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RESEARCH NOTE

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Analysis of *Vibrio parahaemolyticus* OMPs and Production of Antibodies against OMPs

Soo-Min Kim[1], Bong-Soo Noh[2], Hae-Yeong Kim[3], Se Jin Park[4] and Geun Eog Ji[4]*

[1]*Interdisciplinary Program in Bioinformatics, Seoul National University, Seoul 151-742, Korea*
[2]*Dept. of Food and Microbial Technology, Seoul Women's University, Seoul 139-774, Korea*
[3]*Dept. of Food Biotechnology, Institute of Life Science, Kyung Hee University, Suwon 449-701, Korea*
[4]*Dept. of Food Science and Nutrition & Research Institute of Human Ecology, Seoul National University, Seoul 151-742, Korea*

Abstract *Vibrio parahaemolyticus* is a gram-negative bacterium which acts as a causative agent for food poisoning. Studies with respect to specific extracellular proteins of *V. parahaemolyticus* would be useful for the development of specific detection methods against *V. parahaemolyticus*. In our present study, outer membrane proteins (OMPs) of *V. parahaemolyticus* were obtained from insoluble fraction of 1% sarkosyl treated-cell wall materials. SDS-PAGE analysis showed the presence of several conserved outer membrane proteins among five strains of *V. parahaemolyticus*, and three bands were identified as *V. parahaemolyticus* OMPs through MALDI-TOF analysis. Polyclonal antibodies enriched with anti-OmpU were obtained from immunized rabbits. The antibodies against these proteins may be useful for the development of detection methods for *V. parahaemolyticus*.

Key words: *Vibrio parahaemolyticus*, outer membrane proteins, OmpU, antibodies

Introduction

Vibrio parahaemolyticus is a gram-negative, halophilic bacterium that naturally inhabits marine and estuarine environments and causes three major syndromes of clinical illness: gastroenteritis, wound infections, and septicemia (1). Bacteria utilizes a number of external structures to attach to specific host tissues and in some instances, to bring about changes in the biochemical and cellular activity of the host cells to which they adhere (2). Among the best studied of these adhesive structures are pili (fimbriae) and outer membrane proteins (OMPs, or porins), both of which project into the bacterium's environment (3).

The outer membrane of *Vibrio* contains a number of distinct proteins with molecular mass in the range of 30 to 40 kDa. Some of these proteins may be species-specific and possess high antigenicities (4). Especially, cell exterior regions of OMPs are capable of raising protective immune response (5). The identification of proteins located on the outer surface of *V. parahaemolyticus* and antibodies against these outer membrane proteins may have a potential for the development of rapid detection methods for *V. parahaemolyticus*.

In the present study, proteomic techniques were used to identify proteins among the OMPs of *V. parahaemolyticus* by SDS-PAGE analysis and MALDI-TOF. From the analysis, OmpU and OmpA were identified and used to produce specific antibodies against them from an immunized rabbit.

*Corresponding author: Tel: 82-2-880-8749; Fax: 82-2-884-0305
E-mail: geji@bifido.com
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Materials and Methods

Bacterial strains and media *V. parahaemolyticus* ATCC 35117, and ATCC 43996 were obtained from American Type Culture Collection, USA. KCCM 11965 (ATCC 17802) was obtained from Korean Culture Center of Microorganisms, Korea. KCTC 2471 (ATCC 3844) was obtained from Korean Collection for Type Cultures, Korea. KCCM 41664 (ATCC 33844) was supplied from Department of Food and Bioengineering, Kyungwon University, Korea. *V. parahaemolyticus* was grown at 37°C in BHI (Brain Heart Infusion, Difco) with 3% (w/v) NaCl.

Preparation of cell envelopes Cell envelopes were prepared from whole cells according to the reported procedure (6). Cells were harvested by centrifugation with Comi-514R centrifuge (Hanil Science Industrial, Korea) at 3,000 × g for 20 min, washed twice with 3% NaCl solution and suspended in the same solution. The cells were disrupted by sonication withVCX400 (Sonics & Materials Inc., U.S.A) at 4°C. The unbroken cells were removed by centrifugation at 3,500 × g for 10 min, and the supernatant was centrifuged with 55P-72 (HITACHICOREA, Korea) at 30,000 × g for 30 min. The pellet was washed with distilled water and used as cell envelope fraction.

