated separately with olive flounder IgM monoclonal antibody for 4 h at RT. Antigenic spots from whole cell lysates and ECPs were visualized by incubation with goat anti-mouse-HRP (Jackson, USA; 1:4000) for 1 h at RT. Membranes were then washed four times with PBS-T for 15 min, developed using an Enhanced Chemiluminescent (ECL) kit (Amersham Biosciences) then exposed to X-ray film for visualization of the antigenic proteins. Images of stained gels and immunoblotted membranes were digitalized with an Agfa Arcus 1200^{TM} image scanner (Agfa-Gevaert, Belgium) and the acquired images were analyzed using Phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK).

2.6. Vaccination with a combination of killed-cells and concentrated ECPs from Jeju-45

To understand the role of ECPs in protecting olive flounder to Jeju-45, the highly virulent isolate of *S. iniae*, fish were treated with three different antigen preparations from isolate Jeju-45, including formalin killed-cells (0.35 mg ml^{-1}), ECPs and a mixture of killed-cells and ECPs (0.35 mg ml^{-1}). Four groups of fish

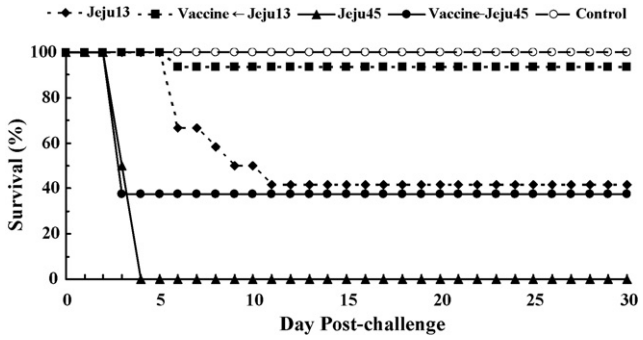

Fig. 1. Virulence of Jeju-13 and Jeju-45, and protectivity of formalin-killed Jeju-45 against Jeju-13 and Jeju-45 in olive flounder.

(15 each), including a control group, were immunized in a vaccine trial. After 1 week of immunization, 0.1 ml (10^8 CFU ml^{-1}) of the cultured broth from the Jeju-45 isolate was injected IP into the fish. The mortality of the fish was observed for 20 days post-challenge.

3. Results

3.1. Virulence of S. iniae isolates

The virulence of the *S. iniae* isolates, Jeju-13 and Jeju-45, was evaluated by challenging olive flounder for 30 days (Fig. 1). Infected fish exhibited dark pigmentation, abdominal extension, hemorrhagic ascites and necrosis of organs, particularly the liver. The control fish showed no clinical signs or mortality during the period. In contrast, initial mortality of fish injected with Jeju-45 appeared on day 3, and total mortality occurred by day 4 post-infection. By comparison, initial mortality of fish injected with Jeju-13 appeared on day 6, and there was only 50% mortality by day 30 post-infection. Fish immunized with formalin-killed Jeju-45 were challenged with Jeju-45 and Jeju-13 at 1 week post-immunization. In the group challenged with Jeju-13, only one fish had died by 6 days post-challenge, and there was no further mortality. There was acute mortality of fish challenged with Jeju-45, with approximately 60% of fish killed by 3–4 days post-challenge. Each group exhibited 89.3% and 37.5% relative percent survivors (RPS) from a challenge with Jeju-13 and Jeju-45, respectively.

3.2. Comparisons of 2-DE immunoblot profiles for whole cell lysates

The 2-DE immunoblot assay was used to investigate the recognition of Jeju-45 antigens by olive flounder sera raised against Jeju-13 and Jeju-45 (Fig. 2). Thirty and 55 antigenic spots were found with homologous and heterologous sera of Jeju-45, respectively, and most antigens were placed below pH 5.5 in 2-DE immunoblot profiles. In addition, antibodies from Jeju-13 appeared to have a lower affinity for antigens of Jeju-45 than for those of its homologous isolate. Eleven common antigenic spots were observed on both 2-DE immunoblot profiles, whereas 19 and 44 spots were found as specific antigens for Jeju-13 and Jeju-45, respectively.

