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NOTE

Separate Expression and *in vitro* Activation of Recombinant *Helicobacter pylori* Urease Structural Subunits

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Abstract Each of the recombinant structural genes of *Helicobacter pylori* urease, *ureA* and *ureB*, was cloned and overexpressed as inclusion bodies. Solubilization and renaturation of the inclusion bodies were carried out, to accelerate the pairing of sulfhydryl groups and the incorporation of nickel ions, which would lead to the native structure with high enzyme activity. Rates of urea hydrolysis were monitored as an indication of *in vitro* activation of renatured ureases. The activation of the apoprotein using 1 mM nickel ion, 100 mM sodium bicarbonate and a 10:1 ratio of reducing power resulted in a weak urease activity (about 11% of the native urease activity encoded by pTZ 19R/*ure-1*). When a sparse matrix screen method originally discovered for the crystallization of proteins was used, the activity increased higher than that obtained using glutathione. The effect of polyethylene glycol (PEG) on the activity was noticeable, giving two-fold increase in the specific activity (about 11 U/mg of protein corresponding to 22% of the native urease activity encoded by pTZ19R/*ure-1*).

Key words: *Helicobacter pylori*, urease, overexpression, *in vitro* activation

Helicobacter pylori is a spiral-shaped and highly motile gram-negative bacterium found in the stomach of patients with active chronic gastritis. *H. pylori* is now recognized as the etiological agent of antral chronic gastritis [2]. The bacterium is sensitive to acidic pH and consequently adopts a strategy to survive in the human gastric mucosa by the production of extracellular ureases [7]. The urease enzyme may secure the survival of the bacterium by neutralizing the local peptic acid. It has been proposed that urease may be an important factor in the colonization of the gastric mucosa [7, 8] and be responsible for the enhancement of the back-diffusion of hydrogen ions or the stimulation of gastrin production [9, 18]. In addition, urease may cause direct or indirect damage to the gastric mucosa, probably due to the accumulation of high concentrations of ammonia [4, 24].

H. pylori urease is multimeric, with an apparent molecular size of 550 kDa, and synthesized at up to 6% of total soluble proteins [12, 19]. The enzyme is composed of two subunits, UreA (29.5 kDa) and UreB (66 kDa), in the native structure with a tentative stoichiometry of $(AB)_6$. Nickel ions are assumed to be present as a component of the active site [13]. *H. pylori* urease genes have been cloned in *Escherichia coli* [3, 5, 11, 15] and it has been found that only UreA and UreB are required for the assembly of apoenzyme [11]. However, its assembly in *H. pylori* appears to be a complex process which requires the function of accessory proteins, UreC, UreD, UreE, UreF, UreG, UreH, and UreI [5, 15]. It was recently reported that *ureC* codes for a phosphoglucosamine mutase [6]. In *Klebsiella aerogenes*, UreD was postulated to be a urease-specific molecular chaperone and UreE to be a nickel-binding protein [17, 21]. Therefore, it has been assumed that the *in vitro* activation of urease is difficult in the absence of these accessory proteins.

Coexpression of soluble forms of two structural subunit genes, *ureA* and *ureB*, of *H. pylori* has been tested for *in vitro* activation, but showed relatively low urease activity recovery [22]. We attempted a separate cloning and expression of each of the two subunits, expecting no growth inhibition of recombinant *E. coli* due to the high pH and the maximum production of gene products for studies of protein structure. However, each subunit was overexpressed as inclusion bodies of which the renaturation turned out to be a major obstacle to purification. After each of the inclusion bodies of UreA and UreB had been solubilized with urea, the mixture was renatured using

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reducing potentials and crystallization screening conditions for *in vitro* activation study. Here, we report the cloning, overexpression and renaturation results of *H. pylori* urease structural subunits.

