Identification of *Edwardsiella tarda* antigens recognized by mucosal natural antibody in olive flounder, *Paralichthys olivaceus*

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ABSTRACT

Olive flounder is an important mariculture fish species in Korea. However, the culture of this species has been threatened due to a variety of infectious agents, especially *Edwardsiella tarda*. The skin mucus of fish, which contains a higher amount of immunity related substances, acts as an innate barrier, protecting them from invasive environmental pathogens. In the present study, we investigated the ability of the natural antibodys (NAbs), secreted in the cutaneous mucus, to recognize *E. tarda* antigens using 2-dimentional gel electrophoresis (2-DE) and immunoblotting. Further, the antigenic proteins recognized by NAbs were identified using electrospray ionization tandem mass spectrometry (ESI-MS/MS) as outer membrane proteins, phosphopyruvate hydratase, phosphoglycerate kinase, Ribose-5-phosphate isomerase A, and triosephosphate isomerases. These proteins recognized by mucosal NAbs are suspected as important epitopes in *E. tarda* to produce the antigen-antibody complexes in innate immunity of mucus.

Keywords: *Edwardsiella tarda*; mucus; natural antibody; *Paralichthys olivaceus*; ESI-MS/MS

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INTRODUCTION

Olive flounder is a type of flatfish, which is regarded as a delicacy in Korea. It is the major aquaculture fish species in Korea with production reaching 54,600 metric tons in 2009 (Korea Statistical Information Service, KOSIS, http://kosis.kr). However, cultured fish are often threatened by infectious agents from the environment resulting in mass mortalities. *Edwardsiella tarda*, a Gram-negative, motile, short rod, causes edwardsiellosis in both freshwater and marine fish, and is a major bacterial pathogen of olive flounder (Thune *et al.,* 1993; Mohanty and Sahoo, 2007).

The olive flounder, which differs from most teleost fish by having a more sedentary bottom-dwelling lifestyle, has an abundance of cutaneous mucus. It is thought that the cutaneous mucus acts as an immunological barrier in these fish, in much the same way as those of the gut, respiratory tract and genital tract (Shephard, 1994; Woof and Mestecky, 2005). The mucus plays a critical role in the defense mechanism of the fish, acting as the first physiological barrier by eliminating pathogens through the continuous production of mucus (Kanno *et al.,* 1989; Ellis, 2001). Furthermore, it acts as a biological barrier because of immune substances within mucus which may include C-reactive proteins, alkaline phosphatase, protease, lysozyme, transferrin, and immunoglobulins (Igs) (Alexander and Ingram, 1992; Fast *et al.,* 2002; Palaksha *et al.,* 2008). Particularly, one of the important molecules in cutaneous mucus of fish is immunoglobulins (Igs) playing a key role in recognizing natural antigens. IgM is known to be the major immunoglobulin in the mucus of fish, which contrasts with the IgA in the mucus of mammals (Woof and Mestecky, 2005; Hatten *et al.,* 2001). The mucosal IgM has also been reported in several fishes; such as catfish, *Ictalurus punctatus* (Di Conza and Halliday, 1971; Zilberg and Klesius, 1997), carp, *Cyprinus carpio* (Rombout *et al.,* 1986) and Atlantic salmon, *Salmo salar* (Hatten *et al.,* 2001). In addition, the IgM has been suggested as a natural antibody (NAb) in olive flounder (Palaksha *et al.,* 2008). However, the knowledge of the interaction of NAbs with bacterial pathogens in flounder mucus is still unknown.

Therefore, the present study aimed to elucidate the interaction between NAb and infectious bacteria such as *E. tarda*. Utilizing a proteomics approach, common antigenic proteins were observed in *E. tarda* which were recognized by NAb of the cutaneous mucus. Subsequently, the recognized proteins were identified by nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS).

