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Cloning, expression and purification flagellar sheath adhesion of *Helicobacter pylori* in *Escherichia coli* host as a vaccination target

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Purpose: *Helicobacter pylori* is a widely distributed gram-negative bacterium that infects the human stomach and duodenum. HpaA is a *H. pylori*–specific lipoprotein that has been shown to be an effective protective antigen against *H. pylori* infection. HpaA of *H. pylori* as a vaccine antigen is fully competent for stimulation of immune responses. The aim of this project is cloning, expression, and purification flagellar sheath adhesion of *H. pylori* in *Escherichia coli* host by fast protein liquid chromatography (FPLC) as a vaccination target.

Materials and Methods: The *hpaA* gene was inserted into pET28a (+) as cloning and expression vectors respectively. The recombinant plasmid (pET-hpaA) was subjected to sequencing other than polymerase chain reaction (PCR) and digestion analysis. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactoside to cultures of *E. coli* strain BL21 transformed with pET-hpaA. Protein expression assessed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein purification of flagellar sheath adhesion was by FPLC.

Results: The restriction endonuclease digestion, PCR amplification analysis showed that the *hpaA* gene of 730 bp was amplified from *H. pylori* DNA and sequencing analysis of the pET-hpaA confirmed the cloning accuracy and in frame insertion of *hpaA* fragment. SDS-PAGE analysis showed the expression of an approximately 29,000 Da protein.

Conclusion: Sequencing results along with SDS-PAGE analysis confirms the expression of recombinant *hpaA* in the heterologous *E. coli* BL21. Conclusion A prokaryotic expression system for *hpaA* gene was successfully constructed. These results indicate that production of a specific recombinant protein is an alternative and potentially more expeditious strategy for development of *H. pylori* vaccine.

Keywords: Recombinant flagellar sheath adhesion, *Helicobacter pylori*, Fast protein liquid chromatography

Introduction

Infection caused by *Helicobacter pylori* is one of the most commonly bacterial infections in the world. *H. pylori* is a gram negative, microaerophilic, and curved rod-shaped bacterium that has ability to colonize and growth in the human stomach. A wide range of digestive problems such as stomach acute inflammation, gastric and duodenal ulcers, chronic peptic ulcer disease, peptic ulcer bleed gastric carcinoma, gastric

lymphoma B cells and extra-intestinal diseases have been caused as a result of the gastric mucosa damage by this bacterium and even is considered as a risk factor for stomach-intestinal metaplasia and stomach cancer development [1-4]. In developed countries, *H. pylori* infection in less than 50% of the adult population and in developing countries such as Iran, the infection rate in the adult population was higher than developed countries and it was close to 80% [5]. This bacterium has been classified recently as class 1 carcinogen by the World Health Organization. In most cases the *H. pylori* infection is not eradicated despite strong immune response against this infection. Despite the relative clearance of bacteria with current treatment options, the occurrence of antibiotic resistance, the incidence of reinfection, lack of affection on passive forms of bacteria and the high cost of treatment has been reported in this case. Therefore, necessity of acquiring an alternative method is proposed to treatment (such as a vaccination) or prevention of this infection [6]. The knowledge of effective and protective immune response type against bacteria identify the suitable bacterial antigens to stimulate immunity and immune response is considered as main points to achieve the effective vaccines [7]. Over the past years a variety of *H. pylori* antigens including bacterial cell lysate, cytotoxin-associated antigen (CagA), vacuolating cytotoxin (VacA), low doses of live bacteria (noninfective dose), purified antigens such as urease and heat shock proteins, configuration of individual or complex forms in combination with toxin or lipopolysaccharide have been evaluated, but there is no vaccine for this purpose yet [8]. According to the available studies, flagellar sheath protein of bacterium (*Helicobacter pylori* adhesion A, HpaA) is proposed for protecting as a suitable antigen because of its high level of conservation [9]. HpaA belongs to a group of bacterial outer membrane proteins that plays an important role in bacterial attachment as an adhesion factor. Binding to sialic acid could be mediated by this adhesion. The tightly bound to cell surface receptors of these molecules lead to the appearance of mucosal lesions. In addition, the increment of toxins and bacterial antigens by epithelial cells is caused by binding the bacteria to epithelial cells. Sialic acid–containing glycoconjugates have been identified on the surface of gastric epithelial cells and human neutrophils. These sialic acid–containing glycoconjugates have been up-regulated upon in contact of *H. pylori*. *hpaA* gene is located in the bacterial genome and its nucleotide and amino acid sequence is significantly conserved. Moreover, antibodies against the HpaA protein are found in approximately 86% in the serum of patients infected with Hp (it is immunogenic in human) that this proportion is much higher than the Hsp (68%) and VacA (68%) and it is like urease B. Production of antibodies against this protein plays the main role in preventing bacterial colonization. Therefore, this protein can be mentioned as a ideal vaccine candidate for *H. pylori* antigen [9-12]. At present, therapeutic strategies and various synthetic medications are used to treat stomach tumors that are accompanied with specified problems, side effects, inefficiencies and high costs. The early gastric clearance of "*Helicobacter pylori*" bacteria can be useful in the prevention of gastric cancer. Therefore, one of the important goals of the vaccine manufacturing and medicine is the necessity of achievement an effective and practical alternative (e.g., vaccination). In this study, we produce the flagellar sheath protein of *H. pylori* (HpaA) with immunogenicity properties that would be effective for vaccines design and production to cancer prevention caused by the *H. pylori* bacterium.