E. coli JM109 and pGEM-T (Promega, Madison, WI) were used for the cloning of PCR products, and *E. coli* BL21(DE3)pLysS and pET-5a (Promega, Madison, WI) were used for gene expression. *E. coli* strains were grown in L broth without glucose (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) at 37°C containing ampicillin (50 μg/ml). Plasmid pTZ19R/*ure-1* encoding the entire gene cluster of *H. pylori* urease was kindly provided by Dr. M. J. Cho (Kyung Sang University, Korea) and used for PCR template. The designs of PCR primers were based on the published DNA sequences of *H. pylori* urease [15]. For amplification of the subunit A gene (*ureA*: 717 bp), the pair of oligonucleotides used were 5'-CATATGAAACTCACCCCAAAA-3' and 5'-TTA-CTCCTTAATTGTTTTTAC-3'. For amplification of the subunit B gene (*ureB*: 1710 bp), the pair of oligonucleotides were 5'-CATATGAAAAAGATTAGCAG-3' and 5'-CTA-GAAAATGCTAAAGAGTT-3'. Each forward PCR primer included an *Nde*I restriction site (CATATG) at the 5' end for cloning into pET-5a. The program for PCR amplification was 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and polymerization at 72°C for 45 sec.

E. coli BL21(pET-5a/*ureA* or *ureB*) was grown at 37°C up to an OD_{600} of 0.5~0.6, induced by adding 0.5 mM IPTG, and incubated for an additional 3~4 h. The culture was centrifuged at 12,000×g for half an hour at 4°C. The cell pellet was dissolved in a lysis buffer (50 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM EDTA, 20 mM PMSF (phenylmethylsulfonyl fluoride), and 5% glycerol) and lysed by ultrasonication(VCX-400, Sonics & Materials Inc, U.S.A.) three times with a 50 sec pulse and a 30 sec interval. After centrifugation at 12,000×g, pellets were resuspended with a buffer containing 0.5% Triton X-100 and incubated for 5 min at room temperature. The supernatant was discarded by centrifugation at 12,000×g for 30 min to remove cell membranes and debris. Inclusion bodies in pellets were dissolved in the working buffer (50 mM Tris, pH 7.9, and 10 mM 2-mercaptoethanol) containing 8 M urea and allowed to remain at room temperature for an hour. After solubilization of the inclusion bodies, the concentration of each subunit fraction was adjusted to less than 2.5 mg/ml with the working buffer. Each fraction was mixed with 1 mM $NiCl_2$ and 2.5 mM glutathione and incubated at room temperature for an hour. Each fraction was then mixed and dialyzed against the refolding buffer (100 mM phosphate, pH 7.5, 1 mM $NiCl_2$, 100 μM EDTA and 100 mM $NaHCO_3$). The dialysates were used for the enzyme activity assay. Protein renaturation was also systematically screened using a sparse matrix crystallization method [10, 14]. Protein samples were tested for renaturation by adding an equal volume of each crystallization buffer (50 μl) and incubated at room temperature for 30 to 60 min. After the first incubation, an equal volume of the crystallization buffer (100 μl) was added and incubated again for another 30~60 min. These steps were repeated twice with 200 and 400 μl of the buffer, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Laemmli method [16]. 10~12% running gels were used. Non-denaturing electrophoresis used 5~8% running gels. The gels were stained with Coomassie Brilliant Blue.

