Purification of a Nickel-containing Urease from the Rumen Anaerobe Selenomonas ruminantium*

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Urease was purified 592-fold to homogeneity from the anaerobic rumen bacterium Selenomonas ruminantium. The urease isolation procedure included a heat step and ion-exchange, hydrophobic, gel filtration, and fast protein liquid chromatography. The purified enzyme exhibited a $K_m$ for urea of $2.2 \pm 0.5$ mM and a $V_{max}$ of 1100 $\mu$mol of urea min$^{-1}$ mg$^{-1}$. The molecular mass estimated for the native enzyme was 360,000 $\pm$ 50,000 daltons, whereas a subunit value of 70,000 $\pm$ 2,000 daltons was determined. These results are in contrast to the findings of Mahadevan et al. (Mahadevan, S., Sauer, F. D., and Erfle, J. D. (1977) Biochem. J. 163, 495-501) in which isolated rumen urease was reported to be one-third this size ($M_r$ 120,000–130,000) and to catalyze urea hydrolysis at a maximum velocity of only 53 $\mu$mol min$^{-1}$ mg$^{-1}$. S. ruminantium urease contained 2.1 $\pm$ 0.4 nickel ions/subunit, comparable to the nickel content in jack bean urease (Dixon, N. E., Gazzola, C., Blakeley, R. L., and Zerner, B. (1975) J. Am. Chem. Soc. 97, 4131–4133). Thus, the active site of bacterial urease is very similar to that found in the plant enzymes.

In 1975, Dixon et al. (1) demonstrated that jack bean urease contained nickel. This plant enzyme has since been extensively studied by Zerner and colleagues (2) who established the presence of two nickel ions/subunit ($M_r$ 96,600) for this hexameric protein ($M_r$ 590,000). Although the detailed nickel active-site structure remains elusive, recent x-ray absorption spectroscopic data are consistent with histidinyl ligation of the nickel centers (3). Nickel has also been shown to copurify with urease from the aerobic soil bacterium Arthrobacter oxydans (4). Urease was purified 121-fold from this microorganism by heat treatment, acetone fractionation, and Sephadex G-200 chromatography. In contrast to the jack bean enzyme, A. oxydans urease was much smaller ($M_r$ 242,000) and possessed a much lower nickel content (0.3 mol of nickel/mol of enzyme) (4). The subunit size was not determined for this bacterial enzyme.

To further assess the nickel content of bacterial urease and to detail the active-site nickel environment, a small, easily purified, nickel-containing bacterial urease was desired. A promising candidate for such an experimentally advantageous

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enzyme was urease from a rumen bacterium. Mahadevan et al. (5) have purified urease from a mixed bovine rumen population by a simple procedure involving (NH$_4$)$_2$SO$_4$ precipitation and chromatography using calcium phosphate gel and hydroxyapatite steps. The 60-fold purified urease from this bacterial consortium was judged to be 80% pure based upon gel electrophoresis. The nickel content of the enzyme was not examined; however, the size of the active urease was demonstrated to be very small ($M_r$ 20,000–30,000) using native gel electrophoresis, sucrose density gradient centrifugation, and gel filtration chromatography on Sephadex G-200. In a different system, other investigators (6, 7) have shown that lamb rumen urease activity exhibited a requirement for dietary nickel. Thus, the rumen enzyme appeared to be a useful prospect for detailed studies of a nickel-containing bacterial urease.

Bacterial urease plays an important role in ruminal nitrogen metabolism (8, 9). Urea, supplied in the diet or recycled from saliva and the bloodstream, is hydrolyzed to yield ammonia, the major source of nitrogen for most rumen bacteria (10–12). Ureolytic bacteria inhabiting the bovine rumen include Succinivibrio dextrinosolvens, Treponema sp., Ruminococcus bromii, Butyryrivibrio fibrisolvens and other Butyryrivibrio sp., Bifidobacterium sp., Bacibacterium ruminicolae, and Peptostreptococcus productus (13). Perhaps the most highly characterized ureolytic bacterium of the bovine rumen is Selenomonas ruminantium (14), which was isolated by using roll-tube medium with urea as the main nitrogen source (15). A simple minimal medium has been devised for this organism (16), and S. ruminantium urease activity has been monitored over a wide range of growth conditions (15, 17).