Materials and Methods

Bacterial strain and culture conditions

In this experimental study, *H. pylori* strain 26695 (prepared from Tarbiat Modarres University) was cultured on Brucella agar plates supplemented with 5% defibrinated sheep blood, 10% fetal calf serum, and antibiotics containing vancomycin, nalidixic acid, and amphotericin B. The plates were incubated under microaerophilic conditions (CO_2 5%) at 37°C for 7 days. The biochemical tests including catalase and urease tests were performed to confirm the obtained colonies. DNA was extracted using a DNA extraction kit (Bioneer, Daejeon, Korea). The agarose gel electrophoresis and spectrophotometer were used for the quantitative and qualitative evaluation of extracted DNA [13].

DNA amplification using polymerase chain reaction

Due to the complete *hpaA* gene sequence obtained from NCBI site (National Center for Biotechnology Information), specific primers were designed by Gene Runner software. Primers were designed with *Nde*I and *Xho*I restriction sites. The sequences of the primers are listed follows: forward primer, 5´-AGCCATATGCTTTTAGGTGCGAGCGTGGTGGC-3´; reverse primer, 5´-GTGCTCGAGTTATTTGCCTTTTAATTCCT TGGCGTC-3´.

Polymerase chain reactions (PCRs) were performed in a total volume of 25 μL using 0.5 mM of each primer, 5 mL of

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PCR buffer (5×), 0.2 mM of each dNTP, 2.5 units of DNA polymerase Pfu enzyme (Takara, Tokyo, Japan) and 200 ng of DNA template. The amplification program was as follows: initial denaturation at 98°C for 2 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and followed by 30 cycles of extension at 72°C for 1 minute, and an additional final extension at 72°C for 8 minutes. PCR products were evaluated on a 1% agarose gel under UV light in the presence of ethidium bromide [14]. After the reaction, the PCR products were analyzed on agarose gel, and the desired gene fragments were excised and purified by gel extraction kit (Bioneer).

hpaA gene cloning in pET28a vector

Vector (pET28a) has a 5,369 bp size, kanamycin resistance gene and different restriction sites for various enzymes. In this step, the plasmid which mentioned above was extracted from bacteria that containing plasmid by using mini prep method. Enzymatic digestion was used for two mentioned enzymes with double digestion (cutting DNA with two restriction enzymes simultaneously). A similar action was replicated on the vector pET28a. Insertion was performed at 4°C using T4 DNA ligase enzyme. The chemical method was used for competent cells preparation of in the presence of calcium chloride and Top10 strain. Transformation was carried out by heat shock (at 4°C for 20 minutes, 42°C for 3 minutes, and 4°C for 20 minutes). Afterward the transformed cells were restored by adding Luria-Bertani (LB) liquid medium without kanamycin to transformants and incubated for 2 hours. Bacteria were collected using low-speed centrifugation. Finally bacterial pellets were added to LB medium containing kanamycin for the recombinant clones containing vector. PCR and restriction enzyme digestion were done on the suspected colonies for cloning confirmation and their products were investigated and on 1% agarose gel in the presence of ethidium bromide and UV light [14,15].

Induction and expression of recombinant protein

The expression host *E. coli* BL21 was used for recombinant protein HpaA expression and the bacteria induction was performed by overnight culture in the presence of 50 μg/mL. Two hundred microliters of bacteria which grown in broth media was transferred to fresh liquid medium. The growth of bacterial culture was monitored by an optical density (OD) at 550 nm of approximately 0.5-0.8 as the time T0. The recombinant protein expression was performed using isopropyl-beta-thiogalactopyranoside (IPTG) to a final concentration of 1.5 mM/mL and incubated in a shaking incubator at 20°C for 4 hours [15]. The best induction time was reached by selecting the induced bacteria samples at time T1, T2, T3, and T4 and the highest expressing colony was in T4.

