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ORIGINAL ARTICLE

Elicitation of IgY in chicken egg yolk by recombinant fragments of UreC and its efficacy against *Helicobacter pylori*

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ABSTRACT

Background: *Helicobacter pylori* multiplies and causes infection in human gastric mucosal layer. New approaches have focused on using specific treatments, such as immunotherapy, to limit this infection. Urease, as one of the most important virulent and antigenic factors of the bacterium, is a suitable target for this purpose.

Patients and methods: In order to prepare recombinant proteins, the synthetic genes for total ureC protein (UreCt) and its N (ureCn) and C (ureCc) terminal fragments were ligated into pET28a. The recombinant proteins were expressed in *E. coli* BL21(DE3). White leghorn hens were injected with the purified recombinant proteins. IgY recovered from egg yolk, using PEG precipitation. Finally, urease neutralizing ability of the antibodies was evaluated by urease activity assay in presence of the purified IgY.

Results: SDS-PAGE analysis revealed a good expression and purification of the recombinant proteins. Indirect ELISA observation demonstrated high antibody titer in sera and egg yolks and high ability of IgY Anti-UreCt and IgY Anti-UreCc antibodies in recognition of urease subunit C. Anti-UreCT and Anti-UreCc IgYs were more potential *H.pylori* urease inhibitors than Anti-UreCn.

Conclusion: While all three UreC fragments induce prophylactic responses. UreCt and UreCc possess almost equal responses. Anti-UreCc IgY has advantage of smaller size and is preferred for its activity and easier protein recovery and purification process. These features emphasize on importance of simpler, easier and cost effective antibody production.

Keywords: *Helicobacter pylori, Urease, UreC, IgY, Recombinant antigen*.
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INTRODUCTION

Helicobacter pylori is a gram-negative, microaerophilic bacterium, that colonizes in the mucus layer associated with gastric-type epithelium in humans. *H. pylori* is the major pathologic agent in the development of gastric and duodenal ulcers, and its eradication is known to reduce recurrence of peptic ulcers (1). Furthermore, epidemiological and statistical studies associated the infection with a higher risk of gastric malignancy leading the World Health Organization International Agency for Research in Cancer to categorize *H. pylori* as a class I carcinogen (2). Infection with *H. pylori* can often be treated with antibiotics. However, increase in antibiotic resistance is starting to affect the efficacy of

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treatment (3). Consequently, it is important to seek new therapies and a broader based means of treating *H. pylori* infection which do not invoke drug resistance problems. It has been shown that oral administration of antimicrobial immunoglobulin is an effective way to establish protective immunity against a variety of microbial pathogens (4).

Chicken egg yolk was recognized as an inexpensive, antibody source whereas hyperimmunized hens could provide a convenient and economic source of immunoglobulin in their egg yolk (5). The efficiency of egg yolk immunoglobulin (IgY) has been assessed for therapeutic application by passive immunization therapy through oral ingestion of IgY. This can be applied in fortified food products for prevention or control of intestinal infections, such as those caused by enterotoxigenic bacteria including *H. pylori* (6,7). As reported by Shin et al. (7) egg yolk IgY against *H. pylori* whole-cell lysates inhibited the growth of *H. pylori* and reduced gastric inflammation. These findings suggest that IgY could be used as a novel modality against *H. pylo*ri-associated gastric mucosal diseases.

However, IgY produced by whole-cell lysates may cross-react with other bacteria, including normal human intestinal flora, possibly decreasing its efficiency and specificity. Immunization using a selective antigen is therefore required. Interestingly, the organism expresses a high level of urease that neutralizes gastric acid by generating ammonium from urea and is therefore required for in vivo colonization of *H. pylori*, because strains deficient in *ureA* (8) or lacking urease activity (9) fail to infect the mammalian stomach. Among different synthetic peptide fragments derived from ureaA and UreB (now termed as UreC), only UreB showed the significant response to anti-*H. pylori* IgY (11). IgYs against UreC obtained from egg yolk showed higher titer and specifically recognize recombinant *H. pylori* urease purified from *E. coli* (12). According to the essentiality of this enzyme in colonizing the mucus layer of the gastric mucosa, a novel approach in prevention and reduction of *H. pylori* infection has been reported based on production of urease-specific IgY that could suppress *H. pylori* colonization through urease-binding by anti-*H. pylori* urease IgY (10).

