

Expression, purification and immuno-characteristics of recombination UreB protein of *H. pylori*

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Subject headings *Helicobacter pylori*; urease; immunology; isolation; recombinant protein; purification

Wu C, Zou QM, Guo H, Yuan XP, Zhang WJ, Lu DS, Mao XH. Expression, purification and immuno-characteristics of recombination UreB protein of *H. pylori*. *World J Gastroenterol*, 2001;7(3):389-393

INTRODUCTION

Helicobacter pylori (*H. pylori*) is associated with the development of chronic gastritis, peptic ulcer and gastric cancer and gastric MALT lymphoma^[1-9]. *H. pylori* has many antigens, including urease, heat shock protein and vacuolating cytotoxin and so on, and urease is an important factor in the colonization of the gastric mucosa and suspected to cause damage to the gastric mucosa^[10-14]. At the same time, urease is also one of the important protective antigens. It consists of two distinct subunits with apparent molecular mass of 29.5 ku (UreA) and 66 ku (UreB), and urease B subunit is nontoxic, highly immunogenic, and an effective component of protective antigens^[15-18]. In our study, Hp ureB gene was cloned in the fusion expression vector and expressed in *Escherichia coli*. We described the immunological characteristics of purified recombination UreB protein.

MATERIALS AND METHODS

Materials

pHp-UreB, plasmid that contains *H. pylori* urease B gene was constructed by Wu^[19] (Third Military Medical University, Chongqing); plasmid PinPointTM Xa-3 (Promega, USA); *E. coli* JM109 (*SupE44endA1 hsdR17gyrA96 relA1thi* Δ (*lac*-

proAB)F'⁺[*traD36 proAB*⁺ *lacI*g *lacZ* Δ M15]) was used as a host for analysis of urease B subunit expression; DNA Purification Kits (Sangon, Shanghai); a polyclonal antibody of rabbit directed against *H. pylori* was prepared by ourselves. Sera from *H. pylori* infected patients and sera from healthy people (Southwest Hospital, Chongqing); six-week-old female Balb/C mice were obtained from Laboratory Animal Centre, Third Military Medical University, Chongqing); mini-cycleTM-PCR amplicon (PE Company, USA); UVP nucleic acid and protein analyser (UVP Company, USA); Bio-Rad mini-protein electrophoresis (Bio-Rad Company).

Construction of the expression vector

A 5' primer (CGTCAAGCTTATGAAA-AAGATTAGCAG) and a 3' primer (CGTCGATATCATCCTAGAAAATGCTAA) were used in a PCR with *Taq* polymerase to amplify the 1.7-kb fragment containing the sequences of ureB flanked by *Hind* III and *Eco*R V restriction enzyme digestion sites. The amplification cycles were an initial denaturation step of 94°C for 5 min, followed by 35 cycles of annealing at 55°C for 2 min, extension at 72°C for 2 min, and denaturation at 94°C for 1 min, then extension at 72°C for 2 min. PCR products were run on 10 g·L⁻¹ agarose gels (containing 0.5 mg·L⁻¹ ethidium bromide). The amplified product purified with DNA purification kits was digested with *Hind* III and *Eco*R V, and ligated into the corresponding sites of PinPointTMXa-3. The recombination plasmid named pPin-UreB was introduced into *E. coli* JM109 by CaCl₂ perforation.

Expression of the ureB gene

E. coli JM109 containing the expression plasmid pPin-UreB was grown in Luria-Bertani broth containing ampicillin (100 mg·L⁻¹) and biotin (2 μ mol·L⁻¹ final concentration). The culture was incubated at 37°C and shaken at 200 r·min⁻¹, until the A600 was 0.8. Prior to adding 1 mmol·L⁻¹ IPTG to cultures, a 1 mL sample was taken (noninduced cell). Cultures were incubated for a further 5 h, at which time another 1 mL sample (induced cell) was taken. The noninduced and induced cell samples were later analyzed by sodium

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Supported by the National Major Science and Technology Projects, No.96-901-01-54.