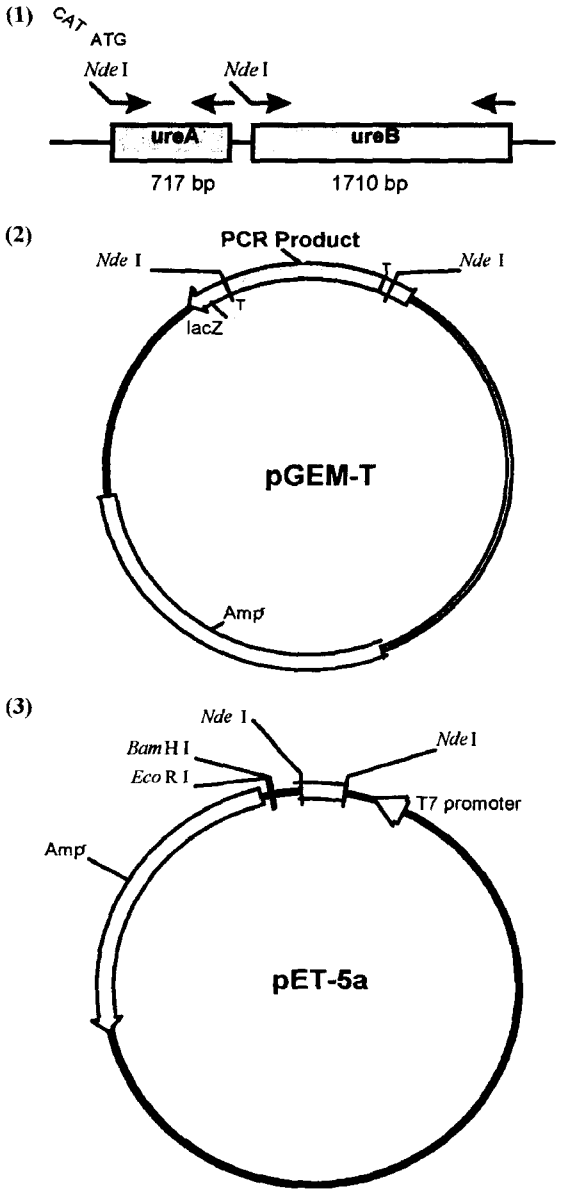

Fig. 1. Diagram of cloning and expression of each *H. pylori* urease subunit gene, *ureA* and *ureB*.

(1) PCR of *ureA* and *ureB* with pTZ19R/*ure*-1, (2) PCR product cloning in JM109, (3) Gene expression in BL21(DE3)pLys (*Nae*I digestion).

Protein concentrations were estimated by Bradford's method [1] using bovine serum albumin as a protein standard. Urease activity was determined by a slight modification of the Berthelot method [20]. The enzyme extract was added to HEPES buffer (50 mM urea, 25 mM HEPES, 0.5 mM EDTA, pH 7.5) and portions of the assay mixture at 37°C were withdrawn at intervals of 15 sec within the first 2 min. Phenol-nitroprusside and alkaline hypochlorite were added, the color development took about 10 min at 50°C, and the absorption at 625 nm was measured to quantitate the rate of ammonia release from urea by formation of indophenol. One unit of urease activity was defined as the amount of enzyme required to hydrolyze 1 μmol of urea per min at 37°C.

Each PCR product (717 bp of *ureA* and 1710 bp of *ureB*) was ligated with pGEM-T and transformed into JM109. After *Nde*I digestion of the cloned plasmids (pGEM-T/*ureA* and pGEM-T/*ureB*), each fragment was ligated with *Nde*I-linearized pET-5a and transformed in to BL21(DE3)pLysS (Fig. 1). The appropriate alignment of *ureA* or *ureB* under T7 promoter of pET-5a was determined by restriction enzyme digestion of the cloned plasmids (data not shown). Double digestion of pET-5a/*ureA* with *Hin*dIII and *Bam*HI produced 650 bp and *Bam*HI digestion of pET-5a/*ureB* produced 740 bp.

To define the culture conditions giving optimal expression of *H. pylori* urease subunit genes in *E. coli*, cells were grown at nickel ion concentrations of 100 μM, 1 mM, and 3 mM, indicating that maximal enzyme activities occurred at 1 mM $NiCl_2$ concentration. Cells were isolated and protein samples from each subunit clone were electrophoresed on SDS-polyacrylamide gels. The large (66 kDa) and small (29.5 kDa) subunit bands were clearly visible (Fig. 2). Each subunit was expressed as inclusion bodies although a small amount of the total *ureA* gene product was also found in the soluble fraction. Urea was used to solubilize the inclusion bodies which were then dialyzed for renaturation of each urease subunit.

The two subunits were mixed at 1:1 equimolar ratio and incubated with nickel ion in bicarbonate-containing buffers. The urease apoprotein complex showed very low urease activity even with 1 mM $NiCl_2$. Alteration of ionic strength and nickel concentrations did not improve the apoprotein activity. When 2.5 mM glutathione was added, however, the activity was significantly enhanced reaching a specific activity of about 5 U/mg of protein (Table 1). For comparison, native urease from pTZ19R/*ure-1* possessed a specific activity of about 49 U/mg of protein.