The present study was designed to evaluate the effect of three IgYs raised against recombinant UreC subunit of *H. pylori* urease and its C-terminal and N-terminal fragments. The ability of these IgYs in interaction by recombinant UreC and neutralization of native *H. pylori* urease were assessed and compared.

PATIENTS and METHODS

Recombinant plasmid construction: Nucleotide sequence of *H. pylori* (j99 strain) ureC gene was optimized by means of *E. coli* codon usage and RNA secondary structure. A full length synthetic gene and the two fragments pertaining to 1-337 aminoacids of N-terminal (1-1011 nucleotides of the gene) and 338-569 aminoacids from C-terminal (1012-1707 nucleotides of the gene) of the UreC, were synthesized and ligated into pET-28a plasmid separately. The sequence of the gene is accessible under the GeneBank accession number GU942733.

Expression and purification of recombinant antigens: *E. coli* BL21(DE3) competent cells (Cinnagen, Iran) were used for transformation of pET28a-ureCt, pET28a-ureCn and pET28a-ureCc plasmid constructs separately. Recombinant bacterial cells were grown overnight on LB broth with 70μg/ml kanamycin and were stored as a glycerol stock in aliquots at -80 °C. LB broth (5ml) containing 50mg/ml kanamycin was inoculated with 10μl of the freezer stocks. Following an overnight incubation at 37°C, these cultures were used to inoculate kanamycin containing-LB broth mediums (300ml) in 1 liter flasks until the optical density at 600nm(OD_{600}) reached 0.6. IPTG (Isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 1.0mM, and the

induction was allowed to proceed at 30°C for 10 hours. The induced *E. coli* cells were harvested by centrifugation at 3000g for 10 min at 4°C. The pellet of the induced cells was resuspended in 6ml lysis buffer (100mM NaH_2PO_4,10mM Tris-Cl, 8M Urea, pH=8). The bacterial suspension was incubated at room temperature for 30 min to lyse cells. After the cells were completely lysed, the lysate was centrifuged at 14000 rpm for 30 min to remove the insoluble cell debris. The supernatant obtained from the above step was allowed to mix with 5ml Ni–NTA (nickel-nitrilotriacetic acid) agarose (Qiagen, USA), pre-equilibrated with the lysis buffer. The mixture of Ni–NTA agarose and supernatant was poured into a plastic column and the flow-through of the soluble fraction was collected. The column was washed with 5ml of washing buffer (100mM NaH_2PO_4,10mM Tris-Cl, 8M Urea, pH=6) followed by 1ml first elution buffer (100mM NaH_2PO_4,10mM Tris-Cl, 8M Urea, pH=5.2) and 1ml second elution buffer (100mM NaH_2PO_4,10mM Tris-Cl, 8M Urea, pH=4.5). Finally, the column was washed by 1ml 2-[N-morpholino] ethanesulfonic acid (MES, Sigma) buffer (20mM, pH=6) to elute any residual protein. Aliquots of all the above fractions were analyzed by SDS–PAGE. The concentrations of these protein solutions were estimated, using the Bradford protein estimation method (13).

Immunization and IgY induction: Immunization of hens was performed in Amirabad Animal Facilities of veterinary faculty of Tehran University. A total number of 16 white Leghorn hens (25 weeks old) were injected subcutaneously with 200μg of purified recombinant UreCt, UreCn, UreCc with an equal volume of Freund's complete adjuvant (Razi Institute, Iran). Freund's complete adjuvant without protein was injected to the negative control group. Three booster injections, with Freund's incomplete adjuvant, were given at 2-week intervals following the first injection. One month after the last dose of injection, the eggs were collected. The yolks (20ml) was carefully separated from the white by washing with deionized water and collected without the yolk skin in a graduated cylinder. Phosphate-buffered saline (pH=7.6) was added twice the volume of egg yolk suspension and mix completely, followed by the addition of polyethylene glycol 6000 (PEG 6000) powder (Merck, Germany) up to 3.5% (w/v) and mixing with magnetic stirrer for 10 min. After centrifugation at 4500g for 30 min, the water-soluble fraction was collected and filtered through a Whatman filter paper (no. 1) to remove solid lipid materials. The resulting IgY-containing filtrate was mixed gently with PEG 6000 up to 12% (w/v) for 15 min and then centrifuged at 12000g for 10 min. Finally, the pellet containing IgY was resuspended in phosphate-buffered saline plus 60% glycerol (pH=7.6) with the equal volume to the egg yolk suspension and stored at -20°C prior to further analysis.