MATERIALS AND METHODS

Preparation of cutaneous mucus

Cutaneous mucus of fish ranging in size from 500-800 g was collected from an edwardsiellosis-free farm in Namhae, Gyeongnam, Korea in autumn 2008. The mucus was prepared by a method modified from Palaksha *et al.* (2008). Briefly, mucus was gathered and mixed from

10 fish by scraping with a soft rubber spatula. The mixed mucus was transferred to sterile 50ml polypropylene conical tube. The samples were transferred to the laboratory on ice and kept at -80°C. To prepare clarified mucus extract (CME); the stored mucus was mixed with 10 volumes of TBS buffer (Tris-buffered saline; 50mM Tris-HCL, pH 8.0, 150mM NaCl); and frozen and thawed repeatedly. The homogenate was centrifuged at 2850×*g* for 60min, and supernatant was filtered with 0.45μm syringe filter (Minisart). The filtered supernatant was lyophilized and dissolved in deionized water (dW, Millipore MilliQ, 18.2 MΩ cm); subsequently, the solution was replaced with TBS buffer and concentrated with Amicon YM3 membrane (Amicon, Danvers, MA). All of the steps were performed on ice or below 4°C, to minimize denaturation or digestion of proteins.

Preparation of bacteria sample

Edwardsiella tarda (ED-45) was previously isolated from Jeju, Korea in 2004. The bacterium was kept at -80°C in 10% (*v/v*) glycerol-trypticase soy broth supplemented with 2% (*w/v*) NaCl (TSB-2). The bacteria were cultured in 50mL TSB-2 at 26°C until an optical density $(OD)_{610nm}$ of 1.0, pelleted by centrifugation at 2,000×*g* for 30min at 4°C, and washed 3 times with PBS (phosphate-buffered saline; 3mM KCL, 137mM NaCl, 1.5mM KH_2PO_4, and 8mM Na_2HPO4, pH7.4). The pellet was suspended in 5ml deionized water and aliquoted to 1ml. The suspensions were sonicated (XL-2020, Misonix) at 120W for 10 min (sonication for 5s, 20s interval) in ice slurry; and centrifuged at 16,000×*g* for 30 min at 4°C. The resulting supernatant was stored at -20°C until use.

1-DE and immunoblotting

The supernatant from the sonicated bacterium was applied to one-dimensional SDS-PAGE (1-DE) according to Laemmli (1970). Ten microgram of protein was applied to two separate 12.5% (*w/v*) SDS-PAGE under denatured condition. One gel was stained with Commassie brilliant blue R-250 for visualization, the other gel was used for immunoblot assay. The proteins in the gel were transferred to PVDF membrane (Millipore, USA) at 60V for 60 min and blocked with 5% (*w/v*) skim milk in PBS-T (3mM KCL, 137mM NaCl, 1.5mM KH_2PO_4, and 8mM Na_2HPO4, pH7.4, and 0.05% (*v/v*) Tween-20) for 60min at RT. Blocked membrane was washed 3 times with PBS-T, and incubated with 200μg of CME protein in 4 mL 5% (*w/v*) skim milk in PBS-T for 2h at 26°C. The membrane was incubated with anti-flounder IgM monoclonal antibody (MAb) (Shin *et al.,* 2007) for 60 min at 26°C after washing 3 times with PBS-T, washed 5 times with PBS-T, and incubated for 60 min with goat anti-mouse IgG-HRP (Jackson. USA; 1:2,000). Finally, proteins recognized by CME were developed with an ECL kit (Enhanced Chemiluminescence; Amersham Biosciences).

