World J Microbiol Biotechnol (2011) 27:969–974
DOI 10.1007/s11274-010-0540-2

ORIGINAL PAPER

Periplasmic expression and one-step purification of urease subunit B of *Helicobacter pylori*

Amirhooshang Alvandi · Ahmad Farajzadeh · Mehri Ghaforian Borojerdnia · Abbass Jelodar · Ehsan Aryan · Abolfazl Gholipour · Abdolrahim Masjedizadeh · Manoochehr Makvandi

Received: 28 April 2010 / Accepted: 13 August 2010 / Published online: 27 August 2010

Abstract *Ure*B is one of the urease subunits of *Helicobacter pylori* and can be used as an excellent antigen candidate for *H. pylori* vaccination. Easy access to highly purified UreB protein, facilitate advances in therapeutic or preventive strategies. To achieve a simplified purification procedure, the present report represents a novel method of producing recombinant urease subunit B extracellularly. *ure*B gene from 26,695 standard strain was amplified by PCR and cloned into pET-26b(+) expression vector. UreB was expressed as a soluble, N-terminal pelB and C-terminal hexahistidine-tagged fusion protein (UreB-6His) and secreted into the periplasmic space of *Escherichia coli*. Expression of the recombinant UreB in *E. coli* BL21 (DE3) was induced by isopropylthio-β-D-galactoside (IPTG). Expression of UreB was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis using anti-His monoclonal antibody. UreB-6His protein was extracted from the periplasm by osmotic shock treatment and was purified in one step by Nickel affinity chromatography. In conclusion, the present protocol is easier to perform; more time effective and low cost than earlier methods.

Keywords *Helicobacter pylori* · Urease subunit B · Cloning · Periplasmic expression

A. Alvandi (✉) · A. Farajzadeh · A. Gholipour · M. Makvandi
Department of Medical Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, 61357-15794 Ahvaz, Iran
e-mail: ah_alvandi@yahoo.com

M. Ghaforian Borojerdnia
Department of Immunology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

A. Jelodar
Department of Microbiology, Faculty of Veterinary, Shahid-Chamran University, Ahvaz, Iran

E. Aryan
Microbiology and Virology Research Center, Avicenna Research Center, and Department of Clinical Bacteriology, Ghaem University Hospital, Mashad University of Medical Sciences, Mashad, Iran

A. Masjedizadeh
Division of Gastroenterology and Hepatology, Department of Internal Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Introduction

Helicobacter pylori causes peptic ulcer disease and some forms of gastric cancer. It is one of the most common chronic bacterial infections of humans (Hatzifoti et al. 2000). The most efficient and economical approach with low risk of adverse reaction to the prevention and control of *H. pylori* infection is vaccination (Del Giudice and Michetti 2004; Rupnow et al. 1999).

Urease and its subunits are currently the most promising vaccine candidates, and their value as vaccine antigens have been confirmed by numerous studies in mice, ferrets, non-human primates and human clinical trials (Cuenca et al. 1996; Del Giudice and Michetti 2004; Ferrero et al. 1994; Haas and Meyer 1997; Stadtlander et al. 1996). *Ure*B one of the four subunits of urease produced by almost all the isolated strains of *H. pylori*. It has been demonstrated to have the strongest antigenicity in all known proteins of *H. pylori* (Corthesy-Theulaz et al. 1995; Pappo et al. 1995). *ure*B gene, responsible for encoding UreB with 569 amino acid residues, is a highly conserved nucleotide sequence with a similarity of approximately 95% in different

H. pylori isolates (Akada et al. 2000; Labigne et al. 1991). On the other hand, it has been proven that UreB is more protective and safer than subunit A in mice; with no signs of gastritis or other side effects after therapeutic vaccination (Corthesy-Theulaz et al. 1995; Ferrero et al. 1995; Hatzifoti et al. 2004). These data strongly indicate that UreB can be used as an excellent antigen candidate for vaccination against *H. pylori*.

Easy access to highly purified UreB protein by investigators should therefore facilitate advances in therapeutic or preventive strategies. Previous purification methods of native or recombinant urease and its subunits invariably required two or several steps involving conventional size exclusion and cation exchange or FPLC combining cation exchange, size exclusion chromatography, and sometimes the conventional hydrophobic interaction gel (Evans et al. 1991; Turbett et al. 1992).

