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ORIGINAL ARTICLE

Cloning, expression, purification and characterization of recombinant $UreB_{229-561}$ from *Helicobacter pylori*

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ABSTRACT

Background: *Helicobacter pylori* is a widely distributed gram negative bacterium that infects the human stomach and duodenum. Some antibiotic regimens are subjected to cure the infection but the cost of drugs, poor patient compliance and emerging of antibiotic-resistant strains are limiting the usefulness of these antibiotic therapies. Therefore, interest in a H. pylori vaccine is growing up rapidly.

Materials and methods: We selected a fragment of B subunit of *H. pylori* urease enzyme consist of four important epitopes, involving in elevating host immune responses. This 1070bp fragment was amplified by PCR from genomic DNA isolated from *H. pylori* 22596 and then cloned into the pET28a expression vector. UreB229-561 was expressed and then affinity-purified by Ni2+-Sepharose resin. The recombinant UreB229-561 was reacted with the serum of *H. pylori*-infected human and rabbit anti-*H. pylori* polyclonal antibody in western-blot analysis.

Results: Having transformed competent *E.coli* DH5α with ligation product of digested ureB fragment and pET28a, plasmid extraction from single colonies appeared in LB-agar plate after 18-24 h incubation at 37°C, using plasmid extraction kit (Bioneer, Korea). Applying both infected human serum and rabbit anti-H. pylori polyclonal antibody, brown strip corresponding to the location of the recombinant protein appeared on PVDF membrane after adding DAB solution, hence confirming the antigenicity of the protein. This recombinant fragment showed urease activity.

Conclusion: Our findings confirmed that a prokaryotic expression system of $rUreB_{229-561}$ was successfully constructed. The results of SDS-PAGE showed that our constructed prokaryotic expression system pET28a- $ureB_{229-561}$ -BL21DE3 efficiently produces target recombinant protein in the form of dissoluble inclusion body. Therefore we can suggest that these epitopes can effectively be a vaccine candidate.

Keywords: *Helicobacter pylori, Recombinant protein, UreB, Cloning.*
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INTRODUCTION

Helicobacter pylori is a widely distributed gram-negative, spiral bacterium that infects the human stomach and duodenum (1). This bacterium appears to transmit from person to person by oral-oral or fecal-oral route. Although most infected individuals are asymptomatic, in some cases the infection is associated with acute/chronic gastritis or development of peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric

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adenocarcinoma (2). Recently, *H. pylori* was defined as a class 1 carcinogen by the World Health Organization (3). Despite of host's vigorous immune response, the organisms evade humoral and cellular immune mechanisms and persist even for decades in the stomach environment (4). The currently most used eradicating treatment strategies for *H. pylori* infection relies on a triple antibiotic therapy, but the cost of these drugs, poor patient compliance and emerging of antibiotic-resistant strains are some of the drawbacks of these therapies. In addition successful eradication therapy does not protect the host from re-infection (5). Vaccination against *H. py*lori becomes an attractive approach for both therapeutic and prophylactic purpose. Various antigens which known to be involved in the pathogenesis of the infection have been proposed as suitable vaccine candidates, such as urease, the cytotoxin-associated antigen (CagA), the vacuolating cytotoxin (VacA), the helicobacter pylori adhesion A (HpaA), and others (6). The urease which is expressed by almost all *H. pylori* isolates is one of the most essential enzymes for virulence and colonization of *H. pylori* in the gastric mucosa by neutralizing the microenvironment of *H. pylori* in the stomach (6). This enzyme consists of two major subunits, UreA (26.5 kD) and UreB (61 kD). The UreB is considered as a reliable vaccine candidate antigen and found to be protective in mice (7). Some important epitopes of the B subunit of *H. pylori* urease and their roles in host's immune induction have been identified such as $U_{546–561}$ and $U_{229–244}$ for induction of Th2-type cytokine responses and $U_{237–251}$ which can induces Th1-type cytokine production. In addition, $U_{327–334}$ induces anti-bodies that neutralize urease activity (8,9).

Since epitope-based vaccines represent a new strategy for eliciting a specific immune response against the selected epitopes, in the present study we choose a fragment of UreB which contains all of these epitopes ($UreB_{229-561}$) to determine its antigenisity as a good vaccine candidate. The results of current study may supply further experimental foundation for the development of *H pylori* vaccine.

MATERIALS and METHODS

Bacterial strains and vector: Standard *H. pylori* strain 26695 was preserved in our laboratory. *E. coli* strains DH5α and BL-21(DE3) were obtained from Invitrogen and Novagen (USA), respectively. pET-28a Plasmid as expression vector was provided from Novagen .