3.3. Comparisons of 2-DE profiles for ECPs

ECPs from Jeju-13 and Jeju-45 were examined using the 2-DE immunoblot assay to determine if differences in virulence and humoral immunity between these *S. iniae* isolates can be explained by differences in ECP (Figs. 3 and 4). The ECPs collected from Jeju-13 after 48 h of culture showed approximately 20 spots clustered into the ranges of pH 4.4–5.2 and MW 10–25 kDa on the 2-DE profiles (Fig. 3A). By comparison, there were approximately 150 spots on the 2-DE profile for Jeju-13 ECPs collected after 60 h of culture (Fig. 3C). For Jeju-45, approximately 170 and 240 spots were observed on 2-DE profiles after 48 and 60 h of culture, respectively (Fig. 3B and D). Three similar patterns were identified on the 2-DE ECP profiles for Jeju-45 cultured 48 and 60 h and Jeju-13 cultured 60 h (Fig. 3B–D).

3.4. Comparisons of 2-DE immunoblot profiles fpr ECPs

The 2-DE profiles for Jeju-45 ECPs collected after 60 h culture revealed 25 and 29 antigenic spots with sera raised against

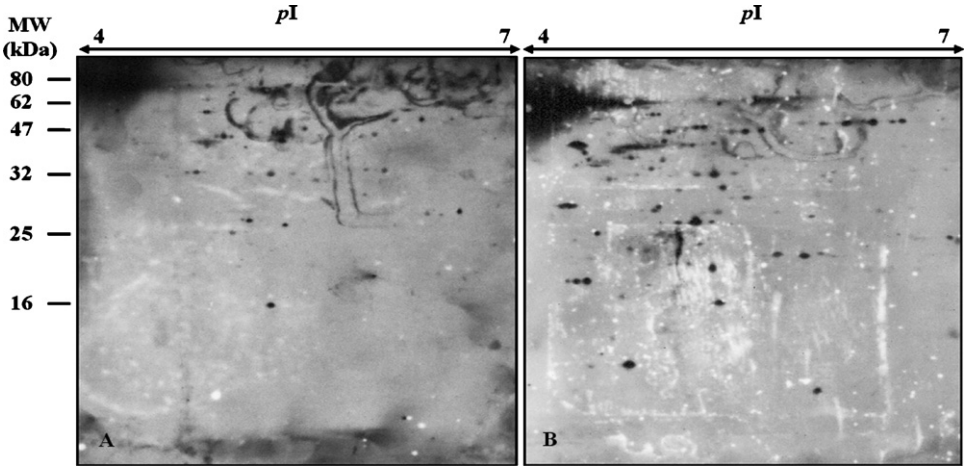

Fig. 2. Comparisons of 2-DE immunoblot profiles for whole cell lysate of Jeju-45 using heterologous (Jeju-13) (A) and homologous (Jeju-45) sera (B) raised from survival olive flounder infected with Jeju-13 and Jeju-45, respectively.

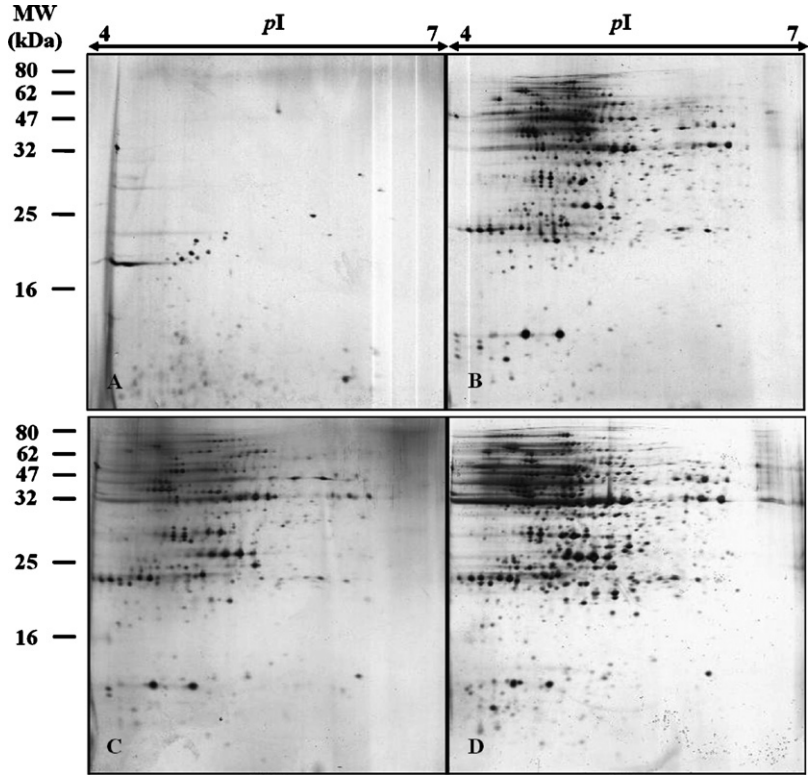

Fig. 3. Comparison of 2-DE profiles for ECPs from Jeju-13 (A and C) and Jeju-45 (B and D) that were cultured and collected for 48 h (A and B) and 60 h (C and D).