Preparation of outer membrane Based on the solubility of bacterial membrane proteins in Sarkosyl (7), outer membrane proteins which were insoluble in the detergent were isolated (8). The cell envelope was treated with 1% Sarkosyl in 10 mM tris-HCl buffer (pH 7.2) at 37 °C for 30 min under constant shaking. The supernatant was decanted and the insoluble materials were collected by centrifugation at 100,000 × g for 60 min, washed with distilled water and used as the outer membrane protein (OMP) samples.

SDS-polyacrylamide gel electrophoresis and in-gel digestion Samples (about 2 mg) were mixed with 20 μL of 2× loading buffer [0.125 M Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8] and boiled for 5 min before loading on the gel, as described by Sambrook et al (9). The boiled samples were loaded into SDS-PAGE gel and electrophoresed at 20 mA for 2 hr in Mini-Protean III cell containing electrophoresis buffer [25 mM Tris, 192 mM glycine, 0.1% SDS]. After electrophoresis, the gels were stained with Bio-SafeTM Coomassie solution (Bio-RAD) for 40 min at 37°C and destained serially with distilled water. The gels were dried in GelDrying Film (Promega, USA) at room temperature, overnight. To estimate protein molecular mass, Kaleidoscope Prestained Standards (Bio-Rad) were used as size marker: myosin (201 kDa), β-galactosidase (125 kDa), bovine serum albumin (81 kDa), carbonic anhydrase (38.5 kDa), soybean trypsin inhibitor (31.3 kDa), lysozyme (18.1 kDa), aprolinin (6.9 kDa). In-gel digestion of protein bands was performed as described by Wilm *et al.* (10).

MALDI-TOF MS and database searches The dried protein sample was dissolved in 0.5% TFA solution. All samples were desalted and concentrated in a 10 μl ZipTipc18 (Millipore) pipette tip. Recovered peptides were contaminant-free and were further concentrated to 1 ~2 μL for direct MS analyses in a Voyager-DETM STR Biospectrometry Workstation (Biosystems Inc., USA). Monoisotopic peptide masses were obtained in the mass range of 3,000-80,000 Da. The data for proteins were matched against MS-Fit program (13) and the National Center for Biotechnology Information (NCBI) protein sequence database.

Production of antibodies Preparation of the anti-OMP polyclonal antibodies and purification of the antibodies were performed at LabFrontier Company Ltd. (Seoul, Korea). Briefly, a rabbit was immunized with 500 μL of OMPs of *V. parahaemolyticus* as antigens. The first injection was followed by two booster injections of 250 μg at intervals of one week. After the immunization protocol, serum was obtained and further purified by affinity chromatography by using commercial Affi-Gel affinity chromatography crosslinked with gel-eluted OmpU as a ligand.

Western blotting Western blot analysis was carried out by using rabbit antiserum. Briefly, OMPs from SDS-PAGE gels were transferred to a PVDF membrane (14) and the membrane was treated with blocking solution [5% skim milk] by incubation for 1 hr or overnight at 4°C without shaking. The membrane was then incubated with primary antibody diluted to 1:2000 with 5% blocking milk for 2 hr. After antibody treatment, the membrane was washed twice in dH_2O for 1 min and thrice in 10 mL of TBS-T for 5 min. Subsequently, the membrane was incubated for 1 hr with anti-rabbit IgG-AP diluted to 1: 2000 with 5% blocking milk. After washing twice in dH_2O for 1 min and thrice in 10 ml of TBS-T for 5 min, the membrane was incubated with BCIP-nitroblue tetrazolium substrate solution for 30 min at room temperature without agitation.

Results and Discussion

Isolation and identification of outer membrane proteins from *V. parahaemolyticus* SDS-PAGE of the isolated OMPs revealed several protein bands with approximate molecular weights in the range of 20,000 to 90,000 Da. OMPs from five different *V. parahaemolyticus* strains contained common major OMPs with molecular weights in the range of 30 to 50 kDa (data not shown). The three major proteins with molecular weights of 48, 36, and 34 kDa were identified as *V. parahaemolyticus* outer membrane proteins -TolC, OmpU, and OmpA -, respectively, by MALDI-TOF MS analysis (Fig. 1, Table 1). The OmpU and OmpA are porin proteins which form water-filled channels across the outer membrane through which many antibiotics and hydrophilic compounds enter cells (15). These porins are constricted by the loops which show a high sequence variation among homologous porins (16). Since the structural variation may play a role of camouflage at the cell surface, OmpU and OmpA might possess *V. parahaemolyticus*-specific antigenic site to some degree.

