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Full Length Research Paper

Expression, purification and antigenic evaluation of toxin-coregulated pilus B protein of *Vibrio cholera*

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The toxin co-regulated pilus B (TcpB) as well as tcpA has been verified as a critical colonization factor for *Vibrio cholera* O1. TcpB is a candidate for making subunit vaccine against cholera; this study aims to produce an oral vaccine by expressing recombinant toxin co-regulated pilus B in *Escherichia coli* (*E. coli*). The toxin co-regulated pilus B (tcpB) gene was amplified by polymerase chain reaction (PCR) method. PCR product was sub-cloned to prokaryotic expression vector PET32a, was transformed in *E. coli* BL21 (DE3) and was induced by Isopropyl_β-D-1-thiogalactopyranoside (IPTG). Recombinant protein was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and purified by Ni-NTA resin. The immune response to TcpB in animal model was studied. PCR product of tcpB gene has 1297 bp and it was confirmed by sequencing. In SDS-PAGE analysis a band with 65 kDa was seen. In patient recovering from cholera and animal model such as Mice, rabbit with oral inoculation, high titer of antibody in serum was detected. These results demonstrated that the recombinant TcpA is antigenic and can be used in a carrier host as an oral vaccine against cholera.

Key words: Antigenicity, *Escherichia coli*, recombinant protein, toxin co-regulated pilus B.

INTRODUCTION

Vibrio cholera is a causative agent of the intestinal disease (Herrington et al., 1988). *V. cholera* is important in gram-negative motile enteric pathogen, which is spread via the fecal-oral route (Manning, 1997) that is, upon passage through the stomach, bacterium wields the single polar flagellum to reach the epithelial surface in the intestinal crypts where it colonies and expression of specific virulence genes occurs (Taylor et al., 1987). Among these virulence factors are the genes encoding the toxin co-regulated pilus (tcp) and tcp biogenesis apparatus, the cholera toxin (CT) genes, as well as activates of these two main virulence factors. Toxin co-regulated pilus (tcp) introduces elementary step to colonization of the bacteria in small intestine (Rhine and Taylor, 1994). Tcp is classified as a type 4 pilus, that are, long, filamentous appendages expressed by a number of Gram-negative bacteria, for example, *Enteropathogenic Escherichia coli* (EPEC), *Enterotoxigenic Escherichia coli* (ETEC) and *Neisseria gonorrhoeae* (Nataro and Kaper, 1998).

Upon colonization the bacterium produces cholera toxin that is composed of two subunits, A and B. The B subunit binds GM1 (monosialotetrahexosylganglioside) on the epithelial cells providing the insertion and cleavage of the A subunit at the membrane (Sandkvist, 2001). Contemporaneous expression of CT and tcp is controlled via the ToxR regulatory protein, that is, ToxR controls expression of another regulator, ToxT, and ToxT directly controls expression of virulence gene (Miller and Mekalanos, 1988). The cholera toxin operons are verified as a section of the genome of the cholera toxin bacteriophage (CTXQ), which utilizes tcp as its receptor (Walder and Mekalanos, 1996). The toxin co-regulated

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pilus (tcp) that is a subtle of polymerized TcpA. The tcp operon is composed of nine tcp-specific proteins but is nearly characterized (Kirn et al., 2003). The 20.5KD tcpA is produced as precursor for the tcp which is processed at the cytoplasmic side of the IM via tcpJ which is a protease (Taylor et al., 2004). The subunits precursors' protein, tcpA is accumulated into tcp via the tcp biogenesis apparatus (Taylor et al., 2004). The biogenesis apparatus Including tcpB, tcpE and tcpJ are thought to form an assembly scaffold; these intermediate proteins are required for tcp assembly. In this report we identify one of the components from tcp biogenesis apparatus, consisted of tcpB. tcpB was originally deduced to amino acid sequence analogously to C of B of ETEC (Taniguchi et al., 1995). The tcpA is the major pilin subunit and 47.5-kD. Tcpb pilin subunit is required for colonization that is also an intermediate subunit as a minor pilin along the shaft, which is perhaps a machinery of the basal structure (Manning, 1997). As mentioned above tcpJ is required for the processing of tcpA, and is also responsible for processing of tcpB (Manning, 1997). However, experimental evidence provides a prediction for tcpB to interact with tcpA in the pili (Manning, 1997). The results of the present study indicate tcpB as a tcp biogenesis apparatus that is required for tcpA assembly and stability.