Jeju-13 and Jeju-45, respectively (Fig. 4A and B). For comparing both 2-DE immunoblot profiles, one (p*I* 5.2 and MW 25 kDa) of common antigenic spots was used as landmark. Although there were 16 common antigenic spots, the visible intensities of these antigenic spots differed between the 2-DE profiles. The major antigenic spots for Jeju-45 sera were identified as spots of (a), (b) and (d) groups at the ranges of p*I* 5.7–6.0 and MW 45 kDa, p*I* 4.5–4.7 and MW 42 kDa, and p*I* 6.0–6.5 and 37 kDa, respectively (Fig. 4B). For Jeju-13 the immunodominant antigenic spots were identified as spots of (c) and (e) groups at the ranges of p*I* 5.1–5.4 and MW 35 kDa and p*I* 5.0–5.2 and MW 27 kDa, respectively (Fig. 4A).

3.5. Vaccine efficacy of ECPs

ECPs as a possible immunogen were investigated by challenging with Jeju-45 (Fig. 5). By day 6 post-challenge, all fish not immunized or immunized with only ECPs had died. There were a few survivors among fish immunized with only formalin-killed Jeju-45 after challenging with its homologous isolate. By

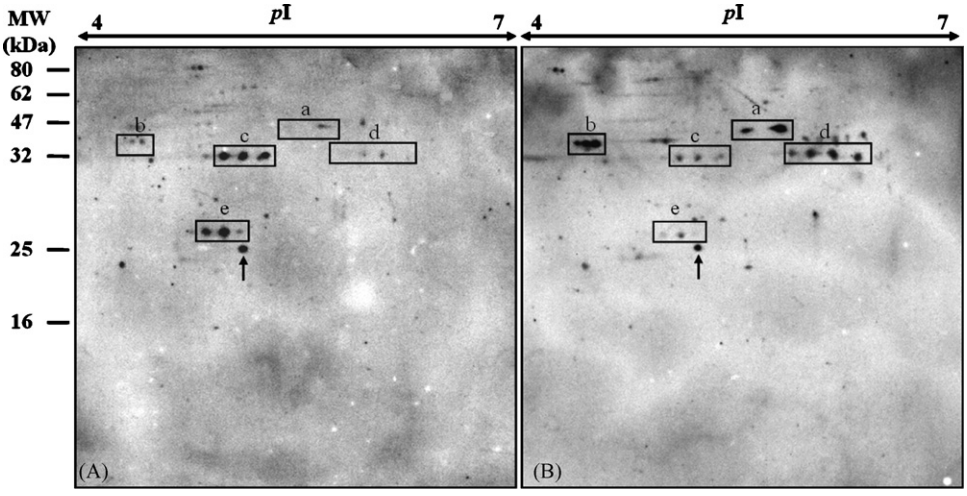

Fig. 4. Comparisons of 2-DE immunoblot profiles for Jeju-45 ECPs cultured for 60 h using heterologous (Jeju-13) (A) and homologous (Jeju-45) sera (B) collected from surviving olive flounder infected with Jeju-13 and Jeju-45, respectively. A spot possessed with p*I* 5.2 and MW 25 kDa (arrow) was used as landmark in image analysis for comparing both 2-DE immunoblot profiles.

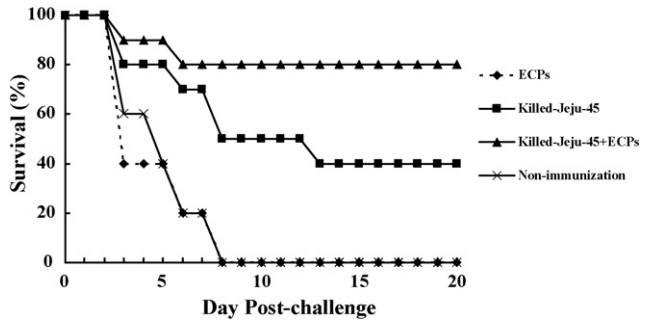

Fig. 5. Efficacy of a mixture of formalin-killed cells and ECPs from Jeju-45 compared with ECPs and killed Jeju-45 cells alone.

comparison, among the 10 fish immunized with a mixture of formalin-killed cells and ECPs from Jeju-45, one fish died at day 3 and another at day 6 post-challenge, but no further mortality was observed up to day 30 post-challenge. These results show that the vaccine mixture of formalin-killed cells and ECPs from Jeju-45 induced a high level of protection in olive flounder against infection by the homologous isolate.