Recovery of a recombinant gene product can be greatly simplified by a secretion strategy that minimises contamination from host proteins and even in the case of periplasmic translocation, a simple osmotic shock or cell wall permeabilization can be used to obtain the product without the release of cytoplasmic protein contaminants. In addition secretion of recombinant proteins to the periplasmic space of *E. coli* by fusing a signal peptide to the N-terminal residue, have several advantages such as reduction of contamination with endotoxins and DNA, higher product stability, solubility and biological activity and correct folding (Baneyx and Mujacic 2004; Cornelis 2000; Mergulhao et al. 2005; Rastgar Jazii et al. 2007; Shokri et al. 2003). Periplasmic expression has been shown to be beneficial in the production of several recombinant proteins.

In order to simplify the procedure steps and further reduce cost in *H. pylori* vaccine production, the present report represents a novel method of producing recombinant Urease subunit B extracellularly. This method is based on expression of *Ure*B sequence containing a NH2-terminal pelB signal peptide in *E. coli*. Therefore periplasmic content is released from the bacterial pellet by a simple EDTA-containing buffer and the protein can be chromatographed in a single step by affinity chromatography.

Materials and methods

Bacteria and culture conditions

H. pylori strain 26,695 was used in this study grown on Brucella agar supplemented with 10% (v/v) defibrinated sheep blood, containing vancomycin (10 μg ml^{-1}), trimethoprim (5 μg ml^{-1}) and amphotericin B (2 μg ml^{-1}) at 37°C under microaerophilic conditions for 3 days. *E. coli* DH5α was grown at 37°C in Luria-Bertani medium (Invitrogen, USA) used for molecular biology manipulations and for maintenance of recombinant plasmid DNA. *E. coli* BL21 (DE3) (Novagen, USA) was used for 6-His-tagged fusion peptide expression was grown at 37°C in LB medium.

Designing of primers

*ure*B nucleotide sequence of *H. pylori* strain 26,695 (GenBank accession number AF507994) was retrieved from the NCBI GenBank (NCBI). Primers were designed with restriction enzyme sites using DNASIS 2.6 software. The stop codon was excluded in designing the reverse primer.

Construction of expression vector

Whole-cell DNA from *H. pylori* strain 26,695 was extracted by Genomic Extraction Kit (MBI Fermentas, Lithuania) according to the manufacture's procedure. *ure*B was amplified by the PCR using the following oligonucleotides (Cinagene, Iran): 5′-CAT GCC ATG GCA TGG AAA AAG ATT AGC AGA AAA G-3′ (forward) and 5′-CCG CTC GAG CGG GAA AAT GCT AAA GAG TTG CG-3′ (reverse). The underlined bases designate *Nco*I and *Xho*I restriction sites. PCR was performed using Expand High Fidelity PCR System (Roche, Germany) following the manufacturer's protocol. PCR product fragment was electrophoresed on the 1% agarose gel stained with ethidium bromide. Subsequently the PCR product was purified using the DNA Extraction kit (MBI Fermentas, Lithuania) and double digested with *Nco*I and *Xho*I. The double digested PCR product was ligated into pET-26b(+), which was digested with the same restriction enzymes to form the over-expression plasmid pET26b-*Ure*B. The ligated plasmids were transformed into the competent *E. coli* DH5α and transformants were selected on LB medium containing kanamycin (50 mg l^{-1}). The presence of inserts was confirmed by colony PCR. Correct orientation and the complete insert sequence were verified by restriction digestion analysis using *Pvu*I and sequencing (TAG Copenhagen, Denmark).

Expression of fusion protein

The pET26b-*Ure*B transformed into the expression host *E. coli* BL21(DE3). To evaluate protein expression a single colony was inoculated into 10 ml of terrific broth containing kanamycin (50 mg l^{-1}) and grown at 37°C overnight. This preculture was then added to 500 ml of the same medium which was incubated on a shaker (200 rpm) at 37°C until the optical density (O.D. 600 nm) of culture reached up to 0.6. Bacterial cultures were induced using

different concentrations of isopropyl thiogalactoside (IPTG) 0.1–1.0 mM and the culture incubated in 22°C or 37°C. 1.5 ml bacterial culture was harvested every 1 h up to 8 h. Similarly, the *E. coli* BL21 containing pET-26b was induced up to 8 h as a negative control. Expression of protein was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions. For purification of the recombinant protein, the *E. coli* culture was grown using the optimized expression conditions (Terrefic Broth medium, 22°C, 1 mM IPTG, 4 h of induction). The bacteria were harvested by centrifugation at 4°C.