This report describes the purification and characterization of urease from the rumen anaerobe S. ruminantium. In contrast to the substoichiometric nickel levels of the A. oxydans enzyme, the S. ruminantium urease possesses a nickel content which is identical to that in urease from jack bean. Furthermore, the S. ruminantium urease differs markedly from a mixed rumen bacterial urease in possessing 15–20-fold greater specific activity and a 3-fold greater size.

**MATERIALS AND METHODS**

**Bacterial Growth Conditions—**S. ruminantium strain D, obtained from Professor M. P. Bryant (Department of Dairy Science, University of Illinois), was cultured at 39 °C on a defined minimal medium (16) in which 1.5 mM urea served as the primary nitrogen source. The medium was modified to provide 10% of the suggested iron content, and the reductant was filter-sterilized cysteine solution (0.025% final concentration) rather than Na$_2$S. The anaerobic techniques for media preparation and culturing of these fastidious anaerobes have been described (18, 19). Large-scale batch culture was performed by using a 22-liter carboy which was continuously gassed with CO$_2$. Cells were grown to an absorbance of over 1 (600 nm) and harvested by using a Sharples steam-driven centrifuge. The cells (typically 30 g, wet weight/22 liters) were washed with 20 mM potassium phosphate, 1
mm EDTA, 1 mm β-mercaptoethanol, pH 7.0 (PEB), buffer and stored frozen at −20 °C until use.

**Assay**—Urease was assayed by measuring the rate of release of ammonia from urea. The released ammonia was converted to indophenol whose absorbance was monitored at 625 nm (20). Specific activity of urease was defined as μmol of urea hydrolyzed min⁻¹ mg⁻¹ at 39 °C in 10 mM HEPES, 5 mM EDTA, pH 8.0, buffer containing 25 mM urea. Protein was assayed as described by Lowry et al. (21) using bovine serum albumin as the standard. All UV-visible absorbance determinations were made by using a Gilford Response spectrophotometer.

The nickel content of samples was quantified by using a Perkin-Elmer PE 5000 atomic absorption spectrophotometer equipped with an HGA 500 graphite furnace and an AS-1 autosampler. Samples were hydrolyzed in 1 N nitric acid, evaporated, and resuspended in 50 mM HNO₃. Nickel standards, which in some cases contained bovine serum albumin to mimic the enzyme matrix, were treated identically to the urease samples. The 20-μl samples were dried at 130 °C, charred at 1200 °C, atomized at 2700 °C, and analyzed for nickel by using the spectrophotometer’s background correction mode.

**Preparation of Crude Extract**—Thawed cells were suspended in an equal volume of PEB buffer and disrupted by two passages through a French pressure cell at 18,000 p.s.i. Cellular debris was removed by centrifugation at 50,000 × g for 120 min at 4 °C. All further isolation procedures, except that using the fast protein liquid chromatography system, were carried out at 4 °C and using PEB buffer with the indicated additions.

**SDS-Polyacrylamide Gel Electrophoresis**—The relative molecular mass for the urease subunit was determined, and the purity of samples were assessed by using SDS-polyacrylamide gel electrophoresis as described by Laemmli (22). The samples were denatured 5 min at 100 °C in 0.0625 M Tris buffer, pH 6.8, containing 3% SDS, 10% glycerol, and 5% β-mercaptoethanol. These samples were then electrophoresed at 50 V overnight through a 3% acrylamide stacking gel and a 7.5% acrylamide running gel, each 1 mm in thickness. Standards used for comparison were phosphorylase b, Mₚ, 92,500; bovine serum albumin, Mₚ, 68,000; pyruvate kinase, Mₚ, 57,000; ovalbumin, Mₚ, 44,000; carbonic anhydrase, Mₚ, 29,000.