ELISA titration and cross-activity of antisera and evaluation of IgYs: Immunization of hens was evaluated, using enzyme-linked immunosorbent assay (ELISA). Recombinant proteins were coated onto microtiter plates (2μg per well) in coating buffer (20mM Na_2CO_3, 35mM $NaHCO_3$, pH=7.2) for overnight in 4°C. After washing the plates four times with PBST and blocking non-specific sites with blocking buffer (3% BSA in PBST) test sera were serially diluted (in PBST) on the plate. The total Ig titer was determined using anti hen HRP conjugates (Sigma, US) at a dilution of 1:30000. The colorimetric detection was carried out using O-phenylenediamin (OPD, Sigma) as a chromogenic substrate of HRP, after washing the plate with PBST. The absorbance of each well was measured at 492nm. Furthermore, IgY preparations were also used in above protocol instead of sera in order to titrate its active immunoglobulin content. The cross activity of antisera, anti-UreCn and anti-UreCc sera were used against recombinant UreCt-coated wells as described above.

Urease- neutralization assay: *H. pylori* were cultured overnight in BHI at 37°C and 10% CO_2. A fresh 10ml BHI media were incubated with 50µl of the overnight culture and incubated at the same condition (37°C, 10% CO_2). When the optical density of these cultures at 600nm (OD_{600}) reached 0.5, several dilutions of the three IgY preparations (IgY-UreCT, IgY-UreCn and IgY-UreCc) were added to them, followed by 6 hours of additional incubation (37°C, 10% CO_2) for IgY-Urease interaction. Urease activity then was assayed with addition of 25µl of urea-phenol red solution (2% urea, 0.03% phenol red) followed by reading the optical absorption in 550nm (OD_{550}).

Statistical analysis: Data of neutralization assay analyses were carried out by one-way ANOVA using SPSS software (Version 16.0, SPSS Inc., Chicago, USA). IgY type was considered as grouping parameter while IgY concentration was considered as covariate. P-values <0.05 were considered to be significant.

RESULTS

Expression and purification of recombinant antigens: To improve the expression level of ureC gene fragments, optimized sequences were completely synthesized, in which the synonymous codons that are frequently employed in *E. coli* were introduced. These synthetic genes were cloned in pET28a expression vector and then transferred into *E. coli* BL21(DE3) competent cells. Whole-cell lysates of IPTG induced cultures were subjected to SDS-PAGE. Specific product could be seen in Coomassie brilliant blue-stained gel (figure 1).

The one-step purification, utilizing Ni-NTA affinity chromatography, was sufficient to yield purifications of each recombinant protein (figure 2). Purification protocols were developed for each protein by adjusting the ionic strength of the buffers in the chromatography steps.

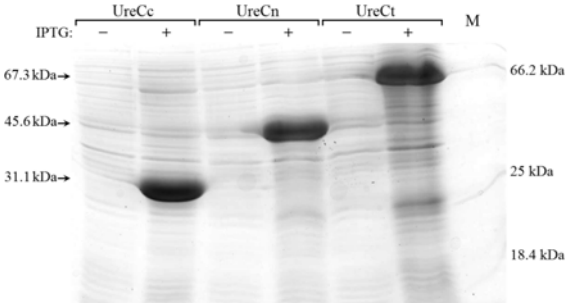


Figure 1. SDS-PAGE analysis of the expression of UreCt, UreCn and UreCc recombinant proteins is shown. Lane M is protein molecular weight marker. Induced and not-induced cell lysates are indicated by + and –, respectively. Positions and predicted molecular weights of the recombinant proteins are shown at left. These molecular weights include the pET28a fusion tags.