Preparation of the periplasmic extract

Osmotic shock was applied according to the Neu and Heppel (1965), with modifications. Cell pellets were resuspended in ice-cold buffer A (0.03 M Tris–HCl, pH 7.3), and harvested by centrifugation at 5,000 rpm (5804R centrifuge, rotor # A-4-44, Eppendorf) for 10 min at 4°C. The pellets were suspended in 40 ml buffer A plus 40 ml ice-cold buffer B [0.3 M Tris–HCl pH 8.0, 1.5 mM EDTA, 40% (w/v) sucrose] for each gram wet weight of cells and was shaken for 30 min on ice. Following centrifugation, the pellet was rapidly resuspended in the same volume of cold water and the suspension shaken for 30 min on ice and then centrifuged again for 10 min. The supernatant (osmotic shock fluid) was collected as the periplasmic fraction.

Purification of recombinant UreB

Recombinant UreB was purified from the periplasm using a published protocol (Loo et al. 2002) with some modifications. Briefly, the periplasmic fraction was adjusted to pH 7 using Mops (Sigma, Germany) to produce a final concentration of 20 mM. The sample was then loaded onto a column containing 10 ml Ni-NTA agarose (nickel-nitrilotriacetic acid) (Qiagen, France), pre-equilibrated with chromatography buffer (20 mM Mops, 0.5 M NaCl, 5 mM imidazole, pH 8.0) and rocked for 3 h. The column was washed with chromatography buffer until the absorbance of the eluate returned to baseline. Recombinant UreB was eluted with a 20 ml 500 mM imidazole in chromatography buffer. Peak fractions were identified by SDS–PAGE. Positive fractions were pooled and dialyzed against PBS buffer at 4°C overnight. Protein concentration was measured by a Bradford assay based on a bovine serum albumin standard curve (Bradford 1976). To determine that periplasmic *Ure*B was not contaminated by cytoplasmic proteins, presence of β-Galactosidase in osmotic shock and cytoplasmic fractions was compared. β-Galactosidase assays were performed using *o*-nitrophenyl-β-D-galactopyranoside (ONGP) as a substrate, as described by Miller (1972).

SDS–PAGE and immunobloting

The resulting bacterial pellet or purified protein were homogenized in SDS sample loading buffer [0.150 M Tris–Cl, pH 6.8; 10% glycerol; 2% SDS; 0.01% bromophenol blue; 0.5 M DTT (Dithiothreitol)]. It was mixed properly and boiled for 5 min and centrifuged at 11,000*g* for 2 min. Electrophoresis was performed in the presence of SDS according to the method of Laemmli (1970). The discontinuous gel consisted of a 5% stacking gel and a 10% separating gel which was run on a vertical electrophoresis unit (Mini-Protean II, BioRad). After SDS–PAGE gel was transferred onto nitrocellulose membrane by Bio-Rad Mini Trans-Blot Cell system following the procedure described. Membrane was blocked using 3% nonfat dry milk in TTBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Tween 20) for 1 h. Following four washes the membrane was dipped into 1:4,000 diluted monoclonal antibody conjugated proxidase anti-His for 1.5 h at room temperature. BM Blue POD substrate precipitating (Roche, Germany) was used to develop the membrane, until dark purple bands appear, following the procedure described (Sambrook and Russell 2001).

Results

Construction of plasmid for periplasmic expression of *Ure*B

To overcome problems associated with cytoplasmic expression of UreB (Evans et al. 1991; Icatlo et al. 1998; Lee et al. 1995), an expression vector for periplasmic expression of UreB was constructed. The full-length *ure*B gene without stop codon was amplified by primers which contains restriction sites. *Nco*I and *Xho*I restriction sites were introduced upstream and downstream of the *ure*B gene respectively, therefore the coding sequence was preceded by a pelB signal sequence at the 5′ region and a 6His-tag at the 3′ of the gene. The target fragment of *ureB* genes with the expected sizes are shown in Fig. 1.

