Original Article

Expression of Recombinant Zonula Occludens Toxin (Zot) ofVibrio Cholerae and Biological Activity on Rabbit Ileal Loops

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Abstract

Introduction: Vibrio cholerae causes potentially lethal disease, cholera, through the elaboration of the intestinal secretion of cholera toxin .The cholera enterotoxin (CT) has been considered as the major virulence factor of V. cholerae. This microorganism also produces other putative toxins such as Zonula Occludens toxin (Zot) and Accessory cholera enterotoxin (Ace). Zot has the ability to reversibly alter intestinal epithelial tight junctions, allowing the passage of macromolecules through the mucosal barrier. The Zot toxin alters ion transport and causes fluid accumulation in ligated rabbit ileal loops which results in mild diarrhea.

Materials and Methods: In this study, gene coding for the Zot toxin was amplified from V. cholera isolate 62013. The PCR product containing the Zot gene was cloned in pET28a expression vector .The recombinant Zot gene was transformed into E. coli (DH5 α) and then retransformed into E. coli Tuner for expression. Expression of recombinant protein induced by isopropythio-β-D-galctoside (IPTG) at different concentration and was examined by SDS-PAGE and Western blotting. Rabbit ileal loops experiment was conducted.

Results: Cloning of Zot was confirmed by colony-PCR and enzymatic digestion. The recombinant Zot with molecular weights of 45KDa and 22kDa was expressed and reacted with rabbit anti-Vibrio cholerae polyclonal antibody in western-blot analysis. Zot protein significantly causes fluid accumulation in ligated rabbit ileal loops test.

Conclusion: Our findings indicated that a prokaryotic expression system for Zot protein was successfully constructed. This expression system can be useful as a tool for production of Zot protein for vaccine purposes.

Keywords: Vibrio cholerae, pET28a, Zonula Occludens toxin, E. coli Tuner, ileal loop.

Introduction

Vibrio cholerae (V. cholerae) is a major human intestinal pathogen which causes significant morbidity and mortality in several developing countries. Currently, cholera is the most important diarrheal disease affecting human worldwide and is endemic in southern Asia and part of Africa and Latin America with 5 million cases each year. Cholera presents with a intense diarrhea caused by toxigenic V. cholerae, colonized in the small intestine (1.2, 4, 7). Cholera toxin (CT) is responsible for severe dehydrating diarrhea associated with this microorganism that produces other recognized toxins such as Zonula Occludens Toxin (Zot) and Accessory Cholera Toxin (ACE) as well.

Zot is the second toxin of Vibrio cholerae that has synergistic role in acute dehydrating diarrhea typical of cholera. The genes encoding the toxins (CT, Zot, ACE) are located on a 4.5 Kb region termed "the core region" or virulence cassette, that is flanked by two copies of repeated sequence (1). The Zot protein seems to be required in the CTXΦ morphogenesis (4, 5, and 10). Zot is a 399 amino acid enterotoxin that is predicted to have a single transmembrane region between residues 226–246 (6). The 153-residue Zot C-terminal domain (Zot -CT) faces the bacterial periplasm with the 225-residue Zot N-terminal fragment (Zot -NT) residing in the cytoplasm (3, 4, 6). Zot is a single polypeptide chain of 44.8 kDa localized in the bacterial outer membrane with subsequent cleavage (4, 11). This cleavage leads to establishment of a 33-kDa amino-terminal fragment that remains associated to the microorganism and a 12-kDa carboxyl-terminal peptide that is secreted in the host intestinal lumen milieu and is responsible for Zot enterotoxic activity (2, 4, 5, 8). Zot has the capability to increase the permeability of the small intestine by affecting the organization of epithelial tight junctions.

The Zot mechanism of action involves a rearrangement of epithelial cell cytoskeleton due to protein kinase Cα-dependent F-actin polymerization which results in opening of tight junctions which depends on binding of Zot to specific receptors (2, 4, 6,). Zot does not cause tissue damage and its effect on intestinal permeability is time and dose dependent and fully reversible. Because of these properties, Zot can be used for mucosal delivery of drugs and macromolecules that do not normally cross the epithelial barrier and may be an ideal adjuvant for development of mucosal vaccines (2).

This paper describes cloning, over expression and

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purification of Zot toxin from E.coli Tuner (host expression). The biological activities of Zot were tested in vivo with rabbit ileal loop assay.

Materials and Methods

2.1 Bacterial strains and vector The V. cholerae - strain 62013 was prepared from Pasteur Institute of Iran. We used E. coli DH5α for cloning and E. coli Tuner for expression of recombinant Zot that were obtained from Invitrogen and Novagen (USA) respectively. Plasmid pET-28a+ as expression vector was provided from Novagen. Bacteria were cultured in LB Broth or on agar (Merck, Germany) with or without 30µg Kanamycin/ml (Sigma, USA).

2.2 Preparation of DNA template and PCR Genomic DNA of V. cholera strain 62013 was extracted by Bioneer kit (South Korea) and concentration and purity of extracted DNA were determined by spectrophotometry. The specific primers were designed according to Zot sequences of V. cholerae from NCBI. The sequence of forward primer with an endonuclease site of BamHI and reverse primer with an endonuclease site of HindIII were 5':TAGGATCCATGAGTATCTTTATTCATCACGGC3 and 5'CGAAGCTTTCAAAATATACTATTTAGTCCTTTT TTATC -3', respectively.