Isolation, characterization and expression of *Vibrio cholera* tcpB gene in *E. coli* as a host are presented in this paper. We also showed that recombinant *V. cholera* tcpB protein is recognized by infected sera using Western blot analysis.

MATERIALS AND METHODS

Bacterial strains, media and vector

V. cholerae EL-Tor (Inaba) was used throughout this study. Bacterial strain was maintained at -70°C in Lysogeny broth (LB) medium containing 25% (vol/vol) glycerol (Miller, 1972). LB and TCBS (Thiosulfate Citrate Bile Salts Sucrose) media were prepared as described previously (Miller, 1972; Mekalanos et al., 1978). Prokaryotic expression vector pET-32a was used. This vector enables the expresses a fusion protein with a histidines tag, a thrombin recognition site and a T7 tag in N-terminus. These additional amino acids increase the size of expressed protein near 20 kDa. The recombinant pET32a (pET-32a-tcpB) is transformed is in *E. coli*, BL21 (DE3) plysS as host strain.

Isolation of chromosomal DNA

After overnight incubation of *V. cholera* subsp. EL-Tor in LB at 37°C, bacterial cells were centrifuged at 5000 rpm for 2 min and the pellet was re-suspended in 567 µl of TE buffer. Chromosomal DNA prepared according to standard CTAB/NACL method. Briefly, re-suspended the pellet of 1.5 ml overnight bacterial culture in TE buffer(Tris 10 mM, EDTA 1 mM, PH 8), the bacterial cell was lysed by SDS and proteinase K, the chromosomal DNA was extracted by CTAB/NACL solution (10% CTAB and 0.7 M NACL). Remove the cell debris and proteins by two times phenol/chloroform/isoamylalcohole (25:24:1) mixture. DNA precipitated by isopropanol and washed in ethanol (70%), air dried and then re-suspend in TE buffer. Quality and quantity of purified genomic DNA was assayed by 0.8% Agarose gel electrophoresis' in 1×TBE buffer and spectrophotometrically (260/280 nm), respectively (Sambrook et al., 2001).

Primers design

The protein product encoded by the tcpB gene was identified by expression It as 6xHis tag and T7 tag fusion proteins. The tcpB open reading frame was amplified with two synthetic primers containing engineered Xho1 and BamHI sites. Primers were designed according to published sequence for tcpb of Cholera (accession number: FJ209011); Forward: (5' TCG AGC TCA TGA GAA AAT ACC AA 3') and Reverse: (5' ACT CGA GAT TTT CAC ACC ATT GA 3'). The PCR product was digested with BamHI and XhoI are cloned into the fusion protein expression vector pET-32a. The nucleotide sequence of the tcpB gene has been deposited in the GenBank data library under accession number FJ209011.

Gen amplification of tcpB

PCR was performed in a 50µL total volume containing 500 ng of template DNA, 1 µM of each primers, 2.5 mM Mg^{2+}, 200 µM (each) deoxynucleoside triphosphates, 10× PCR buffer and 2.5 unit of Pwo DNA polymerase (Roche). The following conditions were used for amplification: hot start at 94°C for 5 min, followed by twenty five cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extention at 72°C for 1 min. The program followed by a final extension at 72°C for 5 min. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1× TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified from the agarose gel by high pure PCR product purification kit (Roche) according to manufacturer recommendation. PCR product was checked by electrophoresis in 1% agarose gel in 1× TBE buffer.

Cloning of tcpB gene in bacterial expression vector

The PCR product was digested with BamHI and XhoI and cloned into the fusion protein expression vector pET-32a, which digested by the same restriction enzymes, that is, by T4 DNA ligase (cinagene). At 16°C over night *E. coli* DH5α and *E. coli* BL21 (DE3) plysS competent cells were prepared by calcium chloride method and used for transformation of pET32a -tcpB plasmid. The transformed bacteria were selected by screening the colonies on antibiotic containing media and plasmid purification. The suspected colony further analyzed by restriction enzymes digestion and PCR.