**Native Molecular Weight Determination**—The relative molecular mass for native urease was deduced by using Superose 6 (Pharmacia) gel filtration chromatography. Standards included ferritin, Mₚ, 540,000; catalase, Mₚ, 240,000; γ-globulin, Mₚ, 158,000; ovalbumin, Mₚ, 44,000; myoglobin, Mₚ, 17,000; and vitamin B₁₂, Mₚ, 1,350. Samples were eluted in PEB buffer containing 0.1 M KCl (0.5 ml/min) while monitoring the absorbance at 280 nm.

**RESULTS**

**Urease Purification**—Cell extracts were heat-treated at 60 °C for 5 min and centrifuged at 39,000 × g for 20 min. The supernatant solution was chromatographed on a column (1.5 × 26 cm) of DEAE-Sepharose (Pharmacia) as shown in Fig. 1A. A 300-ml linear gradient from 0 to 0.6 M KCl in PEB buffer was used to recover urease as a single peak of activity at ~0.5 M KCl. Fractions containing urease activity were adjusted to 1 M KCl and chromatographed on a column (1.5 × 24 cm) of phenyl-Sepharose (Pharmacia). As shown in Fig. 1B, the enzyme bound tightly to the resin and was only partially recovered by chromatography with PEB buffer. Further urease recovery was achieved by washing the resin with PEB buffer containing 10% dimethyl sulfoxide. The fractions containing urease were pooled, dialyzed versus PEB buffer, concentrated by using polyethylene glycol, and chromatographed on a column (2.5 × 50 cm) of Sephadex G-200 (Sigma). As shown in Fig. 1C, urease was recovered in a single peak well separated from a small molecular weight fraction. Final purification of S. ruminantium urease was achieved by fast protein liquid chromatography using a Mono-Q column (0.5 × 5 cm, Pharmacia). As shown in Fig. 2, a two-segment linear KCl gradient was used to resolve trace contaminants from urease activity which was recovered at 0.6 M KCl.

**Subunit Size and Native Molecular Mass**—The subunit relative molecular mass for purified urease was determined by using SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3, the urease is homogeneous and has a subunit molecular weight of Mₚ, 70,000 ± 2,000. The molecular weight of the sample was concentrated to 0.2 ml by using a Centricon 10 (Amicon) and chromatographed on a column (1.0 × 30 cm) of Superose 6 (Pharmacia). This final chromatography served to desalt the enzyme and to provide an estimate of the native molecular weight (see below). The purification of S. ruminantium urease is summarized in Table I. Urease was purified 592-fold with an overall recovery of 50%.

**Kinetic Parameters**—The rate of urea hydrolysis was monitored as a function of urea concentration from 0.5 to 50 mM urea. Lineweaver-Burk analysis by the method of Wilkinson (23) yielded values of 2.2 ± 0.5 mM for the Kₘ and 1100 μmol min⁻¹ mg⁻¹ for Vₘₐₓ. The optimum pH in HEPES buffer was pH 8.
FIG. 2. **Fast protein liquid chromatography of S. ruminantium urease.** The sample from Fig. 1C was chromatographed on a Mono-Q column by using a two-segment linear KCl gradient as indicated (---). The absorbance was monitored at 280 nm (--), and the urease activity was determined (○) for aliquots of the 0.5-ml fractions. The pooled urease fractions are indicated by the bar.

**TABLE I**

| Purification step                  | Specific activity | Purification | Total activity | Enzyme recovery | %  |
|-----------------------------------|------------------|--------------|----------------|-----------------|----|
| *μmol urea min⁻¹ mg⁻¹* -fold µmol min⁻¹ |                  |              |                |                 |    |
| Cell extracts                     | 1.72             | 1            | 4430           | 100             |    |
| Heat treatment                    | 4.33             | 2.5          | 3750           | 84.6            |    |
| DEAE-Sepharose                    | 147              | 85.5         | 3380           | 76.4            |    |
| Phenyl-Sepharose                  | 609              | 354          | 2830           | 64.0            |    |
| Sephadex G-200                    | 905              | 526          | 2890           | 65.2            |    |
| Fast protein liquid chromatography | 1019             | 592          | 2340           | 52.9            |    |

for native *S. ruminantium* urease, deduced from gel filtration chromatography, was $M_r$ 360,000 ± 50,000, as shown in Fig. 4.