The PCR reaction mixture comprised: 0.5µM of each primer, 10µl 5X prime STAR buffer, 0.2 mM of each dNTP, 2.5U of prime STAR DNA polymerase (Takara, Japan) and 200 ng genomic DNA in a final volume of 50µl. PCR amplification was performed with an initial denaturation at 98°C for 4 min followed by 35 cycles of 98°C for 10 sec, 55°C for 10 sec, 72°C for 75 sec and 10 min at 72°C for final extension. PCR products were analyzed by electrophoresis on 1 % (w/v) agarose gel (Fermentas) and then the 1200bp DNA fragments were recovered from the gel using PCR purification kit (Bioneer, South Korea).

2.3 Cloning, Expression and Purification of Zot The PCR product and pET-28a (Novagen United States) expression vector were digested by BamHI and HindIII and then purified from agarose gel. The resultant fragment was ligated by T4 DNA ligase (Fermentas) and the recombinant pET28a was transformed to E. coli DH5α. The integrity of the recovered plasmid was confirmed by restriction endonuclease digestion and sequencing by a commercial facility using universal forward and reverse T7-promoter and T7-terminator primers (TAG Copenhage A/S Symbion, Denmark). The result of sequencing was compared to the sequence of Zot gene in the database using NCBI Blast software. The recombinant plasmids retransformed to expression host (E.coli Tuner).

Several conditions for the expression were tested such as temperature of induction and concentration of IPTG. The E. coli host expression, Novablue and BL21 could not express Zot protein under any of conditions tested. However, the E. coli Tuner (Novagen) was found to be a suitable host to express the Zot protein in presence of kanamycin (30µg/ml) (8). Bacterial cells were cultured in presence of kanamycin at 37º C with shaking (220 rpm) until an optical density at 600nm of 0.6-1 was reached and expression was induced by adding 0.1 mM isopropyl-L-D-1-thio-galactopyranoside (IPTG) and harvested 3 h later. Titration of IPTG concentration versus Zot expression showed that 0.1 mM was optimal. Cells were harvested by centrifugation at 10000g for 10 minutes at 4o C to precipitate the pellet which was frozen at -20o C. The bacterial pellets were lysed by lysis buffer (8M urea) until the solution cleared. After centrifugation, supernatant were examined by SDS-PAGE to verify expressed recombinant protein.

The Zot protein was purified by Ni-NTA affinity chromatography under combination of denaturing and native conditions by binding, washing and eluting steps according to manufacture protocol (Invitrogen). In this protocol, proteins were finally eluted in 20mM imidazole- containing buffer. The eluted proteins were immediately dialyzed against PBS, pH 7.4, to remove imidazole. Protein concentrations were determined by Bradford and NanoDrop analysis and the purity was established by SDS-PAGE (4, 17).

2.4 Producing anti-Zot polyclonal antibodies The overnight culture of V. cholerae 62013 was exposed to formalin (1.5%) at 4° C. After washing and deformalization, it was heated at 65° C for 1 h. The lysate was injected subcutaneously to white New Zealand rabbits weighing about 2 kg. The injection mixture contained ~109 bacteria per 1 ml of physiological serum and 1 ml complete adjuvant (for first injection). After homogenization, 0.5 ml of the injection mixture was injected under shoulder subcutaneously. Incomplete adjuvant had been used as booster on the days 14, 28 and 42. Blood sampling was performed before each injection and blood serum was separated and stored at -20°C until required (12).

2.5 Western blot analysis Following SDS-PAGE, proteins were transferred to 0.45 mm pore size PVDF membrane (Hi-bond Amersham Biosciences, USA) by means of a semidry blotter unit (Bio-Rad). The Non-specific binding sites were blocked by 3% (w/v) skim milk according to standard procedures. The rabbit polyclonal anti-Vibrio cholera serum was diluted to 1:500 in phosphate-buffered saline (PBS) 0.1% (v/v) Tween 20 and incubated for 3 h at 4° C with shaking. Membranes were washed with PBS-Tween 20 and then incubated for 1h with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (HRP) (Sigma) at a 1:2,500 dilution in PBS-Tween 20. Membranes were then washed three times with PBS-Tween 20 and exposed to the substrate DAB solution (Sigma, USA) (13, 17, 18).

2.6 Ligated Rabbit ileal loop New Zealand rabbits weighing 2-2.5kg were starved for 48h before experiments. Rabbits were anesthetized by subcutaneous

injection of a mixture of ketemine (50mg/kg), acepromazine (0.5mg/kg) and the small intestine was tied off 6 segments. One ml (500µg/ml) of the purified Zot protein was injected into the two segments and 1 ml (108 CFU/ml) of the toxigenic V. cholerae 62013 used as a positive control and sterile PBS used as a negative control. Each test was done in two rabbits and all rabbits were sacrificed after 18 h. The enterotoxic response was determined by measuring the fluid accumulation (FA) ratio, which is the ratio of the volume of fluid accumulated in the intestinal loop to the length of the loop. A ratio of greater than 1.0 indicated a strong positive response, while a negative response was defined as FA ratio of less than 0.5 (14, 15, 16).