Expression and purification of recombinant tcpB

E. coli BL21 (DE3) plysS was transformed with pET32a-tcpB and grown in 2 ml LB broth supplemented with Ampicillin (100 µg/ ml) and chloramphenicol (34 µg/ml) at 37°C with agitation. A colony contained with recombinant plasmid was cultured on shaking incubator for overnight at 37°C in 2 ml LB medium containing 100 µg/ml Ampicillin and 34 µg/ml chloramphenicol. The next day, 500 µl of culture was removed and inoculated in 50 ml LB broth (per litre: 10 g yeast extract (Difco), 20 g bactotryptone broth (Difco), 0.2% (mass/vol.) glucose, 10 g NaCl, 1 g KCl, 0.5 g $MgCl_2$, 0.5 g $CaCl_2$) and incubated at 37°C, shaking at 200 rpm with vigorous agitation to an absorbance of 0.5 at 600 rpm. Expression of the tcpB protein was then induced by the addition of Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1mM and

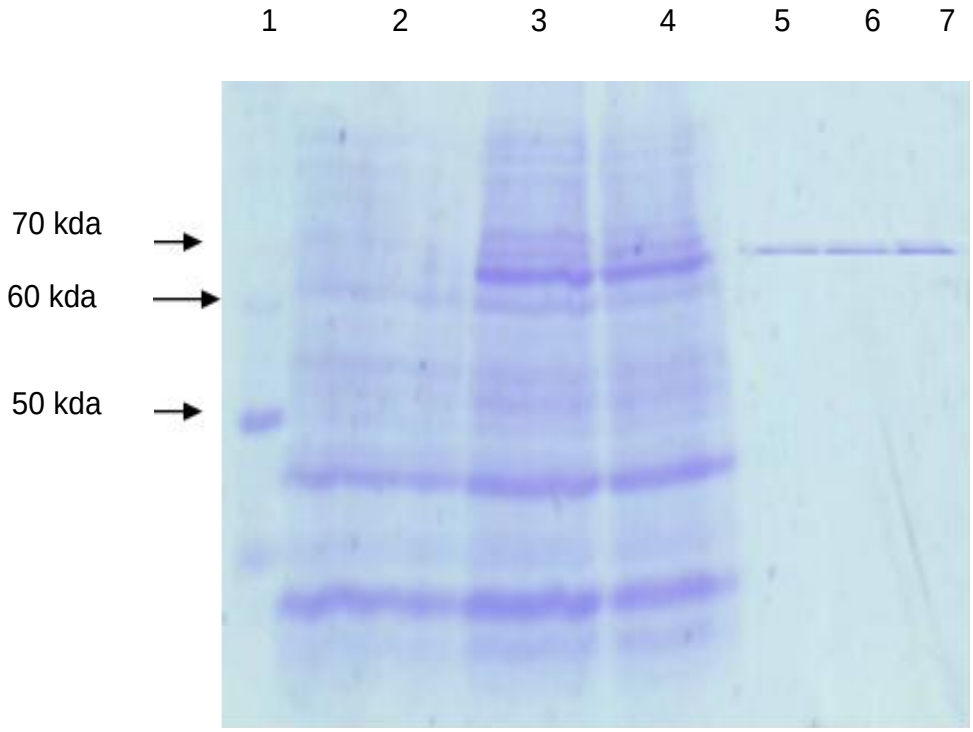

Figure 1. Expression and purification of recombinant tcpB Ni-NTA purification of recombinant toxin co-regulated pilus produced in *E. coli* and stained with coomassie brilliant blue. Lane1: marker, lane 2: uninduced cells of pET32a-tcpB without using IPTG, lane 3, 4: induced cells of pET32a-tcpB with using IPTG at a long time 2 and 4 hours, lane 5, 6 and 7: Extract proteins after Ni-NTA affinity chromatography.

incubation was continued for a further 4 h. The expressed protein was purified using Ni-NTA column according to manufacturer's instruction (Qia gene). The purified protein was dialyzed twice against PBS (pH 7.5) at 4°C overnight. The quality and quantity of purified recombinant tcpB protein was dialyzed by SDS-PAGE (15%) and Bradford methods, respectively.

