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Purification and Characterization of the Recombinant *Bacillus pasteurii* Urease Overexpressed in *Escherichia coli*

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Abstract A 6.9-kb DNA fragment including the minimal *Bacillus pasteurii* urease gene cluster was subcloned into a high-copy-number plasmid vector, pUC19, and the recombinant *B. pasteurii* urease was overexpressed in *Escherichia coli.* The recombinant urease was purified 25.9-fold by using combinations of anion-exchange and gel-filtration chromatography followed by Mono-Q chromatography on a FPLC. N-terminal peptide sequencing analyses revealed that two distinct smaller peptide bands resolved on a 10–18% gradient SDS-PAGE corresponded to UreA and UreB peptides, respectively. It was also shown that the *ureB* gene was translated from a GUG codon and the first methionine residue was post-translationally cleaved off. The native molecular weight of the recombinant urease was 176,000 and 2 nickel atoms were present per catalytic unit. pH stability studies of the purified enzyme showed that the recombinant *Bacillus pasteurii* urease is stable in alkaline pH range, which is similar to the enzyme of the evolutionarily related bacterium, *Sporosarcina ureae.*

Key words: *Bacillus pasteurii,* urease, *Sporosarcina ureae,* nickel

Bacillus pasteurii is an alkalophilic soil bacterium and has been known for its remarkable urease production [6]. *B. pasteurii* urease, like other bacterial and plant ureases, contains nickel at the active site and hydrolyzes urea to yield two molecules of ammonia and a carbonic acid [1, 13]. In addition to being important as a bacterial virulence factor in various human and animal diseases, the enzyme also plays a crucial role in ruminant metabolism and in environmental nitrogen transformations (for a recent review, see Ref. 12). Recent DNA sequence analyses [16] revealed the presence of the urease accessory genes (*ureE, ureF, ureG,* and *ureD*) located immediately downstream of the three structural genes (*ureA, ureB,* and *ureC*). These non-subunit auxiliary genes are thought to be involved in nickel processing and/or nickel incorporation into the apo-urease since the conserved, corresponding accessory genes in the *Klebsiella aerogenes* urease gene cluster were previously shown to be required for urease metallocenter assembly [9]. Lee *et al.* [8] recently demonstrated that the purified *B. pasteurii* urease protein possesses one large subunit and two smaller subunits which is consistent with the DNA sequence data and with the cases of the majority of other bacterial ureases.

Previously cloned recombinant plasmid pBU11 [7] is a pBR322 derivative and contains a 11-kb insert which includes very long unsequenced flanking DNA regions on both sides of the *B. pasteurii* urease gene cluster. In this study, we subcloned a 6.9-kb DNA fragment including the minimal urease gene cluster into the pUC19 vector. The recombinant *B. pasteurii* urease, overexpressed and purified from an *E. coli* strain transformed with this plasmid, possessed the highest specific activity to date and was used for N-terminal peptide sequencing analyses to identify the two small subunits. The purified enzyme was also shown to be stable over the alkaline pH range, which is different from the results of a previous study [7].

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MATERIALS AND METHODS

Plasmid Construction

Plasmid pBU11 [7] was digested with *Pvu*I and the ends were flushed by treating the linearized DNA with T4 DNA polymerase. Subsequent digestion with *Kpn*I yielded a 4.5 kb fragment which includes the structural genes (*ureA, ureB,* and *ureC*). This DNA fragment was isolated by using Gene Clean II kit (Bio101 Inc., CA, U.S.A.), inserted and ligated into the *Hin*cII-*Kpn*I digested, calf intestinal phosphatase-dephorylated pUC19 vector to produce pBU703 (Fig. 1). Separately, a 2.5 kb fragment containing the downstream accessory genes (*ureE, ureF, ureG,* and *ureD*) was isolated by digesting pBU11 with *Apa*LI, followed by treatment with Klenow fragment and *Kpn*I. This was then ligated into the *Nde*I-*Kpn*I fragment (6.9 kb) of pBU703 to