Results

33.1 DNA extraction and PCR amplification of Zot gene

Genomic DNA of V. cholerae 62013 was successfully extracted by using the Bioneer kit. The zonula occludens toxin (Zot) gene was amplified from genomic DNA that produced a single bond of 1200bp (figure 1).

3.2 Identification of the recombinant pET28a-Zot by PCR and enzymatic digestion

The 1200-bp PCR product was digested with BamHI and HindIII restriction enzyme (figure 2) and ligated to the matching sites of digested pET-28a. The recombinant Zot gene was transfected into competent DH5α E. coli. Plasmid extraction was performed from single colonies appeared in LB-agar plate after 18-24 h incubation in 37°C using plasmid extraction kit (Bioneer, South Korea). Restriction enzyme analysis was also carried out on the recombinant plasmids containing the Zot gene, using BamHI and HindIII enzymes. Agarose gel analysis showed that the extracted plasmids contained the goal gene (Fig2).

Additionally, PCR by specific primers and extracted plasmid as template DNA was performed and presence of target gene in recombinant vector was confirmed by electrophoretic detection of amplified 1200 bp DNA fragment from extracted plasmids.

3.3 Expression and purification of target recombinant protein The Tuner competent cells were transformed with confirmed recombinant vectors and induced with IPTG (final concentration=1mmol) to express target recombinant protein. Total protein was electrophoresed on 12% SDS-PAGE gel and stained with coomasi blue. Purification of His-tagged Zot by metal chelating yielded two bands, one at ~22 kDa and the other at ~45 kDa (figure 3A) (8). The large scale culture and induction were performed and the resulted protein was purified by Ni2+affinity chromatography under denaturing and native condition (Figure 3B).

3.4 Western blot analysis To verify protein size and protein purity and to identify the recombinant proteins, Western blot analyses were used. Western blot analysis was performed to detect antigenicity of expressed protein. By using of both V. cholerae anti-Zot antibody and goat anti-rabbit immunoglobulin G horseradish peroxidase (HRP) conjugate antibody, brown strip corresponding to the site of the recombinant protein appeared on PVDF membrane after adding DAB solution which confirmed the antigenicity of our protein (figure 4).

3.5 Rabbit ileal loop test with recombinant protein

Five hundred µg of the purified Zot protein was injected into the rabbit ileum, which induced significant fluid accumulation (FA ratio 1.75+/- 0.2, figure 5). Fluid accumulation of PBS (negative control) loops were not significant (FA ratio 0.5+/- 0.005), however, the fluid accumulation of positive control were significant (FA ratio 2+/-0.2) (figure 5).

Discussion

The second potential enterotoxin in V. cholera, reported by Fasano and named Zot for Zonula Occludens toxin, affects the structure of epithelial tight junctions (7). Baudry et al identified and localized the gene encoding Zot in 1991 and in 2001 gene encoding Zot protein was cloned in plasmid vector pQE30 and expressed in E. coli (4, 18). In 2007 Zot gene was cloned in pQE80 vector and was expressed in E. coli strain UT5600, DH5α and BLR (6, 8). In this study we were capable to clone and express Zot gene in E. coli Tuner, because the E. coli host expression, Novablue & BL21 could not express the Zot protein under any of conditions tested. The 1200bp fragment of Zot was amplified by PCR, cloned in pET-28a and then transformed into E. coli Tuner expression host. Cloning of Zot was confirmed by colony-PCR and enzymatic digestion. The results of SDS-PAGE showed that our constructed prokaryotic expression system pET28a- Zot –Tuner efficiently produces target recombinant protein. Our study indicates rZot cause fluid accumulation (FA) in rabbit ileal loops that was greater than 1. These findings confirm biological activity of recombinant Zot that we have produced (16). Jeandrot et al. revealed that the measurement of CRP and PCT might be noteworthy to distinguish between infected and uninfected diabetic foot ulcers (12). However, the role of PCT as a diagnostic marker in local infections such as soft tissues, bone and joints is uncertain and demands more studies to confirm its diagnostic value (9,10,13,14). Zot displays multiple characters that make it the hopeful tool presently available to increase drug and peptide transport through the intestinal mucosa. Moreover, Zot is a very effective adjuvant that may be useful for improvement of mucosal vaccines against pathogens of the respiratory, gastrointestinal and urogenital systems (1,3, 5, 9, 18,). A mammalian analogue of Zot named zonulin acts as an endogenous regulator of tight junctions. Zot and zonulin bind to the same receptor on intestinal epithelial cells (2, 8, 9,). From this point, producing and understanding activity of this protein is important.

Conclusion

In this study, the Zot gene was successfully cloned in pET 28a+ vector and expressed in E. coli Tuner host strain. The cloning of gene encoding the Zot protein facilitated the production of pure proteins free from other vibrio antigens for research as potential vaccine candidates with the aim of developing therapeutic and preventive strategies.

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