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Expression of the Recombinant *Klebsiella aerogenes* UreF Protein as a MalE Fusion

Keun Young Kim, Chae Ha Yang[1] and Mann Hyung Lee*

College of Pharmacy, Catholic University of Taegu-Hyosung, Hayang, Kyongsan, Kyongbook, Korea and [1]College of Oriental Medicine, Kyungsan University, Kyongsan, Kyongbook, Korea

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Expression of the active urease of the enterobacterium, *Klebsiella aerogenes*, requires the presence of the accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) in addition to the three structural genes (*ureA*, *ureB*, and *ureC*). These accessory genes are involved in functional assembly of the nickel-metallocenter for the enzyme. Characterization of *ureF* gene has been hindered, however, since the UreF protein is produced in only minute amount compared to other urease gene products. In order to overexpress the *ureF* gene, a recombinant pMAL-UreF plasmid was constructed from which the UreF was produced as a fusion with maltose-binding protein. The MBP-UreF fusion protein was purified by using an amylose-affinity column chromatography followed by an anion exchange column chromatography. Polyclonal antibodies raised against the fusion protein were purified and shown to specifically recognize both MBP and UreF peptides. The UreF protein was shown to be unstable when separated from MBP by digestion with factor Xa.

Key words : *Klebsiella aerogenes*, Urease, *ureF*, MalE, Maltose-binding protein, Fusion

INTRODUCTION

Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamate. The carbamate spontaneously decomposes to produce a second molecule of ammonia and carbon dioxide. The bacterial urease has been implicated as a virulence factor in various human and animal diseases (Mobley *et al.*, 1995).

The best-characterized bacterial urease is that from the gram-negative enteric bacterium, *Klebsiella aerogenes*. Crystallographic analysis of this enzyme revealed that the three structural subunits (UreA, UreB, and UreC) associate as a trimer of trimers [(UreA-UreB-UreC)$_3$] containing one dinuclear nickel metallocenter per UreC (Jabri *et al.*, 1995).

Assembly of the nickel metallocenter appears to be a complex process requiring the action of several accessory gene products. For example, deletion analysis of the *Klebsiella aerogenes* urease gene cluster revealed that one gene (*ureD*) located directly upstream and three genes (*ureE*, *ureF*, and *ureG*) found immediately downstream of the three structural subunit-encoding genes (*ureA*, *ureB*, and *ureC*) are involved in the functional assembly of the urease metallocenter (Mulrooney and Hausinger, 1990; Lee *et al.*, 1992). Although detailed and precise function of these accessory proteins has not been established, UreE has been shown to be a nickel-binding protein (Lee *et al.*, 1993), and other accessory proteins are thought to function as complexes with the urease subunits. Both *ureD* and *ureF* possess nonconcensus ribosome binding sites and are expressed at very low levels in the cell. Changing the upstream DNA sequence and initiation codon for *ureD* to improve the ribosome binding site of the gene resulted in the synthesis of high levels of UreD, shown to be present in a series of complexes containing the trimeric apoenzyme [(UreA-UreB-UreC)$_3$] binding one, two, or three UreD peptides (Park *et al.*, 1994). Incubation of UreD-apourease with bicarbonate and nickel ions leads to UreD dissociation and partial activation of apourease (Park and Hausinger, 1995b). Another series of apourease complexes containing UreD, UreF, and UreG was detected and able to be activated *in vitro* (Park and Hausinger, 1995a). Since all of these components are required for generating active urease *in vivo*, the UreD-UreF-UreG-apourease complexes were suggested to serve as the key cellular urease activation machinery. These complexes are present at minute levels in the cell, and their properties have not been well characterized. The specific roles of UreD, UreF, and UreG in these complexes are

Correspondence to: Mann Hyung Lee, College of Pharmacy, Catholic University of Taegu-Hyosung, Hayang, Kyongsan, Kyongbook, Korea

unknown.

In order to better define the function of the *ureF* gene product, we have overexpressed and purified UreF as a fusion with an *E. coli* maltose-binding protein (MBP; MalE), and shown that UreF protein is unstable when cleaved from the fusion protein. Polyclonal antibodies raised against the UreF-MBP fusion protein were shown to recognize both the MBP and UreF, thus could be used for future studies on the interactions between individual accessory proteins.

