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Electrophoretical Analysis of 36-Kilodalton Outer Membrane Protein of *Vibrio vulnificus* ATCC 27562

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Abstract Elecrophoreticl analysis of a 36 kDa protein was runned by SDS-PAGE, isoelectric focusing (IEF) and two dimensional electrophoresis pattern. Major 36 kDa and 25, 46, 48, 66 kDa protein were detected by Coomassie blue stain on SDS-PAGE. Major 36kDa protein was eluted for production of antiserum for serological analysis, IEF and two dimensional electrophoresis. Isoelectric point of 36kDa was aout pH 8.5. Two dimensional electrophoresis of eluted 36kDa showed one point on the gel.

Anti-36 kDa serum made by newzilland rabbit for serological test. In ELISA, final titer of antibody was 100×2^5 : 1. Neutralize ability of serum was examined by slide agglutination test and colonization inhibition test in rat. Anti-36 kDa serum agglutinated whole cell of *V. vulnificus* on slide agglutination test. Serum treated *V. vulnificus* were inhibited colonization on intestine in rat. Accordingly In this paper contain some electrophoretical analysis and serological test of a 36 kDa OMP of *V. vulnificus*.

Key words: Outer membrane protein, *Vibrio vulnificus*, Electrophoretical analysis. Serological test.

Introduction

The family of *Vibrionacea* is one of the most important bacterial group in marine environments. [3] This group presented every place of marine as seawater, intestine of marine animal and so on. In addition, some members of the *Vibrionacae* are important pathogens for marine animals and human. [4]

The marine bacterium *Vibrio vulnificus* caused serious septicemia in human depend on infection modes. [3,4] Wound infections have occured in healthy individulas exposed to marine environments. [3] Ingestion of raw fisheries was associated with primary septicemia way. [4] The motality rate may is above 50%. This species were in common sea envrionment of the wolrd. In Korea, this species were isolated from western and southern sea every year.

Virulence factors of *V. vulnificus* have been described like cytolycine, extracelluar enzymes and so on. [12,22] These have a role in invasion of baceria but not clear their function yet.

The surface materials have been studied for the sake of protective antigens of infection of *V. vulnificus*. [5,15,17] Capsule and lipopolysaccharide have reported as protective materials against phagocytes [2,21] of animal host [7] Outer membrane protein (OMP) was reported as receptor [25] antigen, [11,13,16] and virulence factor in gram negative bacteria. [9,23] 18 kDa OMP have been reported as protective antigen in infection of *V. cholerae*. [5] OMP was associated with ion traslocation and material transfer. [20,24] Under environmental stress like heat and pH, OMP was induced newly for survival of bactria. [1,26]

Recently we have isolated outer membrane protein of *V. vulnificus* as protective antigens. OMPs of *V. vulnificus* were determined by SDS-PAGE. Then we observed that 36 kDa protein was major protein and this protein stimulited production of antibody in host. Here we extrated 36 kDa protein and charaterized with electrophoretic and serological methods. This experiment show basic molecular characterization and vaccine possibility of 36 kDa protein.

Maerials And Methods

Bacterial strains and growth condition

Vibrio vulnificus ATCC 27562 was used for isolation of a 36 kDa outer membrane protein. Isolated *V. vulnificus* were used for immunological tests. Cells were grwoun in brain heart infusion (BHI, Difico) medium at 37℃ with shaking.

Preparation of outer membrnae proteins [10]

One liter culrtures of mid-logarithmic *V. vulnificus* were washed in phosphate-buffered saline (PBS), centrifuged at $4,000\times g$ for 20 min, suspended in 5ml of TEAN (5 mM

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Tris, 10 mM EDTA, 5 mM Azide, 200 mM NaCl, pH 8.0) buffer. Cells were disrupted by ultrasonic homogenizor at 8 of relative output, for 10 min. Bacterial debris were removed by centrifuged at 4,000 × g for 20 min, and the supernatant was resuspended in same volume of TEAN buffer. Centrifugation at 50,000 × g for 1 hour seperated total cell membranes from cytosolic materials. Membrane pellts were suspended in 1% N-lauroylsarcosine (Sigma) solution and stand in room temperature for 30 mins.

Sarcosine insoluble outer membrane proteins were seperated as pellet by centrifugation at 50,000 × g for 1 hour. Pellet was washed and suspened in 100 $\mu\ell$ of detergent free TEAN beffer or deionized water.