Preparation of DNA template and polymerase chain reaction (PCR): Genomic DNA of *H. pylori* strain 26695 was extracted by routine phenol-chloroform method and then was solved in TE buffer; concentration and purity of extracted DNA was determined by spectrophotometry (10). The specific primers were designed by Gene-Runner software according to ureB sequences of *H. pylori* strain 26695 from NCBI. The sequence of forward primer with an endonuclease site of NdeI and reverse primer with an endonuclease site of SacI were:

5'-ACCCATATGTCTGCAATCAATCATGC-3' and 5`-TTGGAGCTCGCTCACTTTATTGGCTG-3`.

By setting up the different items such as heat and concentration of mixture component, suitable PCR condition was achieved. Total volume of each PCR reaction was 25µl which consisted of 0.5µM of each primer, 2.5µl 10X PCR buffer, 0.2mM each dNTP, 1.5mM $MgSO_4$, 2.5U of Pfu DNA polymerase (Takara, Japan) and 200ng genomic DNA. PCR condition consisted of initial denaturing at 98°C for 60s and 30 cycles of amplification consisted of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, polymerization at 72°C for 60 s and additional extension time for 8 min after the last PCR cycle. PCR products were analysis by electrophoresis on 1% (w/v) agarose gel (Fermentas) and then the expected 1017bp bands was recovered from the gel using PCR purification kit (Bioneer, Korea).

Cloning, expression and purification of UreB$_{229-561}$: After enzymatic digestion of purified ureB fragment and pET28a cloning vector with responsible endonucleases, ligation reaction between insert and vector was performed (10) and the recombinant plasmid was amplified in *E.coli* strain DH5α and then extracted by plasmid extraction kit (Bioneer, Korea).

Cloning was confirmed by PCR, enzymatic digestion and sequencing by a commercial facility using universal forward and reverse T7-promoter and T7-terminator primers was performed (TAG Copenhage A/S Symbion, Denmark). Recombinant pET28a-ureB$_{229-561}$ transformed into competent *E. coli* BL-21 (DE3) in the presence of kanamycin (30µg/ml).

Confirmed positive clones was induced by adding of IPTG (1mmol/L) and incubated in 37ºC for further 4 hours. Having centrifuged, bacterial pellet was ultrasonically broken on ice in the presence of PMSF (1mM) as a protease inhibitor and centrifuged (10). Supernatant and precipitate were examined by SDS-PAGE to verify the location of expressed recombinant protein. rUreB$_{229-561}$ was collected by Ni-NTA affinity chromatography under combination of denature and native conditions by binding, washing and eluting steps according to manufacturer's protocol (Qiagen) with some modification which described previously (11). Purified protein was dialyzed against PBS, pH 7.4 to remove imidazole and analyzed by 10% SDS–PAGE.

Antibodies production: To produce *H. pylori* polyclonal antibody, adult females New Zealand white rabbits (Pasteur Research Institute, Tehran, Iran) were immunized subcutaneously with *H. pylori* lysate containing approximately 1mg protein in Freund incomplete adjuvant (Sigma). Booster doses were also given on days 14 and 28 in incomplete Freund's adjuvant. Then, 10 days after the last immunization animals were exsanguinated and the serum was collected and stored at -20°C until required for use.

Western blot analysis: The proteins separated by SDS-PAGE transferred to 0.45µm pore size PVDF membrane. The membrane was blocked by 1% skim milk and then incubated with the serum of patient infected with *H. pylori* (diluted to 1:2000 in PBS–Tween) for 2 hours at RT. After washing, the protein was detected by incubating the membrane with HRP-conjugated goat anti-man IgG antibody (diluted to 1:2500 in PBS–Tween) for 1 h at RT. After 3 washing, the membrane was treated using DAB solution (Sigma, USA). Additional analysis was performed using rabbit anti-*H. pylori* polyclonal antibody (diluted to 1:2500 in PBS–Tween) and goat anti-rabbit immunoglobulin G horseradish peroxidase (HRP) conjugate antibody (Bio-Rad) (at a 1:5000 dilution in PBS-Tween 20) as the first and second antibodies, respectively, with the same method as described above.

Rapid urease test: To determine urease activity of UreB$_{229-561}$, rapid urease test was performed using the commercial kit (Bahar Afshan, Iran).

RESULTS

DNA extraction and PCR amplification for ureB$_{229-561}$: Genomic DNA of *H. pylori* strain 26695 extracted by routine phenol-chloroform method. Figure 1 represents 1017 bp bands resulted from amplification of ureB$_{229-561}$ by specific primers.