Construct transformed into *E. coli* DH5-α cells and selected on LB containing kanamaycin (50 mg l^{-1}). Transformants were characterized by colony PCR against *ure*B primers. The recombinant pET26b-UreB was extracted and its orientation confirmed by digestion with *Pvu*I restriction enzyme. *Pvu*I will have two restriction sites in recombinant plasmid, one in insert and the other one in vector. The two expected bands were observed on gel: a 1585–5442 bp bands (Fig. 2). Sequence analysis of

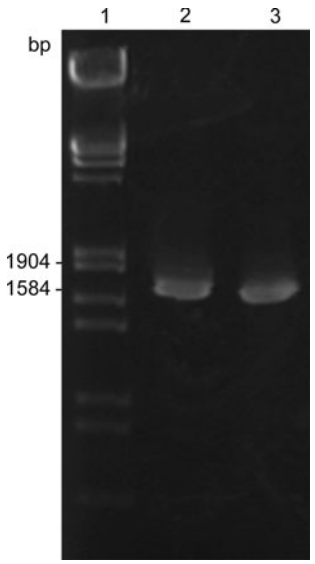

Fig. 1 Target amplification fragment of *ureB* gene and *NcoI and XhoI* double digested *ureB* PCR product. *Lane 1*, Lambda DNA/EcoRI + HindIII Marker; *Lane 2*, Target recovered fragment of *ureB* gene (1731 bp); *Lane 3*, Target recovered fragment *ureB* after digestion with both *Nco*I and *Xho*I (1722 bp)

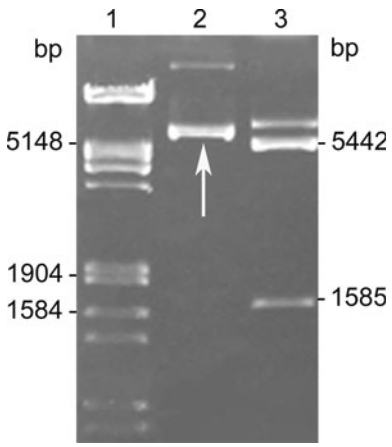

Fig. 2 Agarose gel electrophoresis analysis of recombinant pET26b-*Ure*B with *Pvu*I restriction enzyme digestion. *Lane 1* Lambda DNA/*Eco*RI+*Hind*III Marker, *Lane 2* pET26b-*Ure*B recombinant plasmid (*white arrow*, 7027 bp), *Lane 3 Pvu*I digested recombinant pET26b-*Ure*B. Two expected fragments were observed on the gel (5442–1585 bp bands)

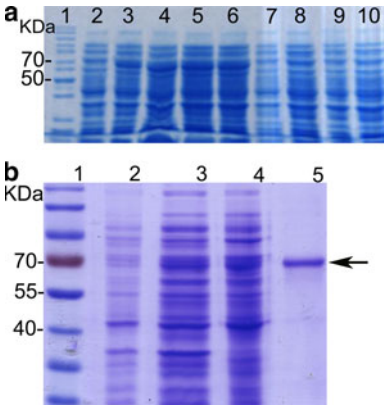

Fig. 3 SDS–PAGE analysis of the control negative bacteria (*E. coli* BL21 with pET21 without insert) (**a**) and the expressed UreB-6His tag protein in *E. coli* BL21 (**b**). A 15 µl volume of each fractions were analyzed by 10% polyacrylamide gel. **a** *Lane 1*, PageRuler™ Unstained Protein Ladder; *lane 2*, total cell lysate of noninduced bacteria; *lane 3–6*, Total cell lysate of bacteria after 1–4 h induction; *lane 7*, total cell lysate of noninduced control negative *E. coli* BL21; *lane 8–10*, total cell lysate of induced control negative *E. coli* BL21 after 1–3 h induction **b** *Lane 1*, PageRuler™ Prestained Protein Ladder; *lane 2*, total cell lysate of noninduced bacteria; *lane 3*. Total cell lysate of bacteria after 4 h induction; *lane 4*, periplasmic proteins extracted by osmotic shock procedure; *lane 5*, purified periplasmic UreB-6His by Ni-NTA agarose. The *arrow* indicates the position of UreB-6His on the gel

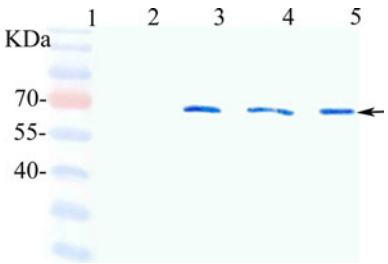

Fig. 4 Western blot analysis of the expressed UreB-6His tag protein in *E. coli* BL21. A 15 µl volume of each fractions were analyzed by 10% polyacrylamide gel and transferred onto nitrocellulose membranes and revealed with an anti-His monoclonal antibody. *Lane 1*, PageRuler™ Prestained Protein Ladder; *lane 2*, total cell lysate of noninduced bacteria; *lane 3*. Total cell lysate of bacteria after 4 h induction; *lane 4*, periplasmic proteins extracted by osmotic shock procedure; *lane 5*, purified periplasmic UreB-6His by Ni-NTA agarose. The *arrow* indicates the position of UreB-6His on the membrane

recombinant pET26b-UreB confirmed that there are no amplification errors and that cloning was accurate.