Evaluation of induction by sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method was used with a concentration of 12.5% polyacrylamide gels, a voltage of at 100 V and in the presence of molecular weight markers to confirm the expressed recombinant plasmid. Finally polyacrylamide gels stained with Coomassie blue G-250 and recombinant protein bands were investigated [16].

Protein purification by fast protein liquid chromatography and urea gradient method

Since the His-tag was embedded in vector and according to the designed primers nickel affinity chromatography resin (Ni-NTA chromatography) can be used for protein purification. For cell lysate preparation, bacterial pellets which cultured in 100-mL lysis buffer were resuspended and bacterium pellet was ultrasonically broken by 10 cycles of 40 seconds with 90% speed at 4°C. The suspension was isolated by centrifugation (4°C, 12,000 rpm, and 20 minutes), the supernatant was collected and filtered through 0.45 μm filter [17]. After passing the supernatant of protein, column washing with B buffer was continued until the output of column was approached zero at OD 280 nm. Then the second washing buffer was added to column and all output was stored in a separate dish. The third wash buffer was added and the column output was collected separately too. After the component protein cumulation, electrophoresis analysis SDS-PAGE was performed to verify their purity. In order to remove imidazole from dissolved protein replaced buffer dialysis was used in the presence of phosphate buffered saline buffer. Fast protein liquid chromatography (FPLC) method was used for purification. Proteins are separated from the stems by FPLC based on isoelectric pH. Fractions were collected and the results of each tube were analyzed on acrylamide gel by SDS-PAGE.

Results

Amplification *hpaA* gene by PCR

HpaA protein gene was amplified using Pfu DNA polymerase enzyme. Desirable fragment with 730 bp length was obtained

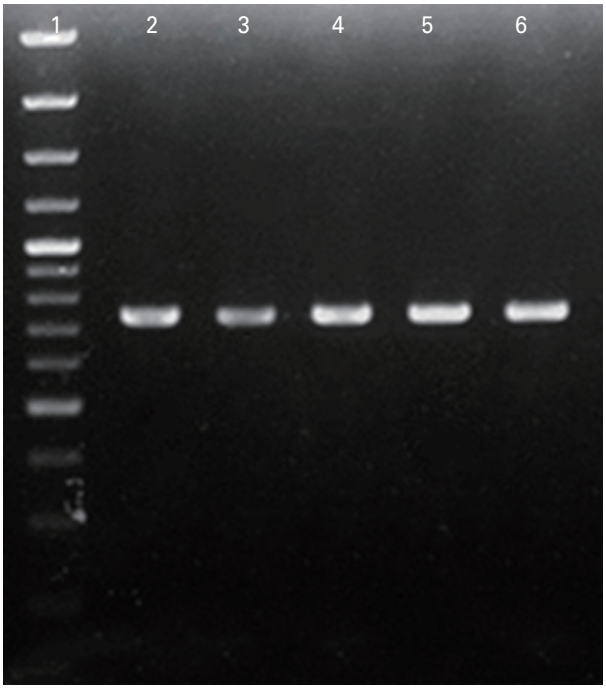

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products stained with ethidium bromide. Lane 1, DNA marker; lanes 2, 3, 4, 5, and 6, amplified *hpaA* gene using pfu DNA polymerase.

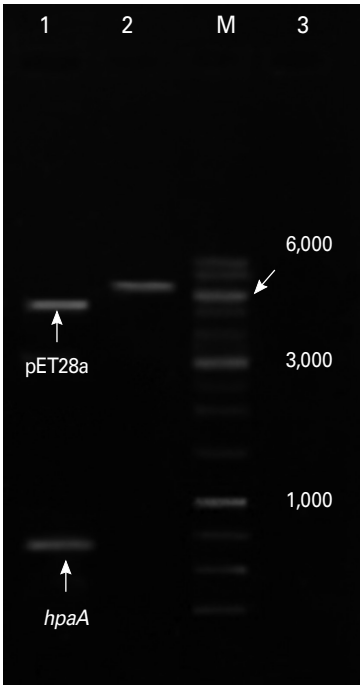

Fig. 2. Evaluation of the recombinant vector enzymatic digestion. Lane 1, the *hpaA* gene and pET28a vector enzymatic digestion; lane 2, pET28a vector carrying the *hpaA* gene; lane M, DNA marker with 10 thousand base pairs length.

on a 1% agarose gel under UV light. An example of agarose gel containing amplified *hpaA* gene is obtained in Figs. 1 and 2.

