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Purification of the Three-subunit, Recombinant *Bacillus pasteurii* Urease Expressed in *Escherichia coli*

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The genes coding for the urease of alkalophilic *Bacillus pasteurii* have been previously cloned and recently sequenced. (You, J. H., B. H. Song, J. H. Kim, M. H. Lee, and S. D. Kim (1995) *Molecules and Cells* 5, 359-369.) The recombinant *Bacillus pasteurii* urease expressed in an *E. coli* HB101 strain was purified 31.2 fold by using combinations of anion-exchange and hydrophobic chromatography followed by Mono-Q chromatography on a FPLC. In spite of the presence of three discrete structural peptide genes in the *Bacillus pasteurii* urease gene cluster, only one or two enzyme subunits have been observed to date. Here we report for the first time that the recombinant *Bacillus pasteurii* urease expressed in a *E. coli* strain consists of three distinct subunits. One large subunit was estimated to be of M_r=65,200 and the two small-subunit peptides are of M_r=14,500 and M_r=13,700, respectively.

Urease (EC 3.5.1.5), a nickel-containing enzyme found in certain plants and many microorganisms, hydrolyzes urea to yield ammonia and carbamate; the carbamate spontaneously decomposes to form a second molecule of ammonia and 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 the environmental nitrogen transformations (for review, see ref. 13).

The most extensively characterized microbial urease is that from the Gram-negative enteric bacterium, *Klebsiella aerogenes* (a non-nitrogen-fixing *Klebsiella pneumoniae*). The urease enzyme possesses three different subunits [M_rs=60,304 (α), 11,695 (β), and 11,086 (γ) (14)] where each catalytic unit is comprised of subunits in an $\alpha\beta\gamma$ stoichiometry and contains a bi-nickel active site (5, 17). Most of the other bacterial ureases show similar three-subunit compositions except the two-subunit *Helicobacter pylori* urease (4), and the apparent single-subunit enzymes that were purified from *Brevibacterium ammoniagenes* (15) and *Bacillus pasteurii* (2, 9).

Since alkalophilic *B. pasteurii* has been known for its remarkable urease production, as reported by Larson and Kallio (8), the urease genes were cloned (6), and the DNA sequence analyses have been recently completed by You *et al.* (19).

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DNA sequence analysis revealed the presence of several additional genes that are part of the *B. pasteurii* urease gene cluster as in the cases of other bacterial urease operons. The three putative urease structural genes (*ureA*, *ureB*, and *ureC*) are immediately followed by the *ureE*, *ureF*, *ureG*, *ureD*, *ureH*, and *ureI* genes (19). These non-subunit auxiliary genes are thought to be involved in nickel processing into the apo-urease since the corresponding accessory genes in the *K. aerogenes* urease gene cluster were previously shown to be required for urease metallocenter assembly (10).

Despite the presence of three putative structural genes (*ureA*, *ureB* and *ureC*), however, Christians *et al.* (2) reported only a single peptide band of M_r=65,500 on a 3-12% gradient polyacrylamide gel, while Lee *et al.* (9) observed a smaller, minor peptide subunit of M_r=20,000 in addition to a single major protein band of larger size (M_r= 67,000) on a 7.5% disc-polyacrylamide gel. Under the experimental conditions used in both studies, however, it is quite possible to overlook one or two small urease subunits if one of the smaller peptides is too small (that is less than 5 kDa) or if the apparent molecular weights of the two peptides are too similar to each other to be resolved on simple polyacrylamide gels.

In this study, we used 10 to 20% gradient SDS-polyacylamide gel electrophoresis to demonstrate that the purified *B. pasteurii* urease protein possesses one large subunit and two smaller subunits which is consistent with the DNA sequence analysis data and with the cases of the majority of other bacterial ureases.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli HB101 cells transformed with the plasmid pBU11 harbouring the entire urease gene cluster of *B. pasteurii* (9) were grown at 37°C in LB medium containing 1 mM nickel chloride and ampicillin (50 mg/ml) as previously described (10). For determination of the optimal nickel ion concentration, various amounts of nickel chloride (0-2 mM range) were added into the otherwise identical media.

Assay

Urease activity was measured by quantitating the rate of ammonia released from urea by formation of indophenol which was monitored at 625 nm (18). The standard assay buffer consisted of 200 mM urea, 50 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), and 0.5 mM EDTA (pH 7.5) Reactions were initiated by the addition of enzyme, the concentration of released ammonia was measured in timed aliquots, and the rates were determined by linear regression analysis. One unit (U) of urease activity is defined as the amount of enzyme required to degrade 1 μmole of urea per min at 37°C in the standard assay buffer.

