

Immunoproteomic analysis of extracellular products (ECPs) obtained from three etiological isolates of streptococcosis (*Streptococcus iniae*, *Streptococcus parauberis* Dongbo and *Streptococcus parauberis* Namhae) of olive flounder (*Paralichthys Olivaceous*) in Korea

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ABSTRACT

In the pathogenesis of *Streptococcus sp.* in mammals, extracellular proteins (ECPs) have been a well critical factor in immunological invasion and evasion. Yet, in fish, there have only been a few reports on the immunological role of ECPs in streptococcosis. Recently, ECPs have been evaluated as an effective immunogenic source against *Streptococcus iniae* infection in olive flounder. Here, we characterize ECPs associated etiological bacteria causing streptococcosis in olive flounder: *Streptococcus iniae*, *Streptococcus parauberis* Dongbo, and *Streptococcus parauberis* Namhae. Immunoproteomic analysis in which ECPs from the respective bacteria reacted with olive flounder antisera against homologous/heterologous bacteria or different strains was carried out. In 2-DE immunoblot profile ECPs, antigenic spots were identified as Secretion protein Bug4, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, Enolase, 60 kDa chaperonin, phosphoglycerate kinase, DNA-directed RNA polymerase alpha chain, and elongation factor Tu. This study might provide useful data to understand ECPs protein composition,

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it's role in pathogenesis, and immunogenicity by using proteomics techniques such as mass spectrometry (MS and MS/MS) and peptide mass fingerprinting (PMF). Further studies will evaluate the efficacy of adding ECPs to *Streptococcus sp.* vaccine.

Key words: Olive flounder; *Streptococcus iniae*; *Streptococcus parauberis*; extracellular proteins; vaccine.

INTRODUCTION

Olive flounder (*Paralichthys olivaceus*) is a commercially important marine species in Korea, China and Japan. Currently, the production of this species yielded 43 metric tons and earned USD 200 M in 2008 (Statistics Korea, www.kostat.go.kr). However, the intensive culture of this species has resulted to severe economic losses by various bacterial diseases, especially streptococcosis (Shin *et al.*, 2007a). The major etiological agents of streptococcosis include *Streptococcus iniae* and *S. parauberis* (Nho *et al.*, 2009). Hence, a general vaccine against *Streptococcus sp.* should be developed to effectively prevent streptococcosis induced by the co-infection of a variety of bacteria (Toranzo *et al.*, 2005). However several attempts using a homologous-inactivated/ killed whole cell vaccine (bacterin) still focused on antigenic variation, since, along with several vaccination conditions (Romalde *et al.*, 2005; Toranzo *et al.*, 2005), the efficacy could also vary with the strains and/or isolates (Bachrach *et al.*, 2001; Eldar *et al.*, 1997; Klesius *et al.*, 2000). In addition, little was known about cross-protection of respective vaccine against such pathogens. Previous studies have shown that streptococcal ECPs could be the key to improve cross-protection; for example, the modified *S. agalactiae* bacterin (containing ECPs) provided protection against homologous and heterologous challenge without booster in tilapia (Evans *et al.*, 2004). It is likely that ECPs could be important materials to provoke cross-protection against various streptococcal agents. Immunoproteomics has been employed in various studies to effectively investigate not only immune response between host and pathogen, but also antigenic proteins of importance for vaccine developments (Ni *et al.*, 2010; Shin *et al.*, 2007a; Vytvytska *et al.*, 2002). In this study, we applied an immunoproteomic approach (the combination of 2-dimensional electrophoresis (2-DE) and immunoblotting with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)) to explore the common antigenic proteins from ECPs of etiological agents, two strains isolated from different regions including *S. iniae* Jeju45 (Shin *et al.*, 2007c), *S. parauberis* Dongbo (Korea Jeju province) (Nho *et al.*, 2009) and *S. parauberis* Namhae (Korea Gyeongnam province) (Korea isolate). The information on secreted antigenic proteins relevant to cross-reactivity might be helpful in understanding the role of ECPs in pathogenicity and antigenicity.