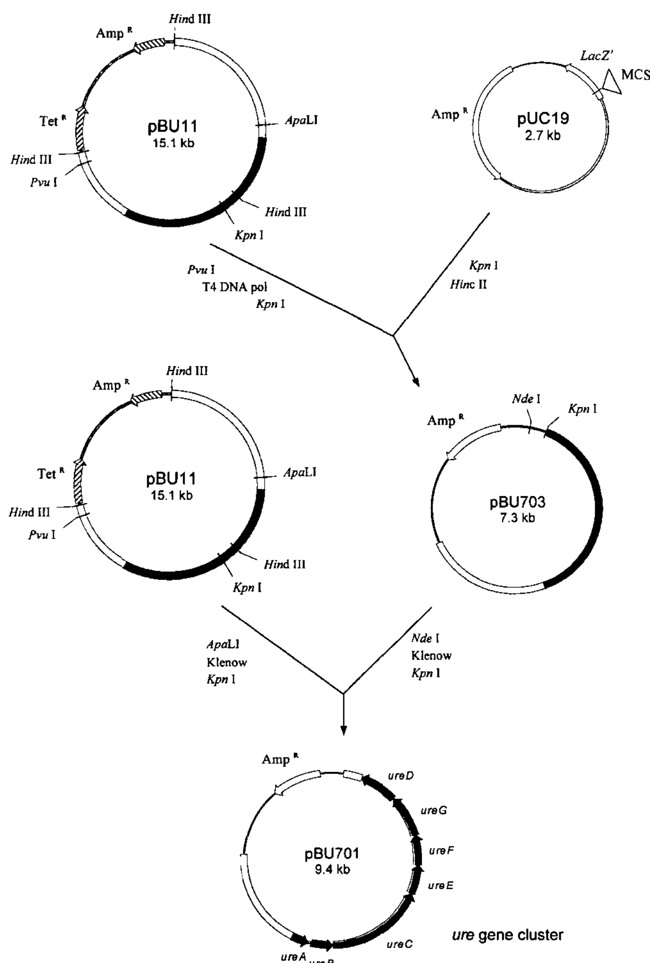

Fig. 1. Scheme for the construction of pBU701 and 703 plasmids. DNA regions containing the urease gene cluster are indicated as gray boxes and the flanking regions by white boxes. The pBU701 carries a complete urease operon and the pBU703 includes only structural genes (*ureA*, *ureB*, and *ureC*).

produce the plasmid pBU701 (9.4 kb) which includes the entire urease gene cluster of *Bacillus pasteurii*. Plasmids pBU701 and pBU703 were transformed into *E. coli* DH5α by standard methods.

Urease Purification

Cultures (2 l) of *E. coli* DH5α (pBU701) were grown to late exponential phase in LB medium containing 1 mM nickel chloride and 100 μg/ml ampicillin. Cells were harvested by centrifugation, washed twice with ice-cold 25 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES, pH 9)-1 mM ethylenediamine tetraacetate (EDTA)-1 mM dithiothreitol (CED) buffer, resuspended in an equal volume of CED containing 1 mM phenylmethylsulfonyl fluoride, disrupted by three passages through a French pressure cell (SLM Instruments, Inc., IL, U.S.A.) at 18,000 lb/in^2, and ultracentrifuged at 100,000×g for 90 min at 4°C in order to remove the membrane fraction.