2-DE and immunoblotting

Two-dimensional electrophoresis (2-DE) samples were prepared using 100μl of supernatant from sonicated bacteria, the supernatant was mixed with 400μl lysis buffer (40mM Tris-HCL, 7M urea, 2M thiourea, 4% (*w/v*) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5% (*v/v*) IPG buffer pH 3-10, and 1.4% (*w/v*) DTT) and vortexed at RT for 30 min. 200 μg of proteins from lysate were precipitated with equal volumes of 20% trichloroacetic acid. The precipitate was washed in acetone and dissolved

in 250 µl rehydration buffer (9M urea, 4% (*w/v*) CHAPS, 5% (*v/*v) IPG buffer pH 4-7, 1.4% (*w/v*) DTT, and trace amount of bromophenol blue); and vortexed for 30 min at RT then centrifuged at 16,000×*g* for 15 min. Isoelectrofocusing (IEF) and 2-DE were performed according to previous study (Shin *et al.,* 2006). Briefly, the supernatants were subjected to IEF (IPGphore™ system, Amersham Bioscience, Uppsala, Sweden) with IPG strips (Immobiline DryStrip™, pH 4-7, 13 Cm; Amersham Bioscience). The IPG strips were equilibrated with equilibration buffer (6M urea, 2% SDS (*w/v*), 30% glycerol (*v/v*), 50 mM Tris-HCL, and 0.002% bromophenol blue, pH 8.8). Equilibrated strips were electrophoresed on 12.5% SDS-PAGE gel. The gel was subjected to 2-DE immunoblotting, which was performed as described in the previous section and visualized by silver staining.

Protein identification using ESI-MS/MS

Recognized protein spots based on 2-DE immunoblotting result were excised from 2-DE gels and extracted by in-gel digestion with trypsin as described previously (Shin *et al.,* 2006). Digested peptides were applied to nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS) according to Lee *et al.* (2004). Briefly, the supernatant of tryptic digestion were purified by a microcolumn packed with a Poros R2 resin (PerSeptive Biosystems) in GELoader tips (Eppendorf, Hamburg, Germany). Purified peptides were applied to a QSTAR pulsar-i MS system (AB/MDS Sciex, Toronto, Canada) equipped with a nano-electrospray ion source (MDS Protana, Odense, Denmark) to obtain the MS/MS data. Ionspray voltage was set to a potential of 850–900 V. Peptide data were acquired from *m/z* range of over 400–1200 Da in positive mode with manually optimized collision energy settings for each peptide. The data were processed and interpreted with the BioAnalyst (PerSeptive Biosystems) software. Mascot (http://www.matrixscience.com) was employed to interpret the raw MS/MS data. The default search parameters used were enzyme = trypsin, variable modification = carbamidomethyl (C), peptide tolerance of parent ion ± 1 Da, MS/MS tolerance ±0.8 Da, and maximum missed cleavage = 1. Proteins with a Mascot score of more than 15 ($p<0.05$) was considered reliable and used in the present study. The identified peptides were aligned by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search to confirm the identity to *E. tarda* protein.

RESULTS

SDS-PAGE and immunoblotting was employed to detect proteins suspected as common antigens in *E. tarda* which can be recognized by natural antibodies in cutaneous mucus. The proteins were separated by 1-DE under denatured condition (Fig. 1a) and was used for immunoblotting. There were several weak bands in *E. tarda* 1-DE immunoblot results. CME and anti-IgM L chain (anti-IgM) MAb (Shin *et al.,* 2007) were used to detect antigenic proteins which can be regarded as specifically recognizable antigens by the natural antibody (NAb) mucus IgM (Fig. 1b). To identify the proteins recognized by mucosal NAb, proteins of *E. tarda* were separated by 2-DE and visualized by immunoblot analysis with CME and anti-IgM MAb. Around 400 spots were visualized in 2-DE of *E. tarda* under a pH range of 4-7 (Fig. 2). The 2-DE gel was transferred to PVDF membrane and applied to immunoblot analysis (Fig. 3). Sixteen spots were recognized by CME, 7 of which were then identified

by ESI-MS/MS analysis (Table 1), spot number 1 and 2 were outer membrane proteins of *E. tarda*; spot 3 was identified as phosphopyruvate hydratase from *Photobacterium profundum ss9*; spot 4 was phosphoglycerate kinase of *Edwardsiella ictaluri*; spot 5 was Ribose-5-phosphate isomerase A of *Enterobacter cloacae*; and spot 6 and 7 were two triosephosphate isomerases from *Gloeobacter violaceus PCC 7421* and *Serratia proteamaculans 568*, respectively (Table 1). The identified proteins from the database of other species were later compared to *E. tarda* aligned by BLAST search, which showed very high sequence similarity except for the 82% of *Gloeobacter violaceus PCC* 7421.