MATERIALS AND METHODS

Bacteria

The *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae used in this study were isolated from spleen of diseased flounder in 2000, 2006 and 2009 (Shin *et al.*, 2007c; Nho *et al.*, 2009). These isolates were incubated in Tryptic Soya Broth (TSB, OXOID) and Tryptic Soya Broth (TSB, OXOID) at 25°C for 24h.

Production of olive flounder antiserum

Olive flounder specific anti- *S. iniae*, *S. parauberis* Dongbo and *S. parauberis* Namhae sera were produced as described by Kang *et al.* (2006). Olive flounder with an average body weight of 150g and an average length of 15 cm were purchased from a commercial fish farm located in Namhae county on the southern coast of Korea. Fish were divided into 4 groups of 5 individuals each and were maintained in 200 L FRP tanks at 25°C. The fishes were intraperitoneally injected with 0.1 ml (approximately 10⁷ CFU ml⁻¹) of *S. iniae*, *S. parauberis* Dongbo and *S. parauberis* Namhae. Live bacteria were used to ensure the immunogenicity of the bacteria is maintained. Fishes from one tank was maintained as negative control. After 2 weeks, blood was collected from the recovered flounder and the upper supernatant was separated by centrifuge at 3,500 rpm for 15 min at 4°C.

ECPs precipitation

Extracellular precipitation was performed as described by Evans *et al.* (2004) and modified. For preparation of bacterial ECPs, *S. iniae* Jeju-45, *S. parauberis* Dongbo and *S. parauberis* Namhae isolates were cultured on TSB for 24 h at 25°C. The supernatants were obtained by centrifugation at 8,000×g for 30 min and the cell-free fluid was sterilized using 0.45 µm disposable filter. The ECPs was dehydrated with a freeze dryer (Heto MAXI dry lyo, Allerod, Denmark) and stored at -70°C until use. The ECPs powder was diluted by sterilized PBS, the protein concentrations were determined by Bradford assay using bovine serum albumin as a standard.

Isoelectric focusing (IEF) and SDS-PAGE

IEF was performed using the IPGphor™ system (Amersham Bioscience, Uppsala, Sweden) with IPG strips (Immobiline DryStrip™, pH 4–7, 13 cm; Amersham Bioscience), according to the previously reported method (Shin *et al.*, 2006). The protein loading volume for ECPs extract was adjusted to 120 µg ml⁻¹ with rehydration buffer (9M urea, 2% CHAPS, 0.4% DTT, 0.5% IPG-buffer, and 0.002% bromophenol blue). Prepared sample was loaded onto the IPG strips and focused at 86.1 kV h at 20 °C using an automated system as follows: rehydration for 12 h (6 h at 30V followed by 6 h at 60 V), and focusing for 17 h (2 h at 200V, 1 h at 500V, 1 h 1000V, 1 h at 2000V, 2 h at 4000V and 10 h at 8000 V). After IEF, the IPG strip was equilibrated with 10 mg ml⁻¹ of DTT in equilibration buffer (6M urea, 2% SDS, 30% glycerol, 0.002% bromophenol blue, and 50mM Tris–HCl, pH 8.8) for 15 min, and then with 25 mg ml⁻¹ of iodoacetamide in the equilibration buffer for another 15 min. Equilibrated IPG strip was placed onto 12.5% SDS-polyacrylamide gels

(18 cm×16 cm×0.1 cm), sealed with 0.5%, w/v low melting agarose (Sigma), then electrophoresis at 10mA gel⁻¹ for 15 min followed by application of 20mA gel⁻¹ until the dye reached the bottom of the gel, and silver-stained.