Protein concentrations were routinely assessed by the spectrophotometric assay of Lowry *et al.* (11) using bovine serum albumin as a standard.

Urease Purification

Cultures (900 ml) of *E. coli* HB101 (pBU11) were grown to late exponential phase and harvested by centrifugation. The cells were washed twice with PEB (20 mM potassium phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.2)) buffer, resuspended in an equal volume of PEB buffer containing 0.5 mM phenyl methylsulfonyl fluoride, disrupted by a sonic dismembrator (Fischer Scientific Co., Pittsburgh, PA, USA) with a maximum output of 70 W for 1 min/ml, and centrifuged at 100,000×g for 90 min at 4°C. The cell extracts were chromatographed on a DEAE-Sepharose column (2.5 by 15 cm) at 4°C, in the same buffer and eluted with a 200 ml-linear salt gradient to 1M KCl. Urease was eluted from the column at approximately 0.35 M KCl. The pooled sample was applied to a phenyl-Sepharose column (2.5 by 17 cm), which had been pre-equilibrated with 2 M KCl and eluted with 0 M KCl-PEB. Pooled fractions were serially dialysed against 3 liters of PEB buffer, then further purified on a FPLC by using a Mono-Q (0.5 by 5 cm) column. The enzyme activity was eluted at 0.5 M KCl by using a multistep KCl gradient. All resins and columns were purchased from Pharmacia. The presence of urease protein in column fractions was assessed by the one-time-point urease assay method based on indophenol production (18).

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (7) and included a 10 to 20% polyacrylamide gradient resolving gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Coomassie brilliant blue R-250.

Subunit Stoichiometry

Stoichiometry of the three enzyme subunits was assessed by scanning vacuum-heat-dried SDS polyacrylamide gels through a densitometer and the Image-Quant™ Software (Molecular Dynamics Co., Sunnyville, CA, USA).

RESULTS AND DISCUSSION

Urease Purification

Purification of the urease from sonic-disrupted cells by a three-column procedure is summarized in Table 1. Based on the increase in the specific activity of the urease, the enzyme was estimated to have been purified 31.2 fold. The enzyme was not purified to homogeneity in this procedure, however, comparison of densitometric scans from several preparations clearly showed that three peptides that were associated with the urease activity always occurred in the same intensity ratio, whereas the intensity of other minor peptides varied among preparations (Fig. 1). The estimated purity of the Mono-Q pool was more than 90% homogeneous and the specific activity reached the level of 836.6 U/mg.

Molecular Weights and Stoichiometry of *Bacillus pasteurii* Urease Subunits

As shown in Fig. 1, the enzyme possessed three subunits of apparent M_rs=65,200 (α), 14,500 (β), and 13,700 (γ), respectively, when calculated according to Fehrnstrom and Moberg (3) on a 10-20% gradient polyacrylamide gel (Fig. 2). The integrated intensities of the three Coomassie blue-stained bands divided by their relative mass yielded an $\alpha:\beta:\gamma$ ratio of 1.0 : 0.9 : 1.1, assuming that the smallest peptide corresponds to UreA (γ) protein.

Christians *et al.* (2) purified *B. pasteurii* urease showing only a single peptide band of M_r=65,500 when denaturing electrophoresis was performed on a 3-12% gradient polyacrylamide gel using the imidazole buffer sys-

Table 1. Purification of *Bacillus pasteurii* urease expressed in *E. coli*.

Purification step	Specific activity (U/mg)	Purification (-fold)	Total activity (U)	Enzyme recovery (%)
Cell extract	26.8	1.0	9781	100
DEAE-Sepharose	213.6	8.0	9675	99
Phenyl-Sepharose	295.7	11.0	5358	55
Mono-Q	836.6	31.2	4250	43

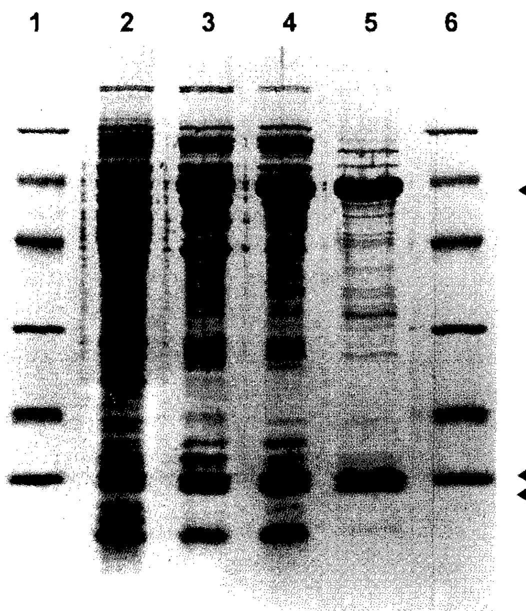

Fig. 1. SDS-polyacrylamide gel electrophoresis of the *B. pasteurii* urease from each purification step.