Verification of cloning by enzymatic digestion

To confirm of the cloning, extracted plasmid from transformed cells were digested by *Xho*I and *Bam*HI and two distinct bands were detected, one at 730 bp and the other at 5,300 bp (Fig. 3).

Induction and expression of HpaA protein in *E. coli* BL21

To evaluate produced protein after induction, first the *E. coli* cells were lysed and dissolved proteins were studied. Dissolved proteins of cells were separated from each other on SDS-PAGE and then assessed after staining with colloidal Coomassie G-250. Since the part of HpaA protein was produced, a protein has obtained with a molecular weight of 29 kD (Fig. 4).

Protein purification

Ni-NTA resin-based affinity chromatography was used to achieve proteins purification. Also the FPLC method was used for purification. Fractions were collected and the results of each tube were determined on acrylamide gel using SDS-PAGE in the presence of a marker (SM0671, Fermentas, Waltham, Ma, USA).

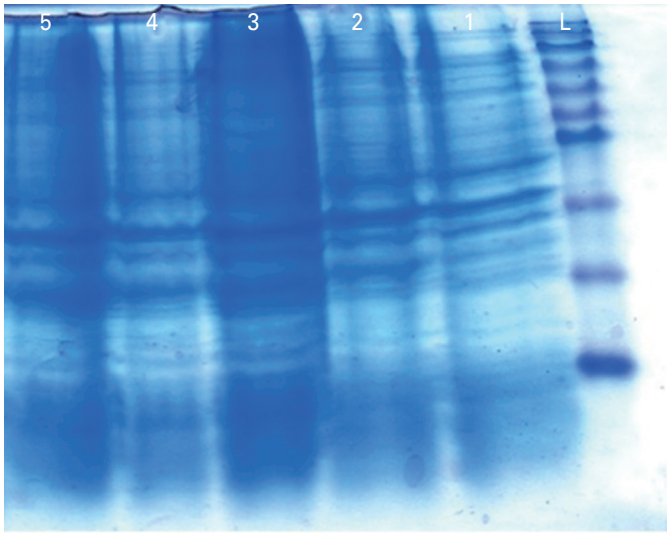

Fig. 3. Assessment of HpaA protein expression on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. Lane L, low molecular weight marker; lane 1, non-induced HpaA; lane 2, induced HpaA by 0.1 mM isopropyl-beta-thiogalactopyranoside.

Discussion

H. pylori infection is associated with wide range of disease such as severe gastrointestinal disease, including stomach

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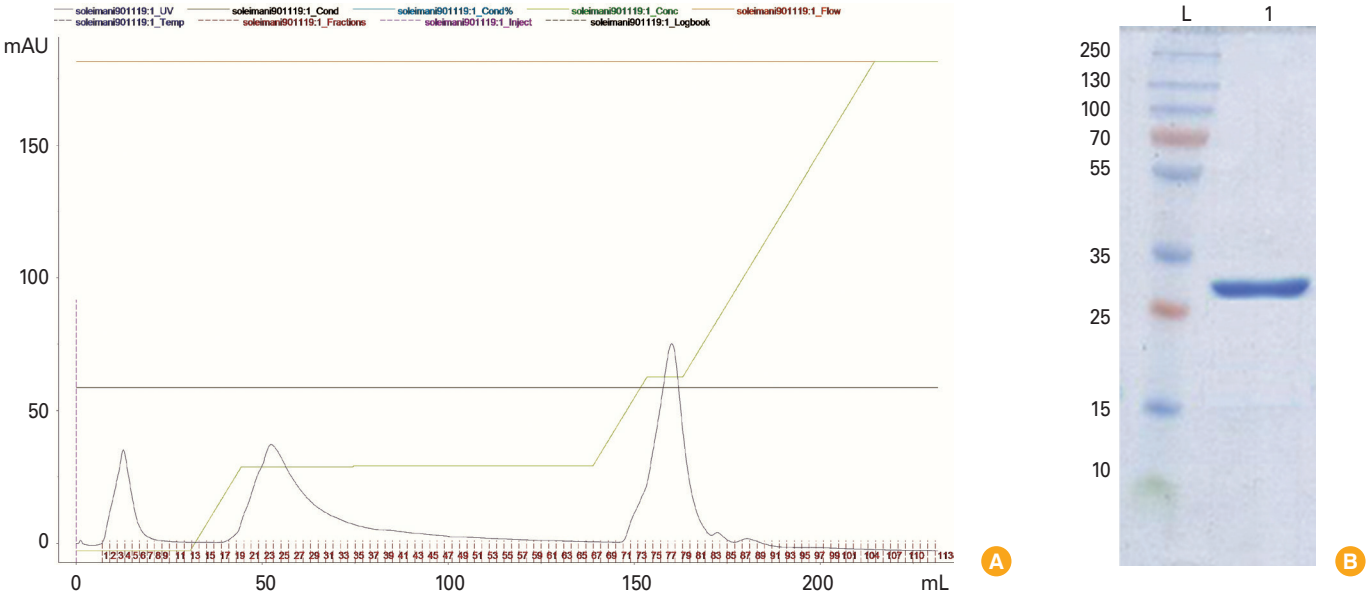