2-DE immunoblot assay

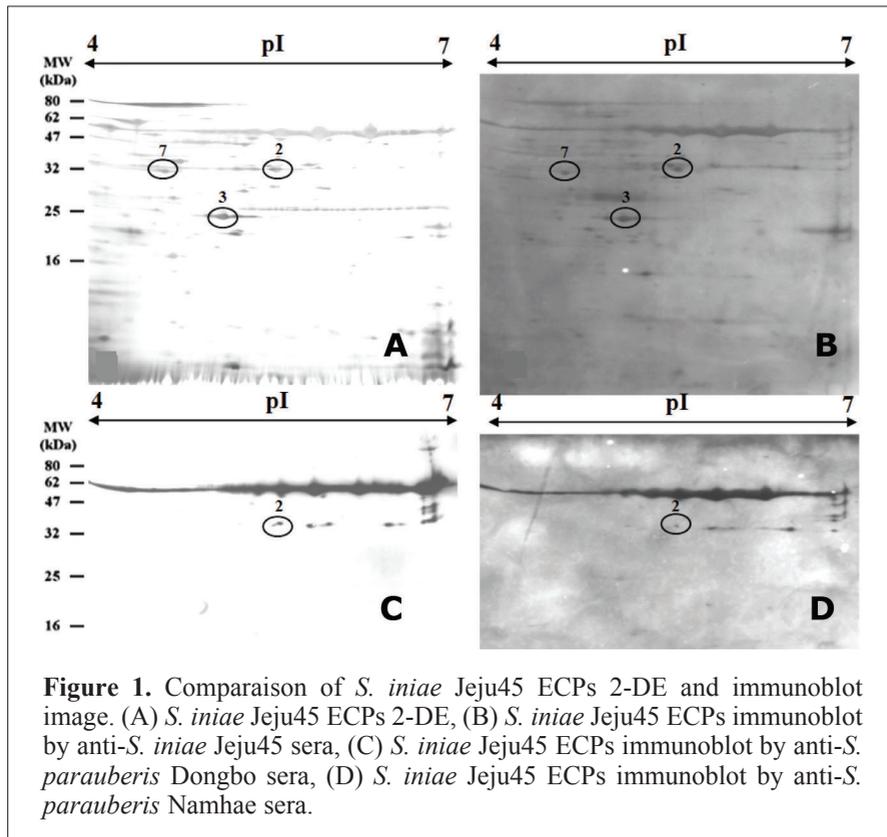
For 2-DE immunoblot assays, each ECPs contained in spots of 2DE gels was transferred to a PVDF membrane (Millipore, USA). The membranes were blocked with 5 % skim milk in PBS-T for 1 h at room temperature (RT), washed three times with PBS-T, incubated with flounder specific anti- *S. iniae* Jeju-45 sera, anti-*S. parauberis* Dongbo sera and anti-*S. parauberis* Namhae sera for 2 h at RT, respectively and washed three times with PBS-T for 15 min. Second antibody, olive flounder IgM monoclonal antibody (Shin *et al.*, 2007b) was incubated for 2 h at RT and washed three times with PBS-T for 15 min. Antigenic spots from ECPs were visualized by incubation with goat anti-mouse-HRP (Jackson, USA; 1:4000) for 1 h at RT. After washing with PBS-T, the membrane was 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 X-ray films were digitalized with an EPSON perfection V700 photo image scanner (SEIKO EPSON CORP, USA) and the acquired images were analyzed using Phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK).

MALDI-TOF MS

Protein spots were identified by Peptide Mass Fingerprinting (PMF) using Mass-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF MS) as described by Shin *et al.* (2006). In brief, protein spots of interest were excised from the silver-stained gels and subjected to in-gel digestion with 12.5 ng/ml porcine trypsin (Promega, Madison, WI, USA) at 37°C overnight (approximately 16 h). The supernatant was recovered and extracted twice with an equal volume of 5% formic acid and acetonitrile, and the extracts were pooled and dried in a vacuum centrifuge. Dried tryptic peptides were redissolved in 1 ml of sample solution (93:5:2, v/v ratio of DW, acetonitrile and TFA), and targeting on MALDI plates was performed using the solution-phase nitrocellulose method (Lee *et al.*, 2003). Alpha-cyano-4-hydroxycinnamic acid (40 mg/ml) and nitrocellulose (20 mg/ml) were prepared separately in acetone and mixed with isopropanol in a ratio of 2:1:1 (v/v). The internal standards, des-Arg-Bradykinin (monoisotopic mass: 904.4681) and angiotensin I (1296.6853) (Sigma–Aldrich, St. Louis, MO, USA) were added to the mixture to generate the matrix solution. The 1 µl matrix solution was spotted onto target circles on the MALDI plate and dried. The dried samples were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). The proteins were identified by comparing the obtained mass spectra to the National Center for Biotechnology Information (NCBI) and SwissProt protein sequence databases using the Prospector (<http://prospector.ucsf.edu>) and Mascot (<http://www.matrixscience.com>) website.