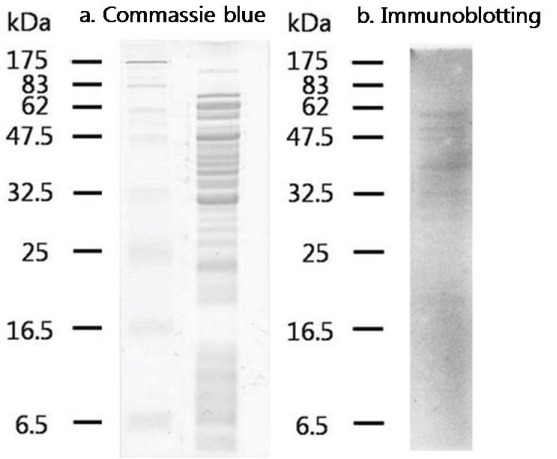

Figure 1 Immunoblot analysis of *E. tarda*. Protein profile of *E. tarda*, were separated on 12.5% SDS-PAGE and stained with coomassie brilliant blue R-250 (a). Several bands were observed in immunoblot analysis of 1-DE, which were recognized by NAb (b).

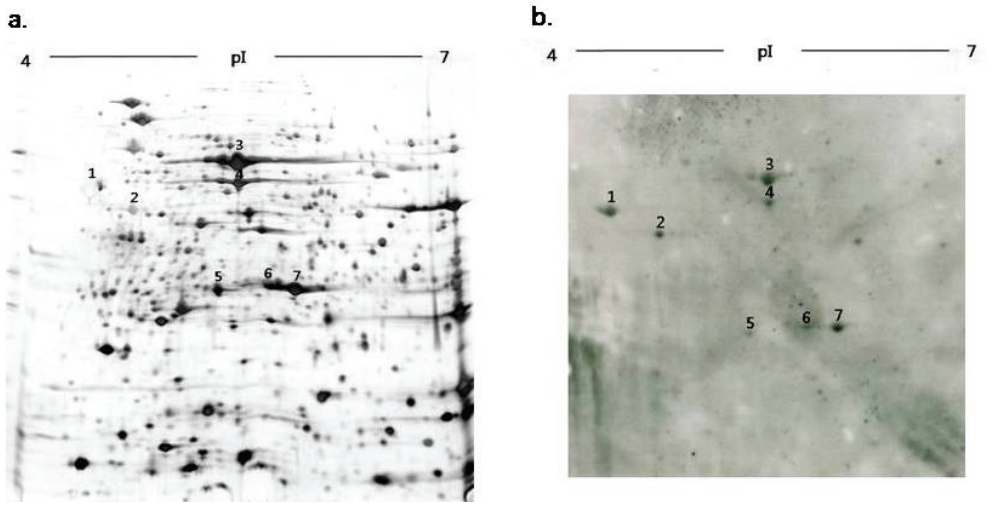

Figure 1a & 1b Proteins of *E. tarda* were separated on 2-DE and stained with silver stain. About 400 proteins were observed in a pH range of 4-7 (12.5% SDS-PAGE), and 16 spot were detected in immunoblot assay with NAb. The 7 proteins recognized by CME were identified by ESI-MS/MS. 2-DE analysis of *E. tarda* (a). Immunblot analysis with NAb (b).