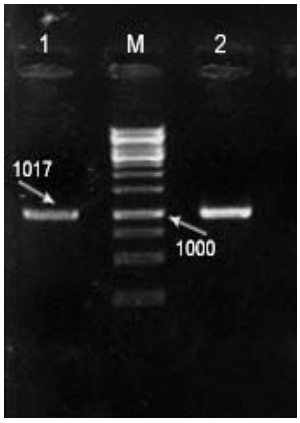

Figure 1. Electrophoresis of PCR product on agarose gel (1% w/v). *Lane* 1&2: expected band of *ureB$_{229-561}$* (1017 bp); *lane* M: 1kb DNA size marker.

Identification of the pET28a- ureB$_{229-561}$ by PCR and enzymatic digestion: Having transformed competent *E.coli* DH5α with ligation product of digested ureB fragment and pET28a, plasmid extraction from single colonies appeared in LB-agar plate after 18-24 h incubation at 37°C, using plasmid extraction kit (Bioneer, Korea). Resultant plasmid double digested with NdeI and SacI agarose gel analysis showed that the extracted plasmids contained the objective gene (figure 2).

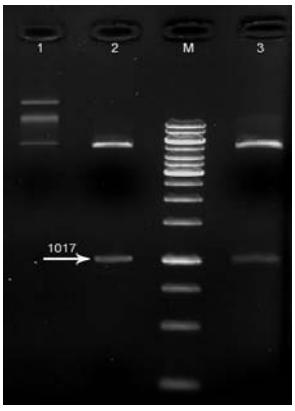

Figure 2. Enzymatic digestion of recombinant vector (electrophoresed on 1% w/v agarose gel). Lane 1: undigested recombinant vector, pET28a- ureB$_{229-561}$; lane 2&3: pET28a- ureB$_{229-561}$ digested with NdeI and SacI; lane M: 1kb DNA size marker.

Additionally, PCR was performed with specific primers and extracted plasmid as template DNA. Presence of target gene in recombinant vector confirmed by detection of amplified 1017bp DNA fragment from extracted plasmids.

Expression and purification of target recombinant protein: The BL21 (DE3) competent cells were transformed with confirmed recombinant vectors and induced with IPTG (1mmol) to express target recombinant protein. Total protein was electrophoresed on 10% SDS-PAGE gel and stained with Coomassie blue and protein of interest with approximate molecular weight of 40KDa was detected (figure 3). The large scale culture and induction was performed and the expressed protein were mainly accumulated as inclusion bodies after sonication which purified by Ni^{2+}affinity chromatography under modified denaturing condition (figure 4).

Western blot analysis: Western blot analysis was performed to detect antigenicity of expressed protein. Applying both infected human serum and rabbit anti-H. pylori polyclonal antibody, brown

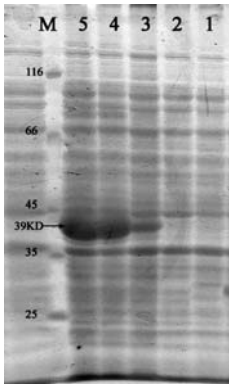

Figure 3. Detection of expressed UreB$_{229-561}$ on SDS-PAGE (12% w/v). The gel was stained with Coomassie blue G-250. Lane 1: pellet of induced bacteria containing pET28a vector without ureB$_{229-561}$ insertion. Lane 2: pellet of un-induced bacteria containing recombinant vector; lane 3: pellet of IPTG induced bacteria after 1 hour incubation; lane 4&5: pellet of IPTG induced bacteria after 4 hours incubation; lane M: standard protein size marker.

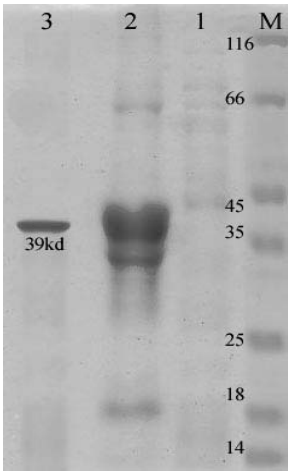

Figure 4. Detection of expressed UreB$_{229-561}$ after sonication and purification on SDS-PAGE (12% w/v). Lane 1: supernatant of sonicated bacteria; lane 2: pellet of sonicated bacteria (inclusion bodies); lane 3: Ni2+-Sepharose purified UreB$_{229-561}$; lane M: standard protein size marker (KDa).

strip corresponding to the location of the recombinant protein appeared on PVDF membrane after adding DAB solution, hence confirming the antigenicity of the protein (figure 5).