The supernatant solution was applied to a column (2.5× 15 cm) of DEAE-Sepharose CL-6B equilibrated in CED buffer at 4°C. The recombinant urease was eluted with a 400-ml linear gradient of 0 to 1 M KCl in CED buffer, resulting in a peak activity at approximately 0.5 M KCl. The pooled sample was concentrated to 3 ml by using an Amicon pressure filtration stirred-cell with a YM30 ultrafiltration membrane and subjected to a Superdex 200HR (1.6×60 cm) gel filtration chromatography in CED buffer supplemented with 0.15 M KCl. Peak fractions were pooled and dialyzed for 18 h against CED buffer at 4°C, then applied to Mono-Q HR10/10 column and eluted with a multi-step gradient of increasing KCl in the same buffer. All resins and columns were purchased from Pharmacia, Inc. (Uppsala, Sweden). The presence of urease protein in column fractions was assessed by the one-time-point urease assay method based on indophenol production [15]. For the studies of specific activity comparison, *E. coli* DH5α (pBU701), *E. coli* DH5α (pBU703), and *E. coli* DH5α (pBU11) were grown in 100 ml LB containing 100 μg/ml ampicillin and 1 mM nickel chloride, cells were disrupted by using a sonic dismembrator (Fisher Scientific Co., PA, U.S.A.) with a maximum output of 70 W for 1 min/ml, and centrifuged at 50,000×g for 30 min.

Urease Assay

Urease activity was measured by quantitating the rate of ammonia released from urea by formation of indophenol, which was monitored at 625 nm as previously described [15]. The assay buffer consisted of 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 200 mM urea, and 0.5 mM EDTA (pH 7.5). The reactions were initiated by the addition of the enzyme, the concentration of released ammonia was measured in time aliquots, and the rates were determined by linear regression analysis. One unit of urease activity is defined as the amount of enzyme required to hydrolyze 1 μmol of urea per min at 37°C under the assay conditions described above. Protein content was measured by the method of Lowry *et al.* [10], with bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis

SDS-PAGE electrophoresis was carried out using the buffers of Laemmli [5] and included a 10-18% polyacrylamide gradient running gel with a 4.5% polyacrylamide stacking gel. The gels were stained with Coomassie brilliant blue R250.

Determination of the Native Molecular Weight of Urease

The native molecular weight of the recombinant *B. pasteurii* urease was estimated by using Superose 12 chromatography in CED buffer (pH 9.0) containing 0.15 M KCl. The column (1.0×30 cm) was standardized by using thyroglobluin, gammaglobulin, ovalbumin, myoglobin, and

vitamin B_{12} (M_rs=670,000, 158,000, 44,000, 17,000, 1350; Bio-Rad Labs, U.S.A.).

Amino Terminal Sequence Analysis

The enzyme subunits from the purified urease were resolved in a 0.75 mm denaturing gel, as described above, and electrophoretically transferred onto a sheet of Immobilon-P membrane (Millipore, CA, U.S.A.) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, pH 10)-10% methanol. The bands were visualized by Coomassie blue staining, cut from the membrane, and analyzed by using an Applied Biosystem 476A automated sequencer in the Korea Basic Science Institute in Taejon.

pH Studies

The enzyme stability was assessed at various pH values by incubating the purified urease in 1 mM EDTA, 10 mM buffer at 0°C for 60 min or 20 h, then diluting the enzyme 20-fold into the standard assay buffer at 37°C. Buffers to test stability included succinate (pH 4.8-5.8), 2-(N-morpholino) ethanesulfonic acid (MES, pH 5.3-6.6), HEPES (pH 6.5-7.8), N-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS, pH 7.7-9.1), CHES (pH 8.9-10.0), CAPS (pH 10.4-11.4), and phosphate (pH 11.5-13.2). The pH of the buffers were adjusted on ice.

Nickel Content of the Recombinant *B. pasteurii* Urease

The purified urease (100 μg) was treated with 20% nitric acid and the sample was vacuum-evaporated a few times with repeated addition of sterile milli-Q water in a rotary vacuum concentrator (MaxiDry-Lyo, Heto-Holten, Denmark). After resuspending in ultra-pure water, the sample was analyzed for nickel content by using an ICP-mass spectrophotometer (VG Elemental, U.K.) in the Korea Basic Science Institute in Taejon.