RESULTS AND DISCUSSION

Proteins were separated based on their isoelectric points using 2-DE. On 2-DE, approximately 87, 98 and 95 spots were observed for *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae ECPs, respectively (Fig. 1A, Fig. 2E and Fig. 3I). In the 2-DE immunoblot assay, serum raised against *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae were used due to its ability to recognize the ECPs containing immune reactive spots three bacteria isolates (Fig. 1, 2 and 3). All the immunoreactive spots were also observed in respective duplicated 2-DE gel.



Analysis of cross reactivity using *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae ECPs and its immunoproteomics contained antigenic proteins such as secretion protein Bug4, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Fructose-bisphosphate aldolase, enlase, 60 kDa chaperonin, phosphoglycerate kinase, DNA-directed RNA polymerase α chain and elongation factor-TU. GAPDH and Fructose-bisphosphate aldolase were commonly elicited against homologous/ heterologous isolates and different genera. These are potentially important antigenic proteins of *Streptococcus* sp. (Table 1).

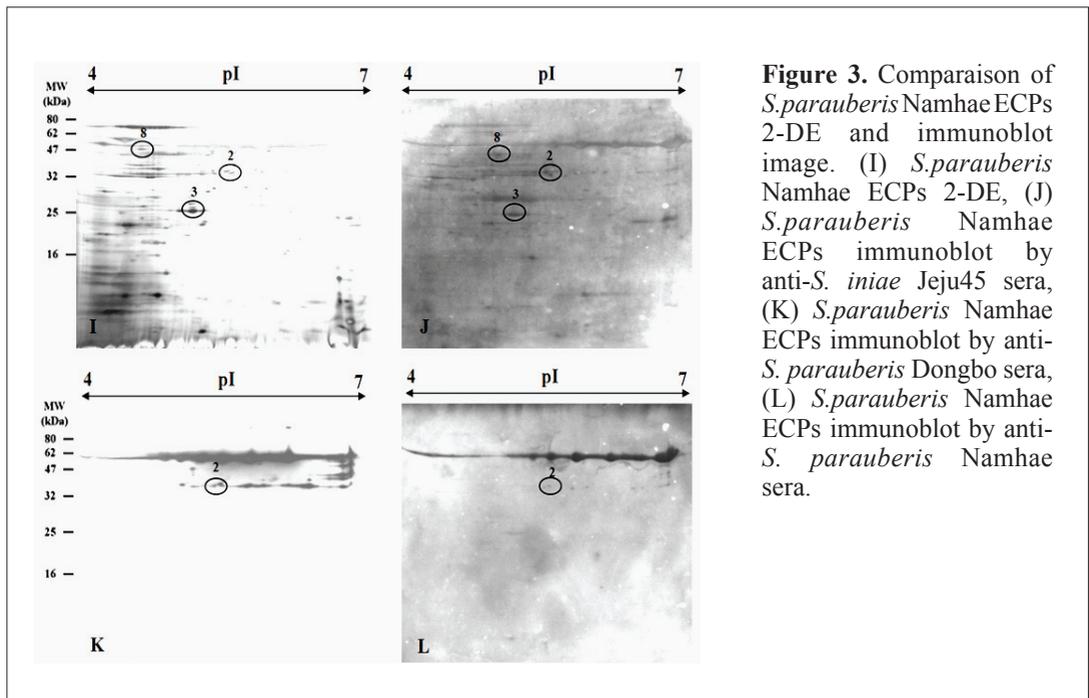
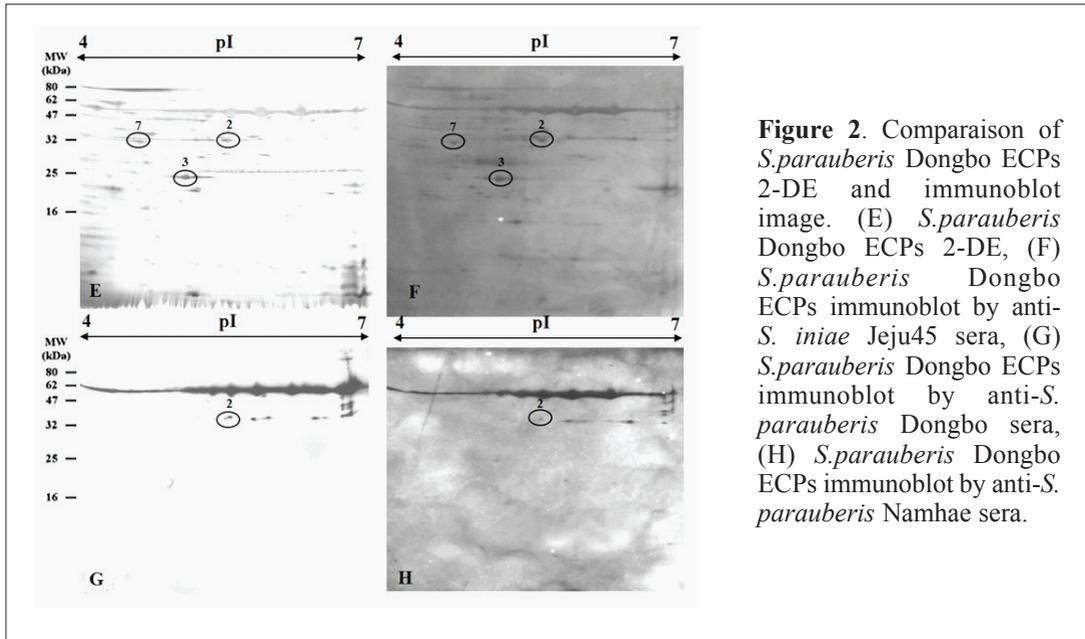