Immunization scheme and isolation of IgYs: Rising of specific IgYs for each recombinant protein was detected with ELISA-titration of immunized hens' sera against three recombinant proteins separately. IgYs, isolated from egg yolks, were also verified by ELISA assay to titrate the amount of active specific IgY in each sample.

Cross-reactivity and urease-neutralization activity: Antisera samples were used to determine the ability of IgY-UreCc and IgY-UreCn antibodies to interact with the intact UreC protein by ELISA technique (figure 3). The urease-neutralization ability of each IgY was assessed in a separated assay in which neutralization of urease activity in a *H. pylori* culture was monitored by color alteration and therefore changing the optical absorption of phenol red as a pH indicator. OD_{550} values of *H. pylori* cultures, coincubated with IgY-UreCc, IgY-UreCn and IgY-UreCt and urea-phenol red containing solution, illustrated in figure 4, shows the activity of urease in presence of each IgY. Results revealed that although all three IgYs significantly decreased urease activity ($p<0.05$), there was a significant difference ($p<0.05$) between neutralization activity.

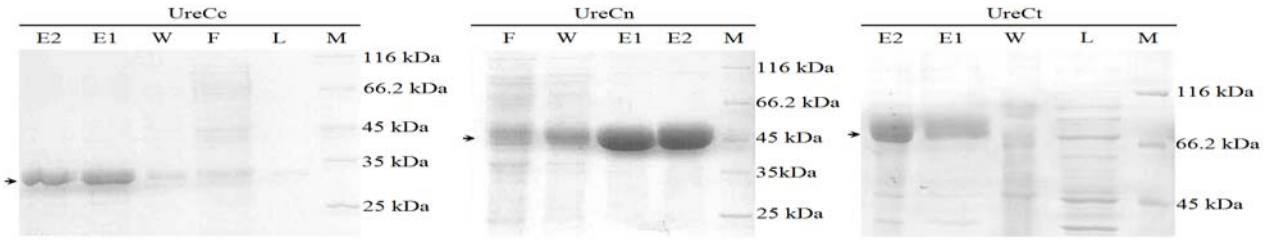


Figure 2. Purification of recombinant UreCt, UreCn and UreCc proteins analyzed in separated SDS-PAGEs. Lanes pertaining to molecular weight marker (M), cell lysate (L), column flow out (F), washed fraction (W) and two fractions of eluted proteins (E1 and E2) are indicated. Positions of recombinant protein bands are pointed by arrows at left of each panel.

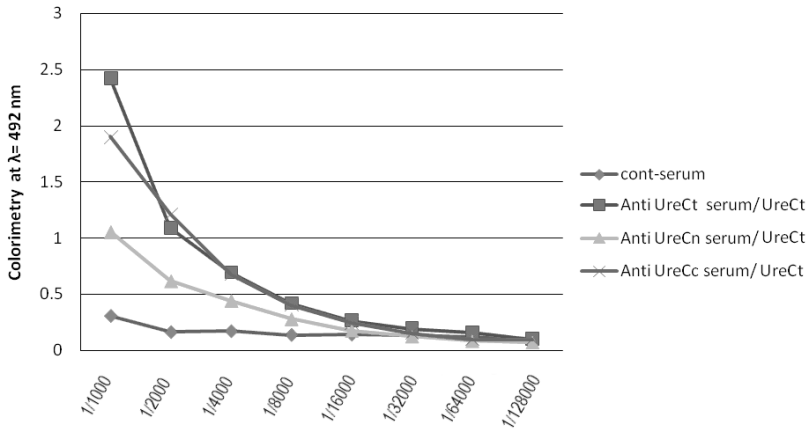
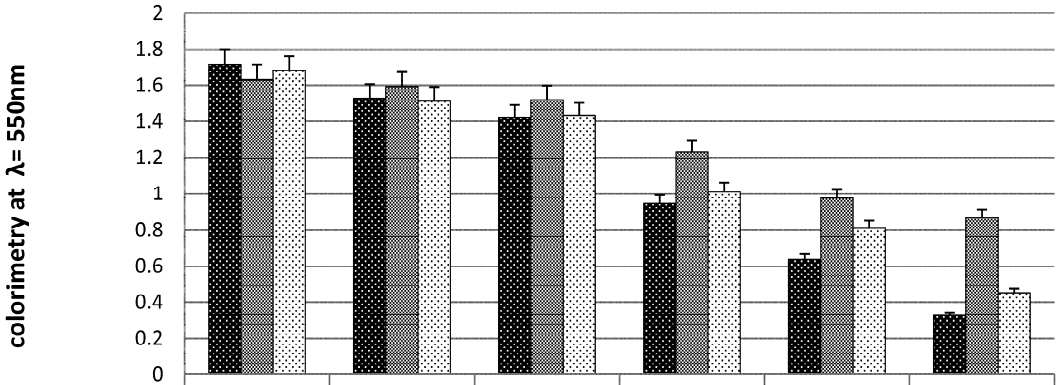