**Fig. 4.** Relative molecular mass determination for native *S. ruminantium* urease. Samples were chromatographed on a Superose 6 column as described under “Materials and Methods.” The molecular weight standards labeled 1–6 were ferritin (540,000), catalase (240,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B₁₂ (1,350).

was determined by atomic absorption analysis to be 2.1 ± 0.4 mol of nickel/mol of subunit when compared to a standard curve which was prepared as described under “Materials and Methods.”

**DISCUSSION**

This work represents the first purification to homogeneity of urease from a rumen bacterium. *S. ruminantium* urease was isolated by using standard techniques resulting in 592-fold purification with a yield of 52.9%. The purification protocol included a heat step and ion-exchange, hydrophobic, and gel filtration chromatographic steps. The rapid and high yield (85%) heat step exploited the stability of urease to short-term incubation at 60 °C. In contrast, inactivation of urease was observed at 70 °C. DEAE-Sepharose chromatography proved to be very effective for purification of this anionic protein. Phenyl-Sepharose chromatography demonstrated such a tight-binding interaction with urease that urease was not fully recovered simply by removal of salt from the elution buffer. The hydrophobic interaction was interrupted by chromatography in buffer which contained 10% dimethyl sulfoxide, and no loss of urease activity was observed in this mixed solvent. The hydrophobic nature of urease may indicate a membrane association of urease within the cell, as was suggested by McLean et al. (24) for a *Staphylococcus* sp. During a concentration step performed preliminary to Sephadex G-200 chromatography, the urease activity was fully recovered, whereas non-urease protein was denatured and removed by centrifugation. Sephadex G-200 chromatography primarily removed small, non-protein components. Mono-Q chromatography followed by Superose 6 desalting completed the purification procedure. In one preparation, a modified elution protocol using DEAE-Sepharose was successfully substituted for Mono-Q chromatography.

The $V_{max}$ for purified *S. ruminantium* urease was calculated to be 1100 $μmol\ min^{-1} \ mg^{-1}$, which greatly exceeds that observed for urease isolated from a mixed rumen population (53 $μmol \ min^{-1} \ mg^{-1}$) (5). This suggests that the latter enzyme may not have been 80% pure as claimed based on the basis of the Amido Black staining pattern of non-denaturing polyacrylamide gels (5), or it may have been in a partially active state. The *S. ruminantium*-specific activity also is far greater than values determined for ureases from *Spirulina maxima* (25), *Klebsiella aerogenes* (26), *Proteus rettgeri* (27), or *A. oxydans* (4) (9.27, 45.4, 30.6, and 219 $μmol \ min^{-1} \ mg^{-1}$, respectively). In contrast, urease from *Proteus morgani* (28) has a 2-fold greater maximum specific activity (2340 $μmol \ min^{-1}$).
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The enzyme nickel-containing urease has a maximum specific activity of 93 (katal/liter)/A_{subunit} where 1 katal is the amount of enzyme which degrades 1 mol of urea/s^{-1} in a defined pH-stat assay. This value corresponds to ~3500 μmol min^{-1} mg^{-1} or 3-fold larger than that reported for the S. ruminantium enzyme.