MATERIALS AND METHODS

Construction of pMAL-UreF plasmid

Previously described plasmid pKAU17 (Mulrooney and Hausinger, 1990) includes the whole urease gene cluster of *K. aerogenes*. To amplify a 766 bp-DNA fragment containing *ureF* open reading frame, a pair of oligonucleotide primers (Fig. 1) were designed such that the 5-end of the resulting DNA product starts at AUG start codon of the *ureF* gene and an artificial *Hin*dIII restriction site could be generated at the 3-end. After being annealed to the denatured pKAU17, the oligonucleotide primers were extended during temperature cycling (94°C, 2 min, 40°C, 2 min, 72°C, 1 min, 35 cycles, then 72°C, 6 min) in the presence of *Taq* DNA polymerase (Promega Co., WI, USA). The amplified DNA fragment was identified on a 1% agarose gel and rendered blunt by using T4 DNA polymerase, cut with *Hin*dIII, then gel-purified by using Gene Clean II kit (Bio101 Inc., CA, USA). This fragment was ligated into the *Xmn*I-*Hin*dIII digested, and calf intestinal phosphatase-dephosphorylated pMAL-c2 vector (New England Biolabs, MA, USA) to produce pMAL-UreF. The recombinant plasmid was transformed into *E. coli* DH5α and the recombinant clones were selected on X-gal-containing agar plates.

Expression of the MBP-UreF fusion protein

E. coli DH5α (pMAL-UreF) cells were grown at 35°C to early exponential phase (OD_{600}=0.5) in tryptone broth or in MOPS (3-[N-morpholino] propanesulfonic acid)-ammonia medium (Neidhardt *et al.*, 1974) containing 0.2% glucose and 60 μg/ml ampicillin. Expression of the fusion protein was induced by adding 0.3 mM isopropyl β-D-thioglucopyranoside (IPTG) and after 2 hour further incubation, cells were harvested by centrifugation, washed twice with ice-cold 20 mM KH_2PO_4 (pH 7.2)-1 mM EDTA-1 mM dithiothreitol (PED) buffer. The optimal induction time was determined with timed-aliquots of the pilot culture by using SDS-polyacrylamide gel electrophoresis and immunoblot analyses as decribed below. Cell pellet was resuspended in an equal volume of PED buffer containing 1 mM phenylmethylsulfonyl fluoride, then disrupted by three passages through a French pressure cell (SLM Instruments, Inc., Urbana, IL, USA) at 18,000 lb/in^2, and centrifuged at 100,000×g for 90 min at 4°C.

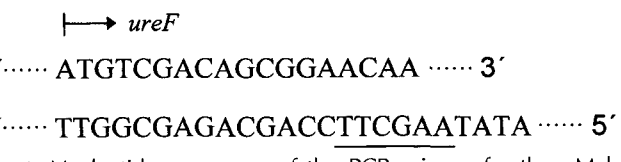

Fig. 1. Nucleotide sequences of the PCR primers for the pMal-c2-*ureF* in-frame fusion cloning. An artificial *Hin*dIII restriction site is shown underlined.

Purification of the fusion protein and isolation of UreF peptide

The membrane-free cell extracts as obtained above were loaded onto an amylose column (2.5×5 cm) which had been pre-equilibrated with the PED buffer and the bound fusion protein was eluted with PED containing 10 mM maltose. The pooled sample was dialyzed for 18 hours against PED buffer at 4°C, then applied to a Resource-Q (6 mL) anion exchange column (Pharmacia Inc., Uppsala, Sweden) and eluted with a multi-step gradient of increasing KCl in the same buffer. The presence of the fusion protein in column fractions was assessed by SDS-polyacrylamide gel electrophoresis. The purified MBP-UreF protein was digested with the factor Xa for 48 hrs at 16°C in the presence of 0.01% sodium dodecyl sulfate. A portion of the resulting hydrolysis mixture was loaded onto a Q-Sepharose column (1 mL), and was eluted with a linear gradient to 1 M KCl.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by using buffers of Laemmli (Laemmli, 1970) and included a 12% polyacrylamide running gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Commassie brilliant blue R250. Protein was measured by the method of Lowry *et al.* (1951) with the bovine serum albumin as the standard.