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Received 2001-02-06 Accepted 2001-04-20

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane by electroblotting. The membrane was incubated in TBST buffer for 60 min firstly, then in 15 mL TBST buffer containing 3 μ L streptavidin-alkaline phosphatase for 30 min at room temperature with gentle agitation. After washed with TBST for 5 min, the membrane was incubated with Promega's Western Blue R stabilized substrate for alkaline phosphatase at room temperature until the bands appear. Dark purple bands will indicate the location of the biotinylated protein species in the lanes containing cellular extracts.

Purification of the recombinant fusion protein

IPTG-induced cultures were spun at 8000 r·min⁻¹ for 10 min at 4°C. Pellets were resuspended in cell lysis buffer (50 mmol·L⁻¹ Tris-HCl pH 7.5, 50 mmol·L⁻¹ NaCl, 50 g·L⁻¹ glycerol) and sonicated on ice. Cellular debris were removed by centrifugation (10 000 r·min⁻¹, 4°C, 15 min). The supernatant was added into the column containing SoftLinkTM- Resin, and the cell extract was captured efficiently. To elute the protein, adding a stabilizing buffer containing 50 mmol·L⁻¹ biotin, when a volume of elution buffer equal to one-half the volume of resin in the column had been applied, stop the flow from the column. Wait 15 min to allow for release of the fusion protein. The fractions containing higher concentration fusion protein were collected in the eluate. The purified fusion protein was cleaved by factor Xa protease at 37°C. The reaction products were added into SoftLinkTM- Resin Column again. In the process of elution, the purified UreB protein was collected.

Immunogenicity of recombinant UreB

Two groups of 10 Balb/C female mice (six week old) including controls were used as follows: ① NS control group was non-immunized mice that received NS; ② UreB group was the mice immunized with 200 μ L NS containing purification rUreB protein (50 g·L⁻¹) each time and once a week for 4 weeks under the skin of the back, and added in Freund's incomplete adjuvant for the first time. Thirty-five days after the immunization, blood was collected from the retro-orbital sinus and the antibody titer was measured with enzyme-linked immunosorbent assay (ELISA). The purified recombinant UreB protein was used to coat 96-well microtiter plates (Corning-Costar Company, USA) and sheep anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) was used in the assay.

Immunoreactivity of recombinant UreB

Microtiter ELISA plates were coated by incubating

the plates with 5 mg·L⁻¹ recombination UreB 0.1 mL diluted in phosphate-buffered saline (PBS) at 37°C for 4 h. After washing plates twice with PBS, the remaining binding sites were blocked by incubating the plates with 10 g·L⁻¹ bovine serum albumin (BSA) at 37°C for 30 min (200 mL·well⁻¹). The human antisera against Hp and control sera from healthy people were added to the coated well and incubated for 2 h at 37°C. After another washing procedure, HRP-conjugated sheep anti-human IgG antibody 100 μ L diluted in PBS (working dilution, 1:5000) was added to each well and incubated for 1 h at 37°C and σ -phenylenediamine in PBS was used as the substrate. The enzyme substrate reaction was read with Spectra Classic spectrophotometer (Tecan, Austria) at 492 nm.

RESULTS

Construction of the expression vector

The PCR product amplified from plasmid pHp-UreB was analyzed under ultraviolet light after 10 g·L⁻¹ agarose gel electrophoresis (Figure 1). The 1.7 kb PCR product was digested with *Hind* III and *Eco*R V restriction enzyme and ligated into the corresponding sites of PinPointTM Xa-3. The recombinant expression plasmid was named pPin-UreB. pPin-UreB was identified by digesting with *Hind* III and *Eco*R V, and the 1.7 kb ureB fragment was separated by electrophoresis in 10 g·L⁻¹ agarose gel (Figure 2). Analysis of DNA sequencing, showed that the nucleotide sequence of ureB gene in plasmid pPin-UreB was the same as reported in the reference^[19].