RESULTS AND DISCUSSION

Overexpression and Purification of Recombinant *B. pasteurii* Urease

A 6.9-kb DNA fragment including the entire *Bacillus pasteurii* urease gene cluster was subcloned into the pUC19 vector in two steps, as described above, resulting in the plasmid pBU701. This resulted in deletion of long DNA regions which are not related with the urease activity and thus many unique restriction enzyme cleavage sites became available for future DNA manipulations in the urease operon region. The specific enzyme activity of the cell extract from *E. coli* DH5α (pBU701) increased 2.5-fold when compared to that from *E. coli* DH5α (pBU11) (Table 1), while deletion of the accessory gene region (*ureE*, *ureF*, *ureG*, and *ureD*) (pBU703) completely abolished the activity, as found in other bacterial cases [9, 13].

Table 1. Comparison of specific activities of cell extracts from *Escherichia coli* DH5α cells carrying pBU11, pBU701, and pBU703.

Strains	Specific activity (U/mg)	% Activity
E. coli (pBU11)	18.2	100
E. coli (pBU701)	50.6	278
E. coli (pBU703)	< 1	< 1

Purification of the urease from French press-disrupted cells by a three-column procedure is summarized in Table 2. Although maximum activity of the *B. pasteurii* urease was observed at pH 7.5, all purification steps were carried out at alkaline pH because of the instability of this enzyme at neutral pH, as shown below. The recombinant urease eluted at 0.5 M KCl from the DEAE Sepharose column and at 0.45 M KCl from the Mono-Q column. Based on the increase in the specific activity of the urease, the enzyme was estimated to have been purified 25.9-fold. The estimated purity of the Mono-Q pool was more than 95% homogeneous as shown in the SDS-polyacrylamide gel (Fig. 2, lane 5) and the specific activity reached the level of 1841 U/mg, which was the highest ever obtained. As reported in the previous study [8], one large subunit (α, M_r=65,200) and two smaller peptides (β and γ, M_rs= 14,500 and 13,700) were clearly shown to be associated with the urease activity throughout the purification steps.

Characterization of the Recombinant *Bacillus pasteurii* Urease

The two smaller peptide bands were blotted onto a Immobilon-P membrane, excised, and subjected to amino terminal sequence analysis. The smallest peptide (M_r= 13,700) was shown to have an N-terminal amino acid sequence (Met-His-Leu-Asn-Pro-Ala-Glu) that is exactly

Table 2. Purification of the recombinant *Bacillus pasteurii* urease from *E. coli* (pBU701).

Purification step	Specific activity (U/mg)	Purification (-fold)	Total activity (U)	Enzyme recovery (%)
Cell extract	71	1.0	91,870	100
DEAE-Sepharose	380	5.4	80,692	88
Superdex HR-200	734	10.3	34,771	38
Mono-Q HR 10/10	1841	25.9	12,297	13

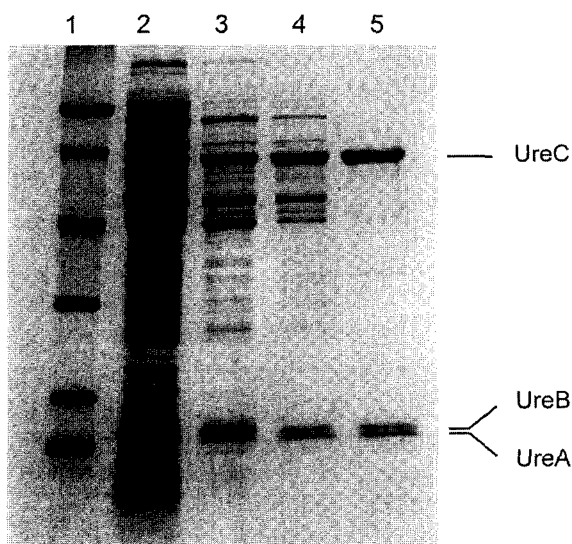

Fig. 2. SDS-PAGE of the recombinant *Bacillus pasteurii* urease from each purification step.