Figure 3. Ability of IgYs to interact with total UreC recombinant protein (UreCt) assessed by ELISA. Control experiment performed by the use of serum from adjuvant-injected hens.



	Cont	1.2mg/ml	2.5mg/ml	5mg/ml	7.5mg/ml	10mg/ml
Anti UreCt IgY	1.714	1.528	1.421	0.946	0.637	0.325
Anti UreCn IgY	1.632	1.592	1.521	1.232	0.975	0.867
Anti UreCc IgY	1.679	1.514	1.432	1.011	0.811	0.451

Figure 4. Prevention of urease activity in *H. pylori* cultures is visualized, using phenol red, which have purple red and orange colors at higher and lower pH, respectively. These color alterations are measured by reading of OD550. Lower OD550 values are pertaining to lower pH which implies the neutralization of urease. Control experiment (cont) was performed with IgYs from adjuvant-injected hens.

DISCUSSION

H. pylori infections are prevalent in humans and although they can be cured with antibacterial therapy, the extensive use of antibiotics leads to the emergence of antibiotic-resistant strains (3). The conceivable alternative is passive immunotherapy with *H. pylori*-specific antibodies. Eggs have been considered a convenient source for the production of polyclonal antibodies, known as IgY (14,15). Large amounts of IgYs can be obtained from the egg yolk by quick and economic purification procedures (16,17). For instance, approximately 1500mg of chicken IgY can be harvested each month, and between 2 and 10% is the specific IgY (18).

Furthermore, IgY has been used successfully against several digestive infectious microbes such as enterotoxigenic *E. coli* (15,19), human rotaviruses (20,21) and *Pseudomonas aeruginosa* (11). Moreover, it has been shown that the egg yolk IgY against *H. pylori* whole-cell lysates inhibits the growth of *H. pylori* and reduces gastric inflammatory cell accumulation in *H. pylori*-infected Mongolian gerbils (7). However, IgY produced by whole-cell lysates can cross-react with other bacteria, naturally found in human intestinal flora, possibly decreasing its efficiency and specificity (22). Specific immunocompetence antigens from *H. pylori* proteins are therefore needed to decrease nonspecific reactions and increase specificity. Urease of *H. pylori* showed an acceptable potential as a vaccine in animal models (23,24). Therefore, we used the urease subunit, UreC, recombinant protein as a *H. pylori* specific antigen to elicit anti-*H. pylori* IgY. Due to application of synthetic genes with *E. coli*-optimized sequences, UreC recombinant proteins are highly expressed in the *E. coli* host (figure 1). Our results regarding more efficiency of C-terminal fragment of UreC in eliciting effective IgYs (figures 3 and 4) are in agreement with a previous study which has been shown that a synthetic peptide of 396-410 UreC amino acids are more efficient antigen than 52-67 and 385-391 peptides (25). Nevertheless, the adequacy of urease inhibition for restriction of *H. pylori* has been showed (26,27), the urease-neutralization ability of anti-UreC IgYs might implies their usefulness for restriction of *H. pylori* infection and prevention of its ruinous effects. However, some aspects of clinical application of IgY, including allergic cross-reactivities and permanence of its effect may require more considerations.

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