Antigenicity and Immunoblot analysis of recombinant tcpB

For preparation of primary antibody 100 µg of emulsion containing bacteria and complete freund´s Adjuvant (50 µg *vibrio cholera*e + 50 µg complete freund´s Adjuvant sigma, St. Louis Ma) at three weeks were injected into five mice, rabbit and acute phase patients and sera received as a gift from Dr. Amozande (Immunology Department, Iran, Arak). After 21 days Injections were repeated by replacing incomplete freund´s Adjuvant (50 µg *vibrio cholera*e + 50 µg incomplete freund´s Adjuvant). 10 days after the second injection, sera separated were used as primary antibodies.

RESULTS

DNA extraction

The Chromosomal DNA of *Vibrio cholera* was extracted and concentration was adjusted to 250 µg/ml. This DNA was used as a template for amplification of tcpB gene. PCR product had expected size of 1297 bp compare to 100 bp DNA ladder (Fermentas). The sequencing result was confirmed by comparing with databases and using basic local alignment search tool (BLAST) soft ware (data not shown).

Expression and purification of recombinant tcpB

pET32a -tcpB in *E. coli* BL21 (DE3) plysS was induced and the expression protein was purified by Ni-NTA column (Figure 1). SDS.PAGE analyses, showed the expected molecular mass of near 65 kDa recombinant protein. The concentration of recombinant protein was Assayed and calculated to 400 mg purified protein per liter of the initial culture.

Western immunoblot analysis

To determine the antigenicity of recombinant tcpB in mice, human and rabbit immunized with *Vibrio cholera*, the recombinant tcpB was assayed by Western-blotting. Figure 2 shows the specific interaction between standardized antibody and purified recombinant tcpB