Cell extracts from *E. coli* DH5α (pBU701) (lane 2), DEAE-Sepharose pool (lane 3), Superdex HR-200 pool (lane 4), and Mono-Q HR10/10 pool (lane 5) were subjected to SDS-PAGE by using a 10-18% gradient gel, followed by Coomassie blue staining. Molecular weight markers (lane 1) were phosphorylase b, M_r=92,500; 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.

matching to that of UreA predicted from the DNA sequence [16]. The peptide which migrated to a slightly higher location on the 10-18% SDS-polyacrylamide gel was revealed to have an N-terminal amino acid sequence (Ser-Asn-Asn-Asn-Tyr) that agreed with that deduced for UreB, except that the first amino acid (methionine) was missing. Apparently, the N-terminal methionine was cleaved by a methionine aminopeptidase coded by the *E. coli* host [2]. This result also confirmed the prediction we made previously [9] that translation of the *ureG* open reading frame might start at an alternative upstream GUG codon [4].

Christians *et al.* [3] reported that the purified *B. pasteurii* urease was an enzyme of a single subunit, and later, Lee *et al.* [7] suggested that it consisted of two peptide subunits. Recently, however, it has been demonstrated that *B. pasteurii* urease was composed of one large and two small subunits like other bacterial ureases [8]. N-terminal peptide sequencing data obtained in this study verified the presence of the three subunits in *B. pasteurii* urease.

The native molecular weight of the recombinant *B. pasteurii* urease was estimated to be 176 kDa by Superose 12 gel-filtration chromatography. This suggests that the native protein exists as a heterodimer of $(\alpha\beta\gamma)_2$, which is distinct from other bacterial ureases, the majority of which are composed of heterotrimers $[(\alpha\beta\gamma)_3]$ [13].

The metal analysis showed that the recombinant *B. pasteurii* urease contains 1.8±0.2 nickel atoms in each catalytic unit, thus suggesting the presence of four nickel atoms in a native enzyme, based on the native molecular weight data as estimated above.

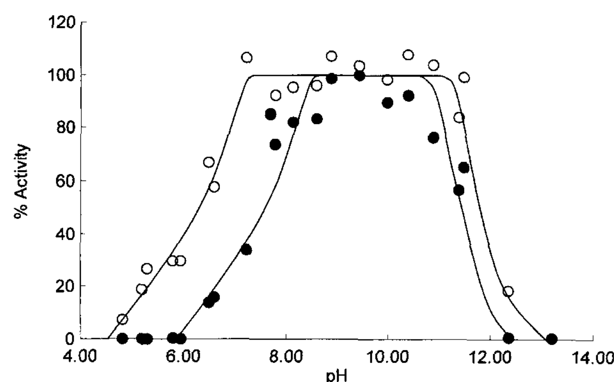

Fig. 3. pH stability of the recombinant *Bacillus pasteurii* urease.

Purified urease was incubated at 0°C in buffers at the indicated pH values for 1 h (○) or 20 h (●), and then the enzyme activity was assessed in the standard assay conditions at pH 7.5.

pH Stability of the *B. pasteurii* Urease

In previous studies, *Bacillus pasteurii* urease showed stability over a broad range of neutral pH [7]. In this study, however, the recombinant *Bacillus pasteurii* urease was shown to be stable over a pH range of 8-11 for up to 20 h at 0°C, but rapid loss of activity was observed at higher or lower pH values (Fig. 3). Incubation of the enzyme on ice at pH 6.6 led to a loss of >40% activity in 1 h and >80% loss after 20 h. By contrast, the enzyme was very stable to elevated pH as shown by the complete retention of activity at pH 11.5. This alkaline stability of *B. pasteurii* urease is similar to the enzyme purified from *Sporosarcina ureae* [11], which is taxonomically closely related to *B. pasteurii* [14].

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