Expression of UreB gene and detection of the fusion protein

The plasmid pPin-UreB was induced to express *H. pylori* UreB protein by IPTG in *E. coli* JM109, and produced the fusion protein with predicted molecular masses of 79 ku (Figure 3). The fusion protein on 100 g·L⁻¹ polyacrylamide gel was transferred by electroblotting onto an NC membrane and was detected with Streptavidin-Alkaline Phosphatase Detection System. The result showed that there was a positive band on the site of the fusion protein in pPin-UreB strain but not in control strains (Figure 4). Measured by UVP Protein Analyser, the biotinylated fusion protein was 150 g·kg⁻¹ in the total bacterium protein.

Purification of recombinant UreB protein

The recombinant fusion protein expressed in *E. coli* was separated and purified by affinity chromatography with the SoftLinkTM- Soft Release Avidin Resin. The biotinylated fusion protein was cleaved into two parts with Factor Xa proteinase: UreB protein (66 ku) and biotin tag protein (13 ku).

With purification of column chromatography, the recombinant UreB protein was obtained and analyzed by SDS-PAGE and Western blotting. The purified UreB protein had predicted molecular masses of approximately 66ku and its purity was more than 95% (Figure 5).

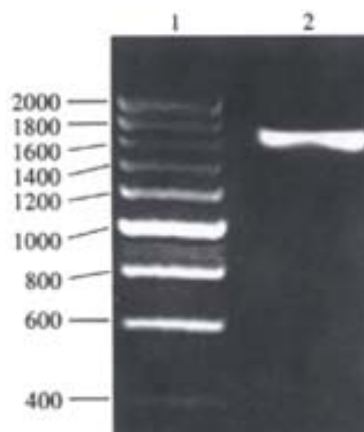


Figure 1 Analysis of the PCR product of *H. pylori* ureB gene by 1.0% agarose gel electrophoresis.
1: 200 bp DNA ladder marker; 2: PCR product of ureB gene



Figure 2 Identification of recombinant plasmids pPin-UreB digested with *Hind* III and *Eco* R V.

1: PCR product of ureB gene; 2: pPin-UreB plasmid digested by *Hind* III and *Eco*RV; 3: PinPoint™Xa-3 plasmid digested with *Hind*III and *Eco* R V; 4:λDNA/*Hind* III marker.

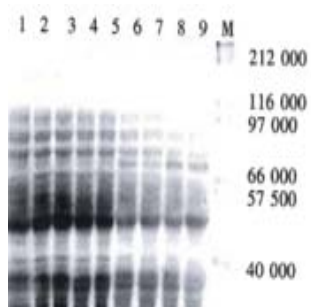


Figure 3 Analysis of expression product of recombinant plasmid pPin-UreB in *E.coli* JM109 by 10% SDS-PAGE.

1,2,3: *E.coli* JM109; 4: *E.coli* JM109/PinPoint™Xa-3 before induction; 5: *E.coli* JM109/PinPoint™Xa-3 after induction with IPTG; 6,7: *E.coli* JM109/pPin-UreB before induction; 8,9: *E.coli* JM109/pPin-UreB after induction with IPTG; M: Molecular weight marker (212, 116, 97, 66.2, 57.5, 40)×10³.

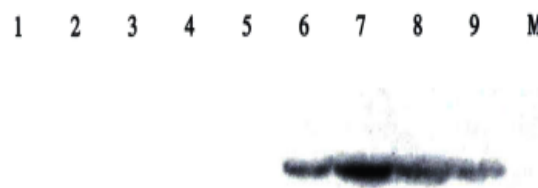


Figure 4 Analysis of recombinant fusion protein by Western-blotting.

1,2,3: *E.coli* JM109; 4: *E.coli* JM109/PinPoint™ Xa-3 before induction; 5: *E.coli* JM109/PinPoint™Xa-3 after induction with IPTG; 6,7: *E.coli* JM109/pPin-UreB before induction; 8,9: *E.coli* JM109/pPin-UreB after induction with IPTG; M: Protein molecular weight marker (212, 116, 97, 66.2, 57.5, 40)×10³.