Overexpression and purification of *UreB*

The pET26b-UreB was transformed into the expression host *E. coli* BL21 (DE3). Transformant was grown at different temperatures, and expression was induced by IPTG and monitored by SDS–PAGE of total protein extracts before and after induction. Highest levels of over-expressed UreB were obtained by allowing the bacterial cultures to grow to an OD 600 nm of 0.6–0.8 before induction and by inducing protein production for 4 h at 25°C with 1 mM IPTG. OD_{600} at harvest was 5.0 and the cell wet weight after harvest was 4 g l^{-1}. The cell fractionation analysis showed that the UreB was mostly detected in the soluble fraction. Figures 3 and 4 show the protein profiles in non-induced and induced bacteria, periplasmic content and purified UreB-6His tag protein by Ni-NTA affinity

chromatography by SDS–PAGE and Western blot analyses. As shown in these figures a protein band migrating to the expected molecular weight for a UreB-6His-tag protein ($\approx$65 kDa) was clearly detected in the induced cells after 4 h and periplasmic fraction extracted by osmotic shock procedure. The yield of purified UreB was about 0.5 mg l^{-1} of culture media.

To prevent release of cytoplasmic content into the periplasmic fraction during extraction (Malik et al. 2007), the purification procedure was carried out within one day at 4°C and in the presence of protease inhibitors. The small quantity of β-galactosidase activity detected in the recovery solutions from osmotic shock (less than 2% of cytoplasmic content) (data not shown) indicates that little cytoplasmic material is released by this procedure.

Discussion

Currently *H. pylori* is recognized as the most widespread human pathogen and approximately half of the world's population is infected (Czinn 2005; Rossi et al. 2004). There is great interest in developing a vaccination method to prevent *H. pylori* infection, given that immunization is always considered the most economic and efficient means for such prevention, especially in developing countries (Del Giudice and Michetti 2004; Rupnow et al. 1999).

The selection of antigenic targets is critical in the design of a *H. pylori* vaccine. A large number of published data showed that UreB might be the most definitive antigen candidate for *H. pylori* vaccine (Corthesy-Theulaz et al. 1995; Morihara et al. 2008; Pappo et al. 1995; Zhao et al. 2007). In order to simplify the procedure steps and further reduce cost in *H. pylori* vaccine production, the present report represents a novel method of producing recombinant urease subunit B extracellularly.

As *E. coli* does not naturally secrete high amounts of proteins (Sandkvist and Bagdasarian 1996), recovery of a recombinant gene product can be greatly simplified by a secretion strategy that minimises contamination from host proteins. Additionally, if the product is secreted to the culture medium, cell disruption is not required for recovery and even in the case of periplasmic translocation, a simple osmotic shock can be used to obtain the product without the release of cytoplasmic protein contaminants (Mergulhao et al. 2005; Shokri et al. 2003). The pET-26b vector produces recombinant protein with signal peptide pelB at the N-terminal for periplasmic secretion and a His-tag at the C-terminal for detection and purification. Therefore, *H. pylori* UreB has been cloned into pET-26b, which places protein over-expression under the control of a lac promoter that is inducible with IPTG. Recombinant UreB-6His has been purified by using a single chromatography step. In this study the possibility of nonspecific exposure of cytoplasmic proteins has been ruled out by comparing periplasm β-gal activity to cytoplasm of *E. coli* transformed with pET26b-UreB-6His. The yield of purified UreB is lower than that in some cytosolic expression systems, however, considering the ease and low costs of scaling up a periplasmic procedure, the protocol can be regarded to be productive. In future the yield of the UreB protein could strongly be increased by use of different signal sequences of prokaryotic origin and optimizing the fermentation process. In conclusion, the present protocol is easier to perform; more time effective and low cost than earlier methods.

Acknowledgments This article has been extracted from an approved research (application No. U-870244) which has financially been supported by Research Deputy of Jundishapur University of Medical Sciences, Ahvaz, Iran. The authors would like to thank Mrs. Neisi and Mrs. Lotfi from Virology Department for assistance with this work, and Mrs. Noorbehbahani and Mrs. Amuzegari from Biochemistry Department of Jundishapur University of Medical Sciences, Ahvaz, Iran for their experience in chromatography throughout this study.

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