Preparation of polyclonal antibodies and purification of the IgG fraction

Antibodies directed against the purified MBP-UreF protein were generated in a white New Zealand rabbit by injecting subcutaneously 200 μl (1.2 mg/ml) of homogeneous protein in PBS emulsified with the same volume of Freunds complete adjuvant (Sigma Chemical Co., St. Louis, Mo. USA). The rabbit was boosted after 28 days, and after an additional 14 days, whole blood was drawn and the serum was separated by centrifugation after 16 hr at 4°C. The IgG fraction was purified from

the serum by using an am-monium sulfate fractionation followed by caprylic acid precipitation method (McKinney and Parkinson, 1987). Purified IgG was concentrated by using an Amicon pressure filtration stirred-cell with a YM10 ultrafiltration membrane (Amicon, USA). Antibodies in the samples were titrated by using standard ELISA methods (Engvall and Perlmann, 1972).

Western blot analysis

The crude extracts of *E. coli* DH5α (pMAL-UreF) cells before and after the IPTG-induction, together with purified fusion protein and factor Xa digestion mixture were loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, the peptide bands were electrically transferred onto a nitrocellulose membrane in Towbins buffer (Towbin *et al.*, 1979) (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and the membrane was stained briefly with 0.2% Fast Green solution in order to confirm the transfer of the peptide bands. Then the membrane was sequentially incubated with Blotto/Tween blocking solution, diluted (1:100,000) anti-MBP-UreF polyclonal IgG, and finally with goat anti-rabbit-alkaline phosphatase conjugate (1:30,000), respectively. Peptide bands which specifically cross-reacted with the IgG molecules were visualized by applying 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution onto the membrane.

Amino terminal sequence analysis

The factor Xa-digested mixture of the purified fusion protein was resolved in a 0.75 mm denaturing gel, as described above, and electrically transferred onto a sheet of Immobilon-P membrane (Millipore, CA, USA) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 10)-10% methanol. The bands were visualized by Coomassie blue staining and cut from the membrane, and analyzed by using an Applied Biosystem 476A automated sequencer in the Korea Basic Science Institute at Taejeon.

RESULTS AND DISCUSSION

Cloning and expression of the MBP-UreF fusion protein

In order to overexpress *K. aerogenes ureF* gene as a MBP-fusion protein, a PCR-amplified, 766 bp DNA fragment containing the ureF open reading frame was inserted into the *XmnI-HindIII* site of the pMALc-2 vector as described above, resulting in the recombinant plasmid pMAL-UreF. The recombinant *E. coli* DH5α (pMAL-UreF) cells grown in tryptone broth or MOPS-ammonia medium produced a large amount of the MBP-UreF fusion protein (Mr≃66,000) after 2 hr induction with IPTG as estimated on 12% SDS polyacrylamide gels (Fig. 2). Induction of the fusion protein, however, was more consistent and reproducible when the recombinant cells were grown in MOPS-ammonia medium than in tryptone broth (data not shown). The level of expression of the protein was estimated by SDS-PAGE and/or immunoblot analyses.

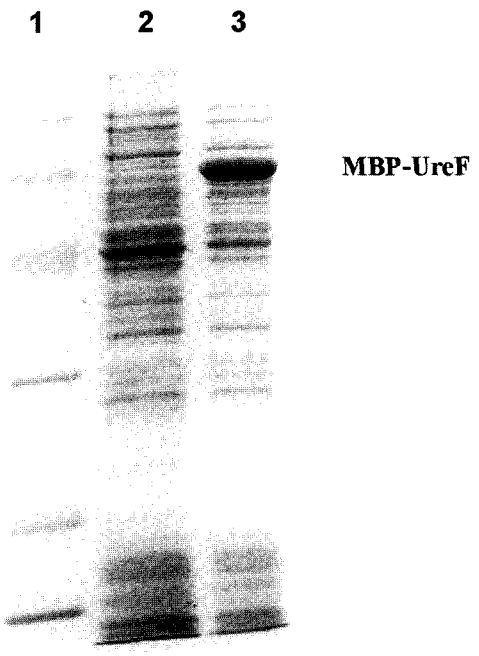

Fig. 2. 12% SDS-polyacrylamide gel electrophoresis of the cell extracts from *E. coli* DH5α (pMAL-UreF) before (lane2) and after (lane 3) IPTG-induction. Molecular weight markers (lane 1) were phosphorylase b, M_r=97,400; bovine serum albumin, M_r=66,200; ovalbumin, M_r=45,000; carbonic anhydrase, M_r=31,000; soybean trypsin inhibitor, M_r=21,500; and lysozyme M_r=14,400.