Rapid urease test: Twenty µl of recombinant UreB$_{229-561}$ containing 10µg protein was added to the test tube, conversion of original color to red due to urea hydrolysis happened which confirmed the enzymatic activity of this recombinant fragment of ureB protein.

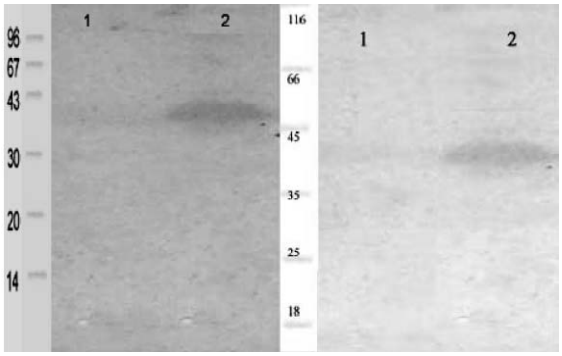

Figure 5. Western blot analysis of recombinant UreB$_{229-561}$ protein probed with rabbit polyclonal anti-*H. pylori* antibody (1:2000). Lane 1: pellet of un-induced bacteria; lane 2: pellet of IPTG induced bacteria; lane M: protein size marker (kDa).

DISCUSSION

Prior investigators showed that UreB might be the most promising antigen candidate for *H. pylori* vaccine (6,12,13). *H. pylori* produces highly active urease, which catalyses the hydrolysis of urea to carbon dioxide and ammonia provides a neutral microenvironment around the organism to resist acidic condition of stomach. Hence, urease-negative mutants fail to colonize gastric tissue (14). Therefore, urease especially UreB became suitable targets in vaccine researches and different investigators used these antigens in their studies. In 1994, Ferrero et al. cloned the genes encoding the respective urease subunits and determined their antigenisity (15). Lu et al. constructed a recombinant protein of the urease B subunit of *H. pylori* and examined its biological properties. In their study, the expression of recombinant UreB was achieved in *E.coli* BL21 and western blotting indicated strong reactivity of the recombinant protein with polyclonal BalB/c mice anti-*H. pylori* sera or human sera infected with *H. pylori* (16). Purification and western blotting methods in these studies were the same as ours and the results were compatible too. An important aspect of current study is using an epitope containing region of ureB instead of whole protein to verify if this shorter fragment confer the suitable antigenisity. The shortness of antigen makes it suitable to fuse with other antigens. Epitope-based vaccine might be an alternative for currently used vaccine candidate such as whole cell or subunit antigens (17). Increased safety and specific immunity are some of the potential advantages of this approach (18).

Shi et al. identified and characterized three $CD4^+$ T epitopes within the UreB antigen by using the RANKPEP prediction algorithm. They were $U_{546–561}$, $U_{229–244}$, and $U_{237–251}$. In their study synthesized peptides were used to explore their anigenicity and immunogenicity. They found that all of these epitopes effectively stimulated proliferation of splenic $CD4^+$ T lymphocytes (8). In addition, it has been reported that a B cell epitope from urease, $U_{327–334}$, induces anti-bodies that neutralize urease activity (9).

The truncated protein in our study contains all of these epitopes in recombinant form which can produce in larger amount and lower cost in contrast to synthetic form. However, to explore the immunogenicity and protective effect of this fragment, further studies such as immunization of animal models and challenging them with *H.pylori* must be achieved.

For the first time, in the present study we constructed a recombinant fragment of UreB protein from aa 229 to aa 561 termed as UreB$_{229-561}$ which contains some important and well characterized epitopes. Our findings confirmed that a prokaryotic expression system of rUreB$_{229-561}$ was successfully constructed. The results of SDS-

PAGE showed that our constructed prokaryotic expression system pET28a- ureB$_{229\text{-}561}$ -BL21DE3 efficiently produces target recombinant protein in the form of dissoluble inclusion body. The output of rUreB$_{229\text{-}561}$ was approximately 40-50% of the total bacterial proteins. This highly expression might be beneficial to industrial production. Western blotting performed in this study confirms that rUreB$_{229\text{-}561}$ could be recognized by both rabbit anti *H. pylori* polyclonal antibody and serum of *H. pylori*-infected human indicating an active and suitable immunoreactivity of the recombinant protein. Different studies demonstrate that the active site of *H. pylori* urease resides on the B subunit (9,19) and structural analysis of the urease showed that the important amino acid residues are Cys321, His322 and His323 which are implicated in catalysis and substrate binding (20). Presence of urease activity in rUreB$_{229\text{-}561}$ suggests that the folding of truncated protein might be in a correct form to confer enzymatic activity. Furthermore according to other studies, our results showed that the urease activity of *H. pylori* is mainly related to the B subunit and the active site of the enzyme resides between aa 229 to aa 561 (21). These results support application of rUreB$_{229\text{-}561}$ as a good candidate for the development of *H. pylori* vaccine.