SDS-PAGE of outer memrane protein and elution [18]

Total protein content was determied by Bradford method with bovine serum albumin as a standard.

Samples were prepared for SDS-PAGE as described by Laemmli. 12% seperating gel and 4.5% stacking gel were casted on Bio-Rad minicell and runned at 170 volt. Protein visualized by stained with 2.5% Coomassie brilliant blue (sigma).

A 36 kDa protein on the gel was eluted by bio-Rad electro eluter (Model 422) at 150 volt.

Isoelectric focusing (IEF) of 36kDa protein [14]

Isoelectric focusing was carried out in reducing slab gels of 12% polyacrylamide containing ampholites (pH 3.5 ~ 10). Samples were suspended in 8M urea, 20% triton X-100, 2.4% ampholyte, 1% β-mercaptoethanol and icubated at 37℃ for 30 min. Focusing was conducted for 3 hours at 200 volt after 30 min rerunning on Bio-Rad unit. The gels soak in 10% trichloroacetic acid (TCA) for 10min and in 1% TCA for overnight. Coomassie blue visualized protein on the gel.

Two-dmensional electrophoresis [1]

For two-dimentional electrophoretical analysis of first IEF gel was incubated in 100ml 100 mM Tris-HCl (pH 6.8) of equilibration buffer containing 0.5% β-mercaptoethanol, 2% SDS, 10% glycerol for 30min at roomtemperature. Prepared gel was placed on 12% of SDS-PAGE and conducted at 200 volt.

Polycolnal antibody production

Newzilland rabbit was immunized with eluted 36 kDa protein for 6 weeks. The antibody titer of anti-36 kDa protein antiserum was determined by ELISA in microtiter plate After 2 weeks from first injection. Western blotting was used for detect anti-36 kDa protein antibody also.

Bacterial agglutination

Grown *V. vulnificus* were harvested and washed. Concentrated suspension of *V. vulnificus* loaded at ten cell slide. One hundred microliters of antiserum was added and dilluted twofold serially. Mixtures were incubated at room temperature for 10minutes.

Inhibition of intestinal colonization

500 $\mu\ell$ of *V. vulnificus* suspension (2×10^7 CFU) and equal volume of dillted antiserum were mixed at 37℃ for 30min in tube. Operate and inject to small intestine of S-D rats. After 16 h of challenge, animals were sacrificed and remove intestine. The intestine was washed in PBS for removing nonadherent bacteria. Then, intestine was homogenized and bacteria were seperated to supernatant. BHI plate and TCBS plate were used for viable cell count.

Results

SDS-PAGE of outer memrane protein and elution

Outer memrbane proteins (OMPs) of *V. vulnificus* were resolved by 12% polyacrylamide gel. The protein profiles of OMPs of *V. vulnificus* was shown at Fig. 1. As illustrated in Fig. 1, we observed sarcosine soluble inner membrane proetin, insoluble OMP and eluted a 36 kDa protein. Molecular size of OMPs were major 36 kDa, 46 kDa, 48 kDa, 66 kDa. Major 36kDa protein was eluted for electrophoretical analysis. Eluted 36 kDa protein was detected by SDS-PAGE and densitometric scan (Fig. 2).

Isoelectric focusing(IEF) of 36 kDa protein

Eluted 36 kDa protein was determined by IEF gel elctro-

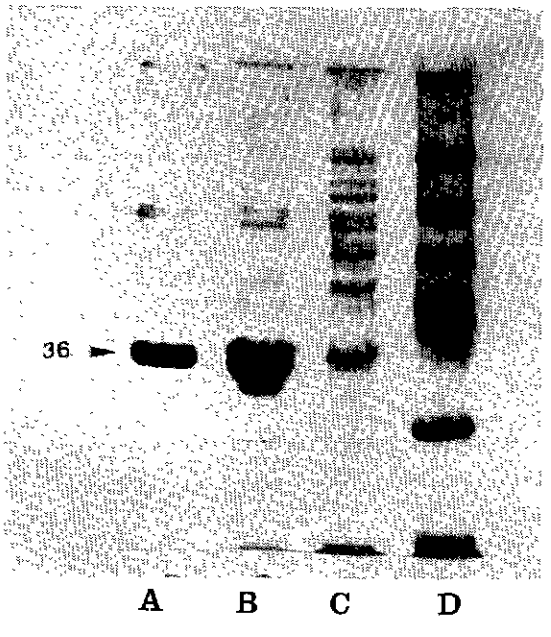

Fig. 1. SDS-PAGE of OMP and eluted 36 kDa OMP of *V. vulnificus*.
A : Eluted 36 kDa, B : Outer membrane proteins, C : Cell Lysate, D : Molecular size Marker (21 kDa ~ 97.4 kDa)