Renaturation studies showed that approximately 12.5% of the urease apoprotein activity *in vitro* was accomplished using 100 mM bicarbonate [21]. A sparse matrix screen method originally discovered for the crystallization of proteins was recently found to promote renaturation to a significant extent [10]. Most enzymes display different optimum conditions for renaturation in the presence of low concentrations of urea or guanidine hydrochloride. For the apoprotein of urease, the activity recovery under several screen conditions increased higher than that obtained using glutathione (Table 1). In particular, the effect of polyethylene glycol (PEG) on the rate of recovery was noticeable, giving two-fold increase in the specific activity (about 11 U/mg of protein). It is interesting to know that β-galactosidase of 540 kDa with multiple subunits also recovered its activity using 30% PEG [10]. It was reported that *E. coli* encoding only *ureA* and *ureB*

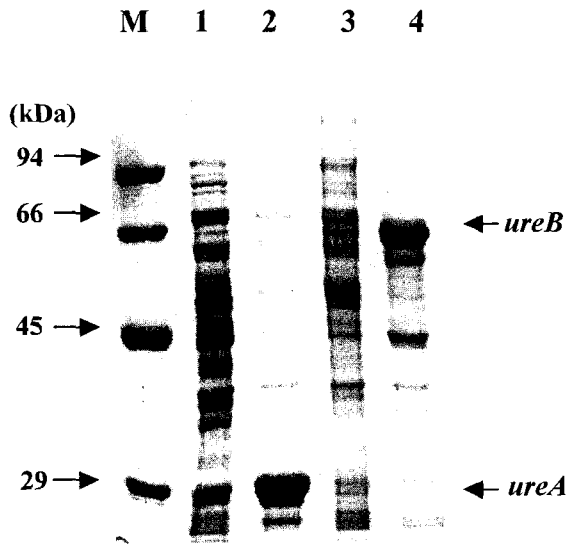

Fig. 2. SDS-PAGE analysis of *H. pylori* urease subunits expressed in *E. coli*.

Lanes: M, molecular weight markers (phosphorylase b, MW 94 kDa; bovine serum albumin, MW 66 kDa; egg albumin, MW 45 kDa; carbonic anhydrase, MW 29 kDa); 1, the soluble fraction in *E. coli* BL21(pET-5a/*ureA*) cell extracts; 2, same as in 1 except for the inclusion bodies instead of the soluble fraction; 3, the soluble fraction in *E. coli* BL21 (pET-5a/*ureB*) cell extracts; 4, same as in 3 except for the inclusion bodies instead of the soluble fraction.

Table 1. Optimized renaturation conditions for the recombinant *H. pylori* urease apoprotein.

Urease	Screening buffer		Activity (μM/min/mg)
	Buffer	Precipitant	
UreA and UreB combined	-	4 M formate	4.9
	citrate	20% PEG 20% 2-propanol	6.1
	-	30% PEG	10.9
	2 mM glutathione		5.4
Native urease from pTZ19R/*ure-1*	-		48.8

synthesized an assembled but inactive urease [11]. The activity of the recombinant urease from *E. coli* was not due to a lack of enzyme assembly, but rather to a failure to incorporate nickel ions into the active site. Renaturation of multiple subunit proteins or multiple domain proteins was slower than those with single domain due to the process of domain pairing and/or independent refolding of each domain [23]. The presence of a precipitant such as PEG may be important for the renaturation of the urease apoprotein complex, suggesting that the subunit assembly is critical for the recombinant urease activation.

Accessory proteins are involved in regulating the rate of folding and association of many proteins in the cell. Their participation is required in the cellular activation of *K. aerogenes* urease [20]. The assembly of *H. pylori* urease apoprotein also requires the function of accessory proteins on which recombinant urease activity was shown to be dependent for activation [5]. A large-scale expression and *in vitro* activation of the recombinant urease are essential for three-dimensional structural determination. Since each subunit of the apoprotein was overexpressed as inclusion bodies, it was partially activated using bicarbonate and nickel ions and further by glutathione which is generally used for disulfide interchange during renaturation. The UreA and UreB subunits contain one and three cysteines, respectively, in their sequences. Thus, it was speculated that the renaturation is independent of refolding of each subunit and that the activity of the recombinant urease depends on the incorporation of nickel ions into the active site. PEG is more effective in the activation of the apoprotein than the other agents studied. Our results to partially activate urease apoprotein in the absence of accessory proteins not only confirm the establishment of the crystallization buffer as initial conditions for renaturation, but also may suggest the importance of subunit pairing of the apoprotein. Purification of urease structural subunits and further analysis of the activation process of the urease apoprotein are underway.

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