Table 1
Protein identification and peptide homologues

Spot No.	Accession no.	Mascot Score	Mass/ pI	Species	Protein name	Peptide	Peptide identity to E. tarda
1	gi|25989456	23	47225/5.3	*Edwardsiella tarda*	outer membrane protein	R.ANSINTDDIVALGLVYQF.-	
2	gi|25989456	59	47225/5.3	*Edwardsiella tarda*	outer membrane protein	K.YVDLGATYYFNK.N	
3	gi|54310177	71	45586/4.9	*Photobacterium profundum ss9*	phosphopyruvate hydratase	K.DVTLAMDCAASEFYDK.E +Carbamidomethyl	100%
4	gi|2708662	71	6426/4.5	*Edwardsiella ictaluri*	phosphoglycerate kinase	K.ISYISTGGGAFLEFVEGK.K	100%
5	gi|30173331	47	22763/5.1	*Enterobacter cloacae*	Ribose-5-phosphate isomerase A	K.QVDILGNFPLPVEVIPMAR.S	100%
6	gi|37520609	35	26591/5.4	*Gloeobacter violaceus PCC 7421*	triosephosphate isomerase	R.LIIAYEPIWAIGTGK.T	82%
7	gi|157373037	38	26626/5.8	*Serratia proteamaculans 568*	triosephosphate isomerase	K.GAVIAYEPIWAIGTGK.S	100%

DISCUSSION

Under normal conditions, natural antibodies are expressed, even if the individuals are not stimulated by specific antigens acting against non-self substance, such as pathogens, cell debris, nuclear proteins or DNA. This NAb has been believed to IgM in humans (Coutinho *et al.,* 1995). Similarly, in olive flounder, two Igs including IgM and IgM precursor have been observed in the serum (Shin *et al.,* 2007; Bang *et al.,* 1996). In addition, IgM has been observed in the cutaneous mucus of olive flounder, and this has been regarded as a NAb (Palaksha *et al.,* 2008). In this study, the mucosal Nab, IgM, was studied to establish its capability to recognize antigenic proteins of *E. tarda*. The result of immunoblot analysis from 1-DE showed several weak bands between 62 and 25 kDa, which were recognized by CME. As described in a previous study, this result shows the property of NAb of reacting to pathogens by non-specific mechanisms (Coutinho *et al.,* 1995). Therefore, the CME of olive flounder contains NAb and could detect common proteins that had not been previously introduced to the host. To detect the common antigenic proteins, the present study carried out 2-DE and immunoblot assay. Seven proteins from 16 spots which were recognized by CME were identified by ESI-MS/MS. The result of protein identification showed two spots belonging to *E. tarda* and five spots identified from other species. The proteins were identified as belonging to other species due to the limited protein database of *E. tarda* at the time of the experiments. Since then, the whole genomic sequence of *E. tarda* has been published (Wang *et al.,* 2009), and these proteins have been shown to have high sequence identity in BLAST alignment analysis against the published *E. tarda* data. The phosphopyruvate hydratase, phosphoglycerate kinase, and Ribose-5-phosphate isomerase A all showed 100% peptide identity to *E. tarda*. Similarly, triosephosphate isomerase showed 82% and 100% peptide identity to *E. tarda* in *G. violaceus PCC 7421* and *S. proteamaculans 568*, respectively.

In the present study, seven common antigenic proteins of *E. tarda* which can be recognized by NAb, were identified using ESI-MS/MS analysis. Until now, infection mechanisms, attaching sites and penetration are not clearly known. However, abraded skin could be suggested as a penetration site of *E. tarda* (Mohanty and Sahoo, 2007). Therefore, these common proteins might be important epitopes of *E. tarda* which are recognized by NAb, and advances the understanding of the pathogen invasion mechanism on the skin of olive flounder; especially, the role of NAb to recognize the specific epitope against *E. tarda* infection. However, the present study only showed *E. tarda* common antigens; thus, information of common antigenic proteins from other infectious species might be required to clearly understand the character of NAb in the cutaneous mucus of olive flounder.

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