Fig. 4. (A) Purification HpaA protein by fast protein liquid chromatography. (B) Purification HpaA protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. Lane L, low molecular weight marker; lane 1, purified HpaA protein.

chronic inflammation, gastric and duodenal ulcers, gastric carcinoma, gastric B-cell lymphoma, and extra-intestinal diseases such as hepatic encephalopathy, vascular diseases, ischemic heart disease, idiopathic thrombocytopenic purpura, autoimmune, and skin disorders [13,18-20]. So far, there is no vaccine against *H. pylori* and efficiency of current antibiotics treatment is not enough. The mainly used treatment is treatment combination consisting of proton-pump inhibitors plus two antibiotics. These antibiotics can cause few side effects and the use of them is limited due to the increasing the rise of antibiotic resistance.

On the other hand, treatment is given only for people who have severe symptoms, it means people who are asymptomatic have a risk of developing a serious *H. pylori* infection yet. Therefore, the immunogenicity for the prevention, control of epidemics and children infections is essential. Therefore, vaccines are the only available option [14,15]. Since *H. pylori* has been found to be difficult for culture and storage, genetic engineered vaccine is the best way to cure for *H. pylori* developing vaccines [16]. Studies on prevention and treatment of *H. pylori* vaccines with treating and preventing capabilities has been started in various animal models in 1993-1994 [4]. Antigenic targets choice is a key point of the *H. pylori* vaccine design. HpaA is flagella sheath protein of *H. pylori*. Because the importance of this protein has been recognized recently, little research was done on that. HpaA belongs to a group of bacterial outer membrane proteins and as an adhesion factor has an important role in the attachment of bacteria to host cells.

In LtB-UreB recombinant protein cloning using *E. coli* in 2004. In the present study, *hpaA* gene was cloned into pET28a expression vector. Also, enzymatic digestion results were confirmed cloned gene. After desired protein induction with IPTG and purified using Ni-NTA agarose, the protein with molecular weight of 29 kDa was obtained respectively. Proteins can be purified by two methods. Recombinant protein was purified by FPLC method and the results of this method have no different In comparison to denaturing purification method and the obtained results from both methods have shown overlaps but FPLC method was easier than denaturing purification method. protein was not down unfold. This method is safe protocol for protein produce And protein folding is preserved. Despite the possibility of therapeutic and prophylactic immunization in these initial studies, the creation of new solutions for safe and effective products are required on a large scale. The use of live attenuated bacteria, despite being efficient on the most intestinal bacteria such as *Salmonella* and *Shigella*, but about *H. pylori* vaccine have been several weak points, such that these vaccines do not provide the safety against the other strains, and high doses of them were required for immunogenicity and it has been the possibility of returning to the wild-type. Also the cross-reactivity between self-antigens of the bacteria and host cells might be occurred [21].

Live vectors were evaluated. The studies showed the im-

mune responses against the vector while no detectable humoral or cellular immune responses were observed to antigens [22]. According to the aforesaid items, due to the abiotic vaccines, it was focused on vaccine such as whole cell bacteria, compounds derived from it and the DNA vaccines. Using whole bacterial cells have their own problems due to some antigenic similarities however, the poorly humoral immune responses have been demonstrated [23,24]. Nowadays, the use of recombinant protein antigens technology for immunization is going to identify the recombinant antigen for stimulating an immune reaction and use them instead of the old vaccine. Although there are many studies on the construction of recombinant proteins and assessment of bacterial vaccine candidate antigens immunogenicity, but it has not been introduced the completely effective vaccine for the *H. pylori* prevention. It is hoped that the obtained results from this study will be useful in this context.

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