Table 1
The list of identified antigenic proteins of *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae ECPs 2-DE and immunoblot.

Spot No.	Figure image	S. iniae Jeju45 ECPs 2-DE and immunoblot image		S. parauberis Dongbo ECPs 2-DE and immunoblot image		S. parauberis Namhae ECPs 2-DE and immunoblot image							
		A	B	C	D	E	F	G	H	I	J	K	L
1.	Secretion protein Bug4	O	O	O	O	X	X	X	X	X	X	X	X
2.	glyceraldehyde-3-phosphate dehydrogenase	O	O	O	X	O	O	O	O	O	O	O	O
3.	fructose-bisphosphate aldolase	O	O	X	X	O	O	X	X	O	O	X	X
4.	Enolase	O	O	X	X	X	X	X	X	X	X	X	X
5.	60 kDa chaperonin	O	O	X	X	X	X	X	X	X	X	X	X
6.	phosphoglycerate kinase	O	O	X	X	X	X	X	X	X	X	X	X
7.	DNA-directed RNA polymerase alpha chain	X	X	X	X	O	O	X	X	X	X	X	X
8.	elongation factor Tu (EF-TU)	X	X	X	X	X	X	X	X	O	O	X	X

GAPDH and enolase known as located on the cell wall in streptococcus species, and transported to the cell surface without recognizable signal sequences. In addition GAPDH and enolase was determined to serve as the streptococcal plasmin receptor involved in bacterial adhesion and signal transduction to host cells (Bisno *et al.*, 2003; Cunningham, 2000). And 60 kDa chaperonin and elongation factor-Tu have a dual function and serves as both a ribosomal structural protein that binds rRNA and a translational repressor for overcome to varied environment (Len *et al.*, 2004). Phosphoglycerate kinase provided a regulatory link between glycolytic activity and signal transduction regulation involving biofilm formation for protective antigen (Iwami and Yamada., 1985) Others also contributed the pathogen survival or/and virulence acquisition such as DNA replication, enzyme synthesis, biosynthesis of metabolic cycle and sugar utilization.

Based on these result, it could be suggested that *S. iniae* Jeju45, *S. parauberis* Dongbo and *S. parauberis* Namhae might share structural constituents of secreted proteins of three bacterial isolates as common immunogens, which could provide cross-protection against etiological agents.

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