Purification of MBP-UreF and separation of UreF

MBP-UreF fusion protein was purified from cell extracts by passing through an amylose column to which MBP has binding affinity. Elution of the fusion protein with 10 mM maltose yielded a nearly homogeneous peptide band as revealed by SDS-PAGE (Fig. 3, lane 4). Fractions containing the fusion protein was further purified to homogeneity by using Resource-Q® anion exchange column chromatography. When purified fusion protein was digested with factor Xa which is supposed to cleave the junction between MBP and UreF, a 27,000±1,000 dalton peptide band matching the approximate size of the predicted UreF molecular weight (25,122 Da) was observed in addition to MBP and the factor Xa peptide bands on the polyacrylamide gel (Fig. 3, lane 5). The identity of this 27 kD-peptide band was confirmed as the genuine UreF peptide [Met-Ser-Thr-Ala-Glu-...] by amino-terminal sequencing analysis as described above.

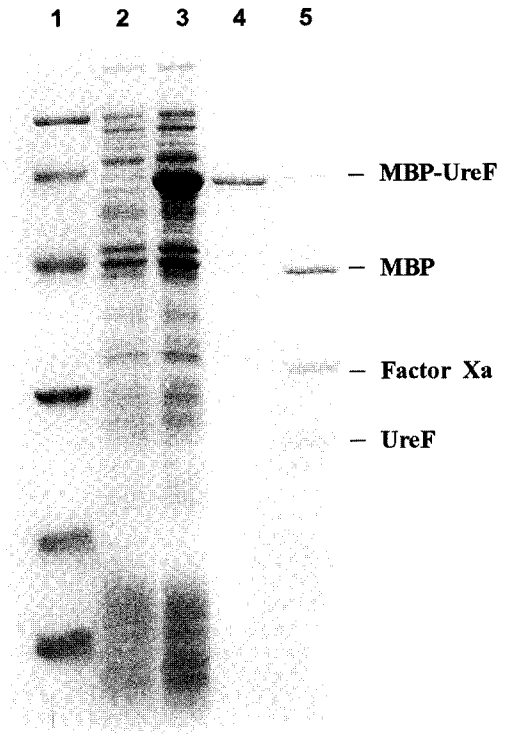

Fig. 3. 12% SDS-polyacrylamide gel electrophoresis of the MBP-UreF fusion protein. Cell extracts from *E. coli* DH5α (pMAL-UreF) before (lane2) and after 2 hr IPTG-induction (lane 3), the purified MBP-UreF fusion protein after amylose column chromatography (lane 4), and the Factor Xa-digested mixture of the purified MBP-UreF fusion protein (lane 5) were subjected subjected to SDS-polyacrylamide gel electrophoresis, followed by Coomassie blue staining. Molecular weight markers (lane 1) used were identical to those in Fig. 2.

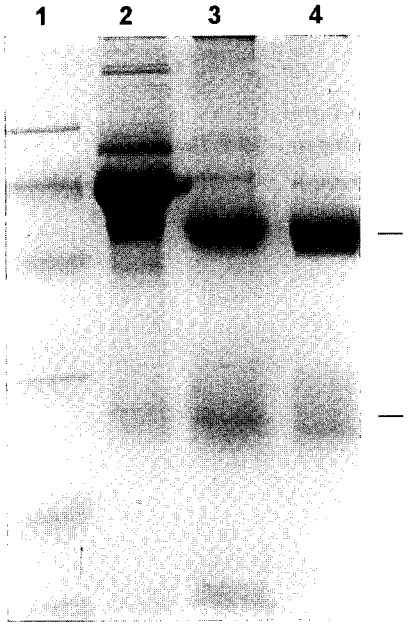

Fig. 4. 12% SDS-polyacrylamide gel electrophoresis of the rabbit anti-MBP-UreF IgG at each purification step. Rabbit serum (lane 2), sample after ammonium sulfate precipitation (lane 3), and the caprylic acid precipitation (lane 4) were subjected to SDS-polyacrylamide gel electrophoresis. Molecular weight markers (lane 1) used were identical to those in Fig. 2. Immunoglobulin heavy chains and light chains are indicated by side bars. The light chain of the purified IgG (lane 4) did not migrate as a discrete band and appeared as a diffused area as observed in the previous studies (McKinney and Parkinson, 1987).