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REFERENCES

1. Marshall BJ. *Helicobacter pylori*. Am J Gastroenterol. 1994;89:S116-S28.

2. Ernst PB, Gold BD. The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu Rev Microbiol. 2000;54:615-40.

3. Honda S, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian Gerbils. Cancer Res. 1998;58:4255-59.

4. Baldari CT, Lanzavecchia A, Telford JL. Immunesubversion by *Helicobacter pylori*. Trends Immunol. 2005;26(4):199–207.

5. Unge P. Antibiotic treatment of *Helicobacter pylori* infection. Curr Top Microbiol Immunol. 1999;241: 261–300

6. Corthesy-Theulaz I, Porta N, Glauser M, Saraga E, Vaney AC, Haas R, et al. Oral immunization with Helicobacter pylori urease B subunit as a treatment against Helicobacter infection in mice. Gastroenterology 1995;109: 115-21.

7. Smythies LE, Novak MJ, Waites KB, Lindsey JR, Morrow CD, Smith PD. Poliovirus replicons encoding the B subunit of *Helicobacter pylori* urease protect mice against *H. pylori* infection. Vaccine 2005;23:901–9.

8. Shi Y, Wu C, Zhou WY, Mao XH, Guo G, Zou QM. Identification of H-2d restricted Th epitopes in Urease B subunit of *Helicobacter pylori*. Vaccine 2007;25(14): 2583–90.

9. Hirota K, Nagata K, Norose Y, Futagami S, Nakagawa Y, Senpuku H, et al. Identification of an antigenic epitope in *Helicobacter pylori* urease that induces neutralizing antibody production. Infect Immun. 2001;69(11):6597-603.

10. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York;2001.

11. Goudarzi G, Sattari M, Habibi M, Montajabi niat M, Zavaran-Hosseini A, Mosavi-Hosseini K. Cloning, expression, purification and characterization of recombinant flagellin isolated from Pseudomonas aeruginosa. Biotechnol Lett. 2009;31:1353–60.

12. Chi CH, Lin CY, Sheu BS, Yang HB, Huang AH, Wu JJ. Quadruple therapy containing amoxicillin and tetracycline is an effective regimen to rescue failed triple therapy by overcoming the antimicrobial resistance of *Helicobacter pylori*. Aliment Pharmacol Ther. 2003;18: 347-53.

13. Rupnow MF, Owens DK, Shachter R, Parsonnet J. *Helicobacter pylori* vaccine development and use: a cost-effectiveness analysis using the Institute of Medicine Methodology. Helicobacter. 1999;4:272-80.

14. Tsuda M, Karita M, Mizote T, Morshed MG, Okita K, Nakazawa T. Essential role of *Helicobacter pylori* urease in gastric colonization: definite proof using a urease-negative mutant constructed by gene replacement. Eur J Gastroenterol Hepatol. 1994;6 (Suppl 1):S49-52.

15. Ferreo RL, Thiberge JM, Huerre M, Labigne A. Recombinant antigens prepared from the urease subunits

of *Helicobacter* spp.: Evidence of protection in a mouse model of gastric Infection. Infect Immun 1994;62:4981-89.

16. Lu DS, Mao XH, Zou QM, Wu C, Yang J, Zhang WJ, et al. Recombinant *Helicobacter pylori* urease B subunit and its biological properties. Di Yi Jun Yi Da Xue Xue Bao. 2003;23(6):549-52.

17. Sette A, Fikes J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Curr Opin Immunol. 2003;15(4):461–70.

18. Sbai H, Mehta A, DeGroot AS. Use of T cell epitopes for vaccine development. Curr Drug Targets Infect Disord 2001;1(3):303–13.

19. Yuan XP, Zhou QM, Bai Y, Yang J, Guo Y, Zhang WJ, et al. Purification and functional analysis of Helicobacter pylori UreB protein fragment. Nan Fang Yi Ke Da Xue Xue Bao. 2007;27(7):959-62.

20. Martin PR, Hausinger RP. Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease. J Biol Chem. 1992;267:20024–27.

21. Shin JH, Roe IH, Kim HG. Production of anti-*Helicobacter pylori* urease-specific immunoglobulin in egg yolk using an antigenic epitope of H. pylori urease. J Med Microbiol. 2004;53(Pt 1):31-4.