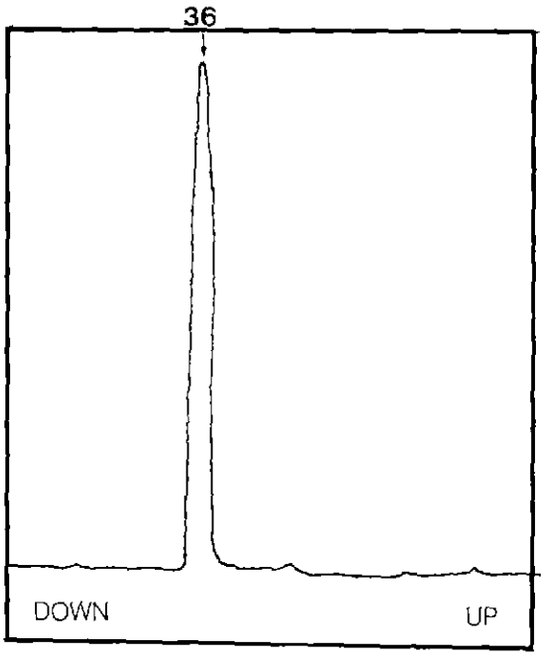

Fig. 2. Densitometric sacn of eluted 36 kDa protein.

phoresis. Used pH range was from pH 3.5 to pH 10. 36 kDa protein appeared upper part of gel and isoelectric point was about pH 8.5 (Fig. 3). So, net charge of 36 kDa was zero at pH 8.5.

Two-dmensional electrophoresis

Primarly IEF gel of 36 kDa was resolved by secondary 12% of SDS-PAGE. Basic 36 kDa protein showed single protein point on the gel (Fig. 4).

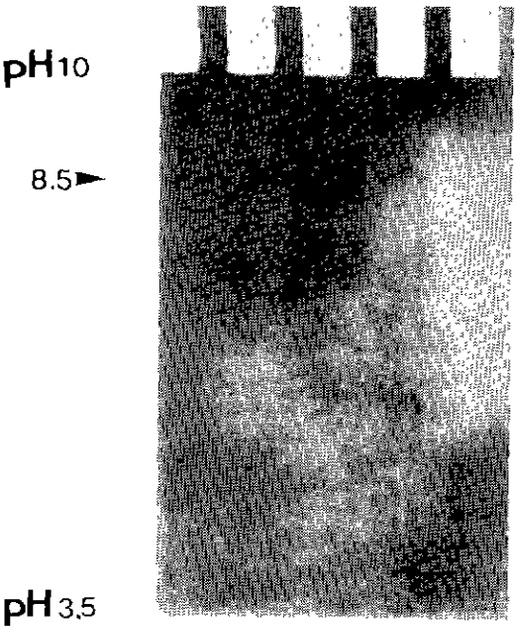

Fig. 3. IEF gel showed ioelectric point of 36 kDa OMP of *V. vulnificus*.

Polyclonal antibody production

Rabbit, immunized with eluted 36 kDa protein produced anti-OMP serum for 5 weeks. Antibody was titrated by ELISA. Final titer of antibody was $100 \times 2^5 : 1$. Anti-36 kDa antiserum determined by wester blotting assay with *V. vulnificus* ATCC 27562, some kinds of isolated *V. vulnificus*. 36kDa protein on membrane reacted with antiserum (Data not shown).

Bacterial agglutination

Anti-36 kDa serum were tested to check agglutination activities toward whole cells of *V. vulnifcus*. Prepared *V. vulnificus* reacted with anti-36 kDa serum on slide. As Table 1, anti-36 kDa serum agglutinated the whole cell of *V. vulnificus*.