4. Discussion

Large quantities of raw olive flounder are traditionally consumed in the form of Hae in Korea or Sashimi in Japan. This has forced the development of a variety of fish culturing methods including land-based culture along the Eastern and Southern coastal regions of the Korea peninsular and Jeju Island. Several epidemiological studies have reported outbreaks of streptococcosis that have led to severe economic losses among flounder farmers in Korea and Japan [3–5]. Our previous study showed that *S. iniae* was a major etiological agent for streptococcosis in olive flounder [4]. At present, there is no available vaccine against *S. iniae* infection. With the intention of developing a vaccine against *S. iniae*, the present study employed an immunoproteomics approach to understand the underlying pathogenicity and immunogenicity of *S. iniae* in olive flounder.

The virulence of the *S. iniae* isolates, Jeju-13 and Jeju-45, was determined by challenging olive flounder. Challenge with Jeju-45 resulted in higher mortality than Jeju-13 and caused total mortality by day 4 post-challenge. By comparison, Jeju-13 led to a cumulative mortality of approximately 60% by day 10 post-challenge. Fish streptococcosis was divided into acute and sub-acute based on the clinical signs. The acute form of the disease is associated with sudden mass mortality of farmed fish without clinical signs. The sub-acute form is always associated with various clinical signs, including the typical signs of exophthalmia, darkened coloration and erratic swimming, and leads to a daily mortality rate of less than 2% [40]. The results of the challenge test in olive flounder showed that Jeju-45 is highly virulent and causes acute infection, whereas Jeju-13 has lower virulence and causes sub-acute infection. The role of whole cell *S. iniae* in fish immunity was explored by challenging fish with Jeju-45 or Jeju-13, followed by immunization with formalin-killed Jeju-45 alone. Immunization with formalin-killed cells induced a higher level of protection in olive flounder against the heterologous isolate than the homologous isolate. This result demonstrates that whole cell Jeju-45 is not an effective immunogenic source and not suitable for the development of a vaccine against *S. iniae*.

S. iniae can be classified into two serotypes, serotype I (ADH positive) and II (ADH negative), based on serological, phenotypic and genetic studies. Both serotypes possess capsular structure on the cell surface, but differences are observed in resistance to phagocytosis by phagocytic cells, serum-mediated killing, and bacterial dissemination. Serotype II is known to induce apoptosis in macrophages, which leads to high mortality [7,12,15–17]. In previous studies, Jeju-13 and Jeju-45 did not exhibit a reaction to ADH using a API strep 20 kit [4], suggesting that both isolates are serotype II. There is little information on differences in antigenic profiles among serotype II isolates. The present study explored the antigenic variation between Jeju-13 and Jeju-45 using 2-DE immunoblot assays. Olive flounder sera raised against Jeju-45 and Jeju-13 reacted weakly with antigenic spots from whole cell lysates of Jeju-45. Moreover, sera from Jeju-13 exhibited lower intensity antigenicity and fewer antigenic spots for Jeju-45 compared with sera from Jeju-45. As a result, whole cell from both isolates of serotype II appeared to possess weak and different immunogenicity. It became clear from these results that other immunogens were required to protect against the pathogen after vaccination, and further studies focused on analyzing the immunogenicity of ECPs from Jeju-45 and Jeju-13.

The 2-DE assay was employed to analyze the expression of ECPs from Jeju-45 and Jeju-13 in order to ascertain whether differences in ECP expression are related to virulence. The composition of secreted ECPs depends on the isolate and growth phase. Similar investigations were performed for *S. pyogenens* and *Streptococcus mutans*. The expression of exoprotein differed according to strain and/or culture conditions, such as O_2 and CO_2 concentrations, temperature, and growth phase [20–22]. Group A streptococci, *S. pyogenes*, is known to release a variety of soluble proteins that contribute to its virulence, for example, hyaluronidase, streptokinase, streptococcal pyrogenic exotoxin B (SpeB) and DNases, streptolysin O and S [18,19], that appear to correlate with severe disease. In the present study, the 2-DE profiles of *S. iniae* ECPs appear to relate to the mortality of groups immunized and non-immunized with formalin-killed Jeju-45 alone. It is possible that Jeju-45 induces a more severe acute infection in olive flounder than Jeju-13 due to the secretion of higher levels of ECPs.