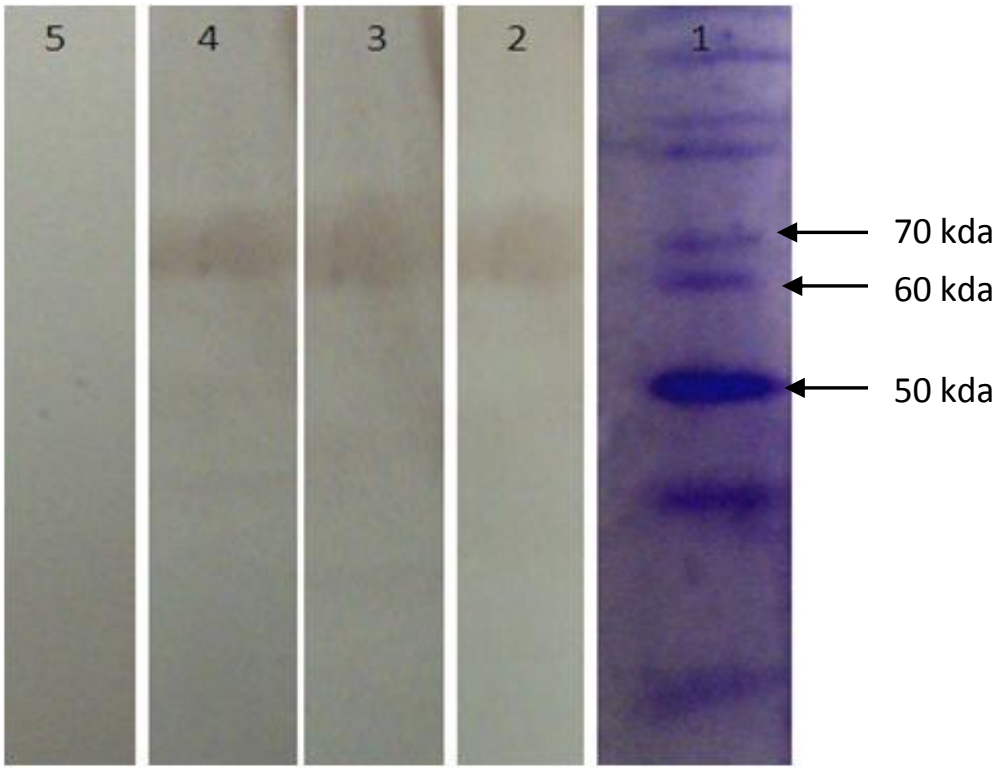

Figure 2. Western blotting analysis of tcpB extracts using anti- tcpB rabbit, human and rabbit antiserums. lane 1 : Protein marker, lane 2 : interaction between serum of Immunized mice with purified recombinant tcpB protein , lane 3 : interaction between serum of Infected human with purified recombinant tcpB protein, lane 4: interaction between serum of Immunized rabbit with purified recombinant tcpB protein, lane 5 : control negative.

protein.

DISCUSSION

Cholera is an acute diarrheal disease leading to death by severe dehydration without appropriate treatment, especially in developing countries. *Vibrio cholerae* is mainly a fecal-orally transmitted and humans are the only known natural host. Cholera has been endemic in southern Asia. Cholera has spread in seven pandemic since 1817. In 2008, the WHO reported 190,130 cholera cases worldwide, associated with 5143 deaths (98% in Africa), but cholera is globally under-reported and the true disease burden is estimated to be in the millions. In addition to endemic outbreaks, sporadic outbreaks can occur whenever sanitation and clean water provisions are lacking, such as occurred in Zimbabwe between 2008 to 2009. The ability of *V. cholerae* to persist in water will continue to confound our ability to eradicate cholera and thus cholera vaccines are needed. *V. cholerae* utilizes adhesion factors some of which may remain to be elucidated, but may include O1 LPS; GlcNAc-binding protein (GbpA); a protein (tcpF) secreted by the toxin coregulated pilus (tcp) biogenesis apparatus; outer membrane protein OmpU and cholera toxin (CT), although this has only been implicated in an adult rabbit model. Tcp facilitates inter-bacterial interactions that are important for colonization. An effective cholera vaccine could prevent colonization by inducing the production of antibodies that directly neutralize the function of key colonization factors and/or facilitate phagocytosis and killing through bacterial opsinization (Bishop and Camilli, 2011).

Tcp of *V. cholera* belongs to a subgroup of type-4 fimbriae and is expressed by both classical and ELT or strains of the O1 serotype, as well as O139 Bengal (Manning, 1997). In the present study, we sought Antigenic activity the tcpB, within the virulence factors from *V. cholerae* serotype inaba. Toxin-Coregulated Pilus B (tcpB) would be expected to be important element in pathogenesis in *V. cholera*. Tcpb is a predicated major pilin subunit protein with a molecular mass of 49.5 kDa and some similarity to tcpA in this pilus (Manning, 1997). In this study, recombinant protein TcpB was expressed in *E. coli* BL-21 (DE3) plysS bacteria through expression vector pET32a mediated transformation. The presence of 6xHis tag and T7 tag to the N or C terminal of recombinant peptide causes increase near 20 kDa. Therefore, molecular weight of tcpB-pET32a fusion

proteins was found to be 65 kDa. Recent studies (karaolis et al., 1998, 1999; Walder and Mekalanos, 1996) of *V. cholera* have demonstrated that tcp gene cluster occupied a *V. cholera* pathogenicity island which genes of lysogenic filamentous phage were included and tcp, specific tcpA acts as a receptor foe cholera toxin phage (CTXQ). On the other hand, this would show a marker for tcpB to indicate more importance of its function in pathogenicity pilus. As simple as this sound tcpB can be evaluated as a vaccine antigen. These results have demonstrated the value of tcpB in the genetic analysis of bacterial virulence and its potential application in the field of vaccine development. We suggest that tcpB in combination with other molecular subunits of *V.cholera* would provide superior protection to infection because solid protective immunity requires immunization with several parasite proteins rather than a single moiety.

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