Inhibition of intestinal colonization

Anti-36 kDa serum produced protection against colonization of *V. vulnificus* to intestine of animal (Table 2). Serum treated *V. vulnificus* were reisolated from animal intestine less than untreated *V. vulnificus*.

Discussion

The surface materials of *Vibrio vulnificus* was important function in invasion to host aniaml. [9] Especailly outer membrane protein (OMP) have been reported as apparatus for adherence and invasion. [23] On previously study, we observed that OMP of *V. vunificus* associated with pathogenecity and antigenecity of this bacteria. The important one of OMPs was 36 kDa major protein.

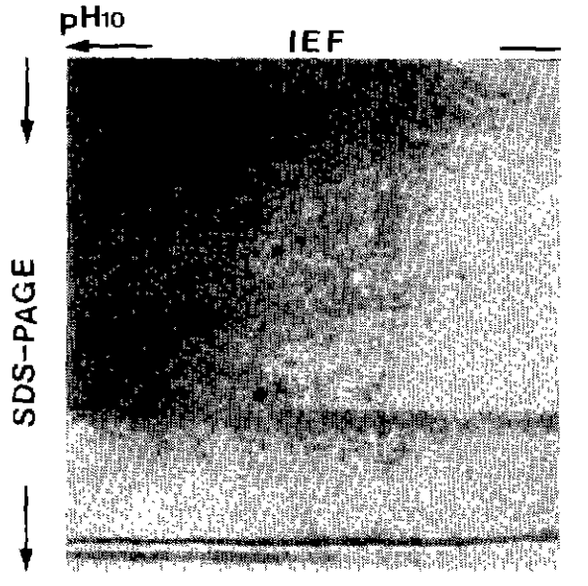

Fig. 4. Two dimentional electrophoresis of 36 kDa OMP of *V. vulnificus*.

Table 1. Bacteiral agglutination test of *Vibrio vulnificus* with anti-36 kDa OMP serum.

	Two fold dilution of antiserum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Agglutination[a]	+++	+++	++	++	+	+	+	-

[a]Cell suspension in dilluted serum was incubated for 10 min at roomtemperature.

Table 2. Iinhibition of colonization test of *Vibrio vlnificus* with anti-36 kDa OMP serum.

	Total Number	*V. vlnificus* on TCBS
Serum treated *V. vlnificus*[a]	200 ± 3	8
Serum untreated *V. vlnificus*[b]	296 ± 3	76
Control(Saline)[c]	178 ± 3	

[a]*V. vlnificus*(2×107, CFU) were injected to small intestine by operate, [b]*V. vlnificus*(2×107, CFU) were injected after incubation with anti-36 kDa protein serum for 30 min at 37℃, [c]Saline was injected without bacteria.

Here, we examed electophoretical and serological characteristics of this 36kDa protein. Elecrophoreticl analysis of 36 kDa protein was runned by SDS-PAGE, isoelectric focusing (IEF) and two dimensional electrophoresis pattern. Major 36kDa and 25, 46, 48, 66kDa protein were detected by Coomassie blue stain on SDS-PAGE. These protein were sarcosine insoluble phase and the profile of OMPs was simillar with report of Elana [8] *et al.*

Major 36 kDa protein was eluted for production of antiserum for serological analysis, IEF and two dimensional electrophoresis. Isoelectric point of 36 kDa was aout pH 8.5 which is simillar with OMP of *V. cholerae.* Two dimensional electrophoresis of eluted 36 kDa showed one point on the gel. So, we conclude 36 kDa protein contain one molecule.

Anti-36 kDa serum made by newzilland rabbit for serological test. Titer of serum was determined by ELISA and western blotting assay. In ELISA, final titer of antibody was 100×2^5 : 1. Neutralize ability of serum was examined by slide agglutination test and colonization inhibition test in rat. The former was associated with in vitro test and the later was associated with in vivo test. Anti-36 kDa serum agglutinated whole cell of *V. vulnificus* on slide agglutination test. Serum treated *V. vulnificus* were inhibited colonization on intestine in rat. So, we expect that 36 kDa protein was useable material for protection against infection of *V. vulnificus.*

Accordingly In this paper contain some electrophoretical analysis and serological test of 36 kDa OMP of *V. vulnificus.* For this protein was applied to active vaccine or serum passive vaccine, more examination for molecular charaterization still remain.

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