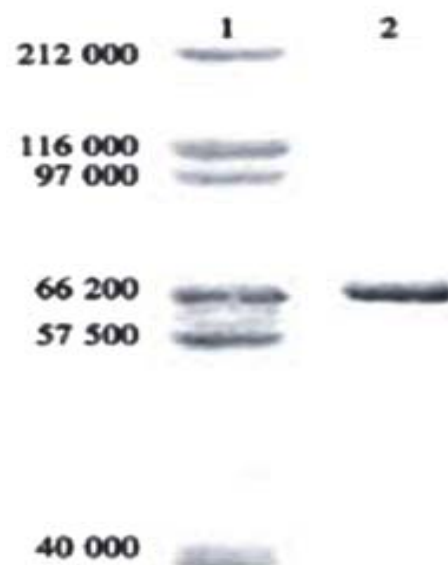


Figure 5 Determination of the purified rUreB by 10% SDS-PAGE.

1: Protein molecular weight marker; 2: The purified rUreB

Immunology character of recombinant UreB protein

Balb/C mice, immunized with recombinant UreB, generated anti-UreB antibody and the titer was detected to be 1:3000 with ELISA. However, in the mice of the control group no antibody was found. Forty antisera against *H. pylori* and 20 control sera from healthy people were detected by ELISA with recombinant UreB protein, the coincidence ratio being 100%.

DISCUSSION

H. pylori urease is a nickel metalloenzyme, which hydrolyzes urea and is 50-100 g·kg⁻¹ in total protein of the cell. *H. pylori* urease is a 550ku enzyme, consisting of two distinct subunits: UreA (29.5ku) and UreB (66ku). The ratio of subunits is approximately 1:1, suggesting a stoichiometry of

(29.5ku-66ku)6 for the native enzyme. The research of *H. pylori* urease genes suggest that ureA and ureB are structural genes, which are required for the synthesis and assembly of the 550ku apoenzyme^[13], and these additional genes (ureC, ureD, ureE, ureF, ureG and ureH) are required for the expression of urease activity^[13,20,21]. To express recombinant UreB protein (rUreB), we cloned *H. pylori* ureB gene by PCR and constructed the expression plasmid pPin-UreB containing *H. pylori* ureB gene. The purified rUreB was absent of urease activity in our study, which is different from what Li *et al*^[22] reported. At the same time, the cloned ureB gene was sequenced and the sequence homology of nucleotide and predicted amino acid were 96.44% and 99.65% with those reported by Labigne *et al*^[23]. The difference of nucleotide was due to the difference strains and gene diversity, but the obvious DNA homology suggested that the main antigenic determinants which encode *H. pylori* UreB protein were similar in difference strains^[13,24,25].

Urease is the important antigen of *H. pylori*, which can stimulate significant immune response. These results suggested that the significant immunoprotection against *H. pylori* infection was induced after the purified native urease from *H. pylori* and recombinant urease were used to immune mice, and that the recombinant UreB but not rUreA protein produced immunoprotective response against *H. pylori* infection in mice after immunization separately^[26-30]. In our study, the purified rUreB protein was used to immune Balb/C mice under the skin, and the antibody against rUreB was produced successfully. These demonstrated that the rUreB protein had obvious immunogenicity, and could be used in *H. pylori* vaccine research^[31-33].

Individuals infected with *H. pylori* produce vast quantities of specific immunoglobulin G (IgG) antibodies in the serum^[34-36]. Serum antibodies against *H. pylori* can be detected by a variety of methods including complement fixation, bacterial agglutination and immunofluorescence but enzyme-linked immunosorbent assay (ELISA) is usually used due to speed, simplicity and reproducibility^[37-41]. *H. pylori* urease is a key protein used for detection of the organism by measuring serum antibody against the protein^[13]. We discovered that the purified rUreB protein had a positive reaction with specific antibodies against *H. pylori* in sera of patients and negative reaction with the control sera from healthy people. The specificity and sensitivity of rUreB to *H. pylori* antisera of patients will be used in the diagnosis, assessment of curative effect and epidemiological investigation of *H. pylori*.

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Edited by Ma JY