The relative molecular mass for native S. ruminantium urease was found to be 360,000 daltons. This value is smaller than that (Mr, 590,000) of the well-characterized jack bean urease (29) and 3-fold larger than that (Mr, 120,000-130,000) found for urease isolated from a mixed rumen population (5). The latter finding may indicate that S. ruminantium urease differs significantly from other rumen bacterial ureases or that proteolysis may have occurred in the mixed rumen bacterial urease preparation. Values of Mr, 230,000-242,000 have been reported for ureases from S. maxima, K. aerogenes, and A. oxydans (4, 25, 26); whereas, four strains of Proteus were reported to possess urease with Mr, 560,000-800,000 (30). The subunit relative molecular mass for the S. ruminantium urease was determined to be 70,000 daltons, suggesting a possible pentameric structure for the native enzyme. More likely, a tetrameric or hexameric structure is present, and the protein possesses nonspherical hydrodynamic properties. The latter configuration is consistent with jack bean urease, a hexamer comprised of subunits with Mr, 96,600 (29), and S. maxima urease, a hexamer of Mr, 38,000 subunits (25).

The Km of 2.2 mM determined for S. ruminantium urease is similar to that (0.83 mM) calculated for urease from the mixed rumen bacterial population (5) and is typical of other bacterial ureases (26, 30). The turnover number (kcat) for this enzyme is 1280 s^{-1}, attesting to its very high catalytic efficiency.

As in the archetype urease from jack bean (1) and as recently found in A. oxydans (4), the S. ruminantium urease contains nickel. In contrast to the trace nickel content observed for the A. oxydans enzyme (0.3 mol of nickel/mol of enzyme) (4), the S. ruminantium urease contains 2 mol of nickel/mol of subunit. This result is identical to that of the well-studied jack bean enzyme. The presence of nickel in urease may relate to an observed inhibition of urease activity for S. ruminantium when grown using histidine as the nitrogen source. Smith et al. (2) had observed that this histidine-induced urease inhibition is relieved by NiCl2 supplementation, suggesting a depletion of nickel by amino acid chelation. Others (6, 7) had previously shown that expression of urease activity in sheep rumen required nickel ion. In contrast to these results indicating nickel involvement in rumen bacterial urease, Mahadevan et al. (5) reported an 84% inhibition of urease activity for purified, mixed rumen bacterial enzyme in the presence of 20 μM nickel ion. These nickel inhibition results may be unrelated to the nickel content of their enzyme preparation, which was not determined. To preclude enzyme inhibition by exogenous trace metals during S. ruminantium urease purification, all buffers contained 1 mM EDTA. Nickel dependence for urease activity has also been observed in the fungus Aspergillus nidulans (31). These results support the concept of nickel being a universal component of microbial ureases in addition to its requirement for plant ureases (32). In contrast to these nickel-containing ureases, the ATP:urea amidohydrolase from yeast and green algae degrades urea by an alternate ATP- and biotin-dependent reaction (33). Besides urease, the only other known nickel-containing enzymes include methyl coenzyme M reductase from methanogenic bacteria (34, 35), carbon-monoxide dehydrogenase from methanogens (36) or acetogens (37), and uptake hydrogenases from a number of microorganisms (38, 39). The detailed roles for nickel in catalysis for these enzymes must await further investigation.

The initial goal in this investigation was to purify and characterize a small, nickel-containing, bacterial urease in order to define the nickel center. Toward this end, the nickel-containing urease from S. ruminantium was successfully purified to homogeneity and shown to contain an active-site nickel content very similar to the jack bean enzyme. However, the purified enzyme was found to be 3-fold the size expected from earlier work (5) examining urease from a mixed bacterial rumen population. Thus, there is no significant advantage over the jack bean enzyme in further probing the S. ruminantium urease nickel center. Rather than continuing to study urease from an anerobic rumen bacteria, current efforts are directed toward urease characterization in other aerobic bacteria.

Note Added in Proof—Nakano et al. (40) have recently described the purification to homogeneity of urease from Brevisbacterium ammoniagenes. This enzyme was found to possess 1 mol of nickel/mol of subunit (Mr, 67,000). Similarly, the urease from Bacillus pasteurii has been purified and shown to contain 1 mol of nickel/mol of subunit (Mr, 65,000) (S. Christians and H. Kaltwasser, personal communication).

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