The band intensity of the UreF peptide on the SDS-polyacrylamide gel, however, was much weaker than that of the MBP which was supposed to be expressed in 1:1 stoichiometric ratio. Q-sepaharose column chromatography of the factor Xa-digestion mixture did not resolve the free UreF peptide, either. Recently, *ureF* has been overexpressed under the influence of a T7 promoter in a background containing all or most of the other urease genes (Moncrief and Hausinger, 1996). With this construct, the authors identified and purified a series of complexes containing UreD, UreF, and the urease subunits, thus showing the interactions between urease accessory proteins and structural subunits. The UreF peptide, however, was shown to be nearly insoluble when separated from other urease protein subunits. These results suggest that in our experiment, significant amount of UreF peptide might have been degraded upon releasing from the fusion protein or a certain proportion of the protein might have precipitated out of the reaction buffer. The latter possibility was ruled out since centrifuged and trichloroacetic acid-precipitated sample did not show the UreF peptide on the SDS-polyacrylamide gel (data not shown).

Specificity of the antibody directed against the fusion protein

Polyclonal antibody against the purified MBP-UreF fusion protein was raised in a rabbit and purified by non-chromatographic method as described above. The purified antibodies were sufficiently pure for the purpose of our experiments as shown in lane 4 of Fig. 4. When factor Xa-digested mixture of the fusion protein was subjected to SDS-PAGE and subsequently to Western blot analysis, the polyclonal antibody specifically recognized both MBP and the UreF protein as shown in Fig. 5 (lane 4). A peptide band corresponding to a certain portion of the undigested fusion protein was also visible. Cell extracts from uninduced *E. coli* DH5α (pMAL-UreF) showed a faint band of the fusion protein which indicates a basal level of expression (lane1), while crude extracts from induced cells (lane 2) contained some additional peptide bands which are probably some degradation products.

These results suggest the potential applicability of this polyclonal antibody to studies of protein-protein interactions among urease accessory proteins including the

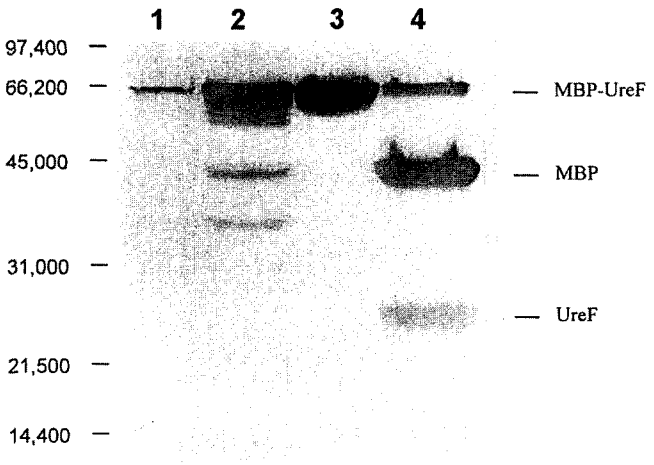

Fig. 5. Western blot analysis of the various MBP-UreF or UreF samples with the purified rabbit anti-MBP-UreF IgG. Cell extracts from *E. coli* DH5α (pMAL-UreF) before (lane 1) and after 2 hr IPTG-induction (lane 2), the purified MBP-UreF fusion protein after Resource-Q column chromatography (lane 3), and the Factor Xa-digested mixture of the purified MBP-UreF fusion protein (lane 4) were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis as described in Materials and Methods.

UreF peptide by using, for example, affinity chromatography and/or immunoprecipitation. Alternatively, this polyclonal antibody could be gold particle-conjugated and used to localize the UreF or accessory protein complex including UreF within the bacterial cellular structure.

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