Cellular and extracellular antigens of *S. mutans* were investigated by SDS-PAGE immunoblot assays that showed human sera reacted strongly with many antigens of ECPs (30 μg/lane) compared with antigens of cellular proteins (200 μg/lane) [39]. This result provided evidence that *S. mutans* produced immunologically distinct antibodies against both extracellular and cellular proteins [39]. Sera from Jeju-13 and Jeju-45 detected several antigens from Jeju-45 ECPs, and moreover, the intensity of spots was stronger compared with those generated from whole cells. These results were consistent with those for *S. mutans* [39]. The ECPs of *S. iniae* appear to play an important role in the development of an immune response.

Some antibodies in sera raised against Jeju-13 and Jeju-45 recognized antigenic spots on the 2-DE immunoblot profiles for Jeju-45 ECPs, supporting the finding that the respective isolates possess different pathogenic pathways. In 2-DE immunoblot profiles for ECPs, spots of (c) and (e) groups were distributed in the ranges of p*I* 5.1–5.4 and MW 37 kDa, and p*I* 5.0–5.2 and MW 27 kDa, respectively, and were immunodominant antigens for Jeju-13 sera. Spots of group (b) on the 2-DE immunoblot profiles using Jeju-45 sera were observed at p*I* 4.5–4.7 and MW 42.0 kDa. Although the identity of these spots was not ascertained in the present study, they might correspond to glyceraldehyde-3-phosphate dehydrogenase, fructose-biphosphate aldolase and enolase on 2-DE profiles generated from *S. iniae* whole cell lysates in our previous study [36]. In addition, these proteins showed common characteristics with respect to p*I*, MW and pattern (multiple isoforms) on 2-DE profiles for *Streptococcus* sp. [20–22,26–28,36,38]. Therefore, GAPDH and FBA, and enolase are thought to be the major Jeju-13 and Jeju-45 antigens, respectively. In GAS, GAPDH is known to have multiple binding activities to various mammalian proteins, such as fibronectin, lysozyme and cytoskeletal proteins myosin and actin of the host [18,19]. These properties suggest that GAPDH may play a key role in adhering to and colonizing the host cell. In addition, GAPDH has been reported as an ADP-ribosylating enzyme [19]. Enolase possesses stronger plasminogen-binding activity than GAPDH, which may aid host cell invasion [18,19]. Although the role of FBA in the pathogenicity of *Streptococcus* sp. is unknown, it is possible that FBA and GAPDH function as protective antigens in *S. pneumonia* infection [28]. Based on the present and previous studies [18–22,26–28,38], the spots suspected as GAPDH, FBA and enolase in the present study appear to correlate with virulence and protective antigens for the *S. iniae* isolates, Jeju-13 and Jeju-45.

There have been several studies focused on the development of a vaccine against streptococcosis caused by *S. iniae* [12,13]. Among the vaccines developed to date, those generated from formalin-killed cells and concentrated ECPs are reported to have the greatest efficacy against tilapia streptococcosis. An *S. iniae* isolate used for immunization gave lower protection in fish challenged with the homologous isolate than with the heterologous isolate [13]. However, this study did not report the virulence and ECP characteristics of the *S. iniae* isolates. Similar findings were obtained in the present study in fish groups immunized with formalin-killed Jeju-45 in the absence of ECPs. In contrast, high vaccine efficacy was observed in olive flounder challenged with the highly virulent isolate followed by immunizing with blended vaccine, killed-cells and concentrated ECPs from the homologous isolate. The success of this vaccine may be attributed to the Jeju-45 ECPs eliciting a better immune response.

In summary, the present study investigated the immunogenicity of the *S. iniae* isolates, Jeju-13 and Jeju-45, using 2-DE immunoblot assays and challenge experiments to develop an effective vaccine in olive flounder. We found that Jeju-13 and Jeju-45 differed in virulence and secreted ECPs that correlated with differences in antigenicity. The 2-DE immunoblot assays showed that *S. iniae* ECPs had the capacity to induce distinct antibodies, depending on the isolate causing infection in olive flounder. ECPs were found to be an essential in the development of a vaccine that offers a high level of protection against *S. iniae* infection in olive flounder. Finally, this study provides a model for the use immunoproteomics in the development of vaccines.

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