Cell extracts from *E. coli* HB101 (pBU11) (lane 2), DEAE-Sepharose pool (lane 3), Phenyl-Sepharose pool (lane 4) and Mono-Q HR5/5 pool (lane 5) were subjected to SDS-polyacrylamide gel electrophoresis by using a 10-20% gradient gel, followed by Coomassie blue staining. Molecular weight markers (lane 1 and 6) 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. Arrow heads are indicating the locations of the three subunits.

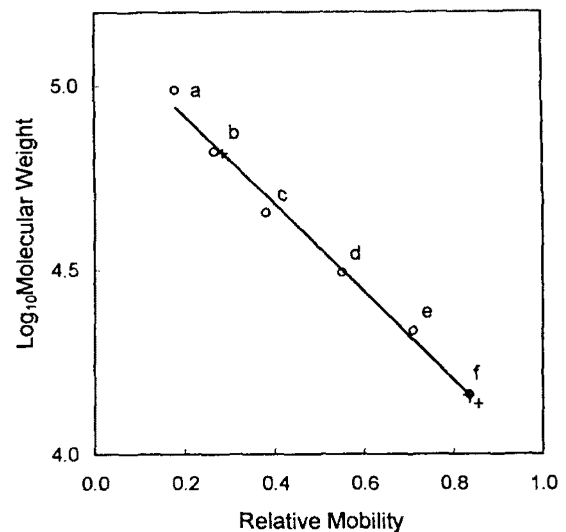

Fig. 2. Determination of the apparent molecular weights of the *B. pasteurii* urease subunits (+) according to Fehrnstrom and Moberg method (3).

(a) phosphorylase b, M_r=92,500; (b) bovine serum albumin, M_r=66,200; (c) ovalbumin, M_r=45,000; (d) carbonic anhydrase, M_r=31,000; (e) soybean trypsin inhibitor, M_r=21,500; and (f) lysozyme M_r=14,400.

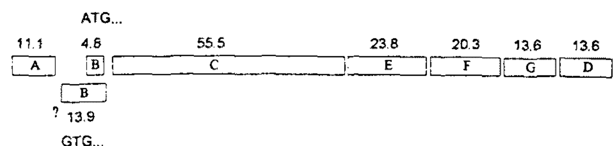

Fig. 3. Schematic drawing of the *B. pasteurii* urease gene cluster showing two possible alternative open reading frames for the *ureB* gene (19). Unit for the numbers is kilodalton (kDa).

tem, while many years later, Lee *et al.* (9) observed a smaller, minor peptide subunit of M_r=20,000 in addition to a single major protein band of larger size (M_r=67,000) on a 7.5% disc-polyacrylamide gel. Thus *B. pasteurii* urease seemed to belong to unusual class of bacterial ureases. As proven in the case of urease from *Sporosarcina ureae* (12) that is taxonomically closely related to *B. pasteurii* (16), however, it is quite possible to overlook one or two small urease subunits depending on the SDS-PAGE conditions. The authors initially thought *S. ureae* urease to be a single-subunit enzyme, but it was later shown to consist of three peptides like most other bacterial enzymes.

The apparent discrepancy between the DNA sequence analysis data which has been recently completed and the peptide patterns of the purified urease preparations on polyacrylamide gels prompted us to further examine the presence of the additional peptides by using a broad range (10-20%) gradient resolving gel in SDS-PAGE analyses. As described above, this report clearly demonstrated that *B. pasteurii* urease consists of three subunits which is consistent with the DNA sequence data.

DNA sequence data of the *B. pasteurii* urease gene cluster revealed three structural open reading frames (*ureA*, *ureB*, and *ureC*) in its upstream region. The predicted molecular weights of the peptides are 11.1, 4.8, and 55.5 kDa, respectively. However, it is possible that the translation of the second ORF (*ureB*) might start at an alternative start codon (GTG) located further upstream resulting in a larger peptide of 13.9 kDa (Fig. 3). In this case, the two smaller peptide bands could possibly be overlapped on a polyacrylamide gel since the apparent molecular weight of a peptide can vary significantly from the predicted one depending on the intrinsic charge of the peptide even in the presence of SDS molecules. We think this is the case, since the two smaller peptides were frequently observed as a single, overlapped band on a test-run using 10-15% gradient resolving gels (data not shown).

Determination of the nickel content of the purified urease by atomic absorption spectroscopy, and N-ter-

minal amino acid sequencing analysis of the two smaller peptides to confirm these peptides are indeed the *ureA* and *ureB* gene products are currently under progress and will clarify this issue.

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