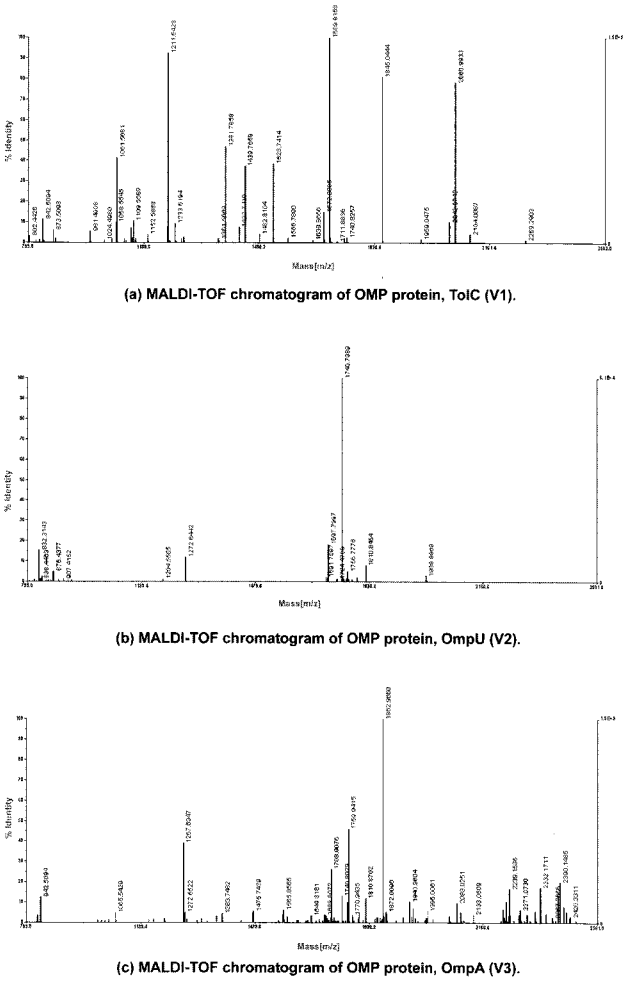

Fig. 1. MALDI-TOF chromatograms of three major OMP proteins.

Table 1. Identification of three major OMPs of *V. parahaemolyticus* by MALDI-TOF analysis

Band number	Protein MW(Da)	Protein pI	Masses Matched	Cov %	Accession number	Protein Name
V1	47983	4.7	20/76 (26%)	40	28897199M	*V. parahaemolyticus* outer membran protein TolC
V2	36286	4.3	7/38 (38%)	19	28899241M	*V. parahaemolyticus* outer membran protein OmpU
V3	34073	4.4	6/35 (17%)	28	28897538M	*V. parahaemolyticus* outer membran protein OmpA

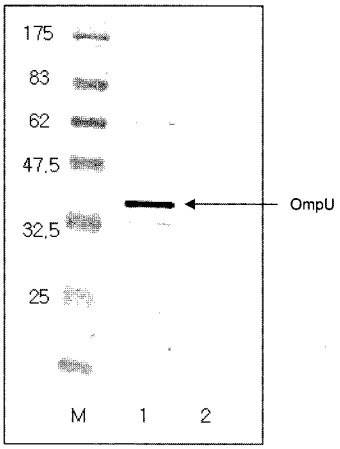

Fig. 2. Western immunoblot analysis with specific polyclonal antibody against OmpU.
M, molecular mass markers; Lane 1, 25ng of OMPs of *V. parahaemolyticus* . ; Lane 2, 50ng of OMPs of *Staphylococcus aureus* .

Production of polyclonal antibodies against Omps We produced specific polyclonal antibodies against Omps of *V. parahaemolyticus* through rabbit immunization with OMPs of *V. parahaemolyticus*. We further purified the antibodies by using OmpU-linked affinity-chromatography as described in Materials and Methods. Fig. 2 shows that the polyclonal antibodies raised against OmpU was able to detect OmpU antigen. In the previous study (4), affinity-purified antibodies against two outer membrane proteins (molecular mass 36 and 34 kDa, respectively) of *V. parahaemolyticus* were used as the primary antibodies to label *Vibrio* sp., and fluorescein isothiocynate-conjugated goat anti-rabbit immunoglobulin G was used to reveal the antigen-antibody reaction. However, in their studies, the employed antigens were not identified. The antibodies prepared in this study may be useful for the development of detection methods for *V. parahaemolyticus.*

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