Urease from a Potentially Pathogenic Coccoid Isolate: Purification, Characterization, and Comparison to Other Microbial Ureases

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Strain SL100 is a gram-positive coccoid isolate prototype with an adhesin specific for gastric mucin and is representative of potentially pathogenic organisms obtained at biopsy from patients with gastric disorders. The urease of this isolate constitutes a significant fraction of the total cell protein, and the outcome of the purification strategy described herein suggests that it is associated with a cell wall fraction. The urease was purified 138-fold to apparent homogeneity, as indicated by gel electrophoresis, to a specific activity of 1,120 U/mg. The urease was unstable during purification in the absence of nickel, which is present in a metallocenter in other microbial ureases. When nickel sulfate was present during growth (5 μ M) and in buffers during sonication and purification (100 μ M), the urease was completely stable at room temperature during the purification procedure. The native urease was approximately 260 kDa and was composed of three subunits of 65 kDa and three subunits of 21 kDa. The purified urease was relatively stable in acid and retained most of its activity after incubation for 30 min at pH 1.3. The K_m s for urease measured from whole cells and for the purified enzyme were 0.56 and 1.7 mM, respectively, indicating that some cell wall component(s) affects the affinity of the enzyme for urea. The V_{max} s for urea hydrolysis measured from whole cells and for the purified enzyme were 8.1 and 1,120 mol/min/mg of protein, respectively. The kinetic parameters, relative abundance, and subunit composition are more similar to those of the ureases of *Helicobacter* than to those of the ureases of other microbial species. These similarities are consistent with an adaptation of this organism to colonization of the stomach and indicate that the urease may be a virulence factor during colonization.

It is known that many cases of gastritis and gastric ulceration in humans are caused by *Helicobacter pylori* infection (6, 27, 39). Similar gastric disorders are caused by *Helicobacter felis* infection in cats, dogs, and mice (3, 36) and by *Helicobacter mustelae* infection in ferrets (13, 14, 35, 36), while *Helicobacter heilmannii* causes gastric disorders in a wide range of animals (29, 42). One shared feature among the *Helicobacter* species is high urease activity. Evidence has been presented which indicates that ureases protect these gastric organisms from gastric acid by neutralizing the acid with the ammonia generated through urea hydrolysis (12, 21). In addition, ureases have been shown to promote colonization of the stomachs of gnotobiotic piglets and ferrets by *H. pylori* (9, 10) and *H. mustelae* (1), respectively. Various toxic effects of ureases have also been documented (17, 40, 45).

There are many bacterial species with strong ureases (30, 31), and several of these species infect the urinary tract. They include *Proteus mirabilis* (28), *Ureaplasma urealyticum* (44), *Staphylococcus saprophyticus* (15, 34), and others (32). Some urease-positive bacteria can infect other human organs (33, 37, 43). The ureases of *Helicobacter* species are composed of two subunits of approximately 26.5 (UreA) and 60.3 (UreB) kDa. The ureases of non-*Helicobacter* bacterial species have three subunits of 6 to 14 kDa (UreA), 11 to 20 kDa (UreB), and 60

to 70 kDa (UreC) (2, 4, 5, 7, 15, 20, 23, 25, 33, 41). The amino acid sequence of the large UreC subunit of the non-*Helicobacter* bacterial species is homologous to that of the large UreB subunit of *Helicobacter* species. The nucleotide and amino acid sequence homologies are consistent with fusion of the genes encoding the UreA and UreB of non-*Helicobacter* bacterial species to generate the *Helicobacter* urease subunit of 26.5 kDa (31).

The isolation of a mucinophilic gram-positive staphylococcal species from the stomachs of gastric patients was recently reported, and these isolates were shown to exhibit a strong urease activity (24). We describe here the purification and characterization of the urease of this organism. Using purified urease, we found that the enzyme retained 75% of its original activity after exposure to pH 1.3, indicating that this urease is well-suited to an acidic environment. In addition, the urease was found to be composed of subunits of 21 and 65 kDa. As the only other bacterial ureases with two subunits are those of *Helicobacter* species, this urease may be more closely related to the urease of *H. pylori* than to the ureases of gram-positive bacteria such as *Staphylococcus* species.

MATERIALS AND METHODS

Cell growth and disruption. The SL100 prototype of the coccoid organism isolated from the antra of human gastric patients was grown as described previously (24) except for the inclusion of 5 μ M nickel sulfate in the medium. Cells scraped from 40 confluent-culture plates were suspended in 240 ml of distilled water and were pelleted by 10 min of centrifugation at 12,000 rpm with an SS34 rotor and a Sorval centrifuge. The cell pellets were then suspended in 100 ml of 20 mM Tris-HCl buffer (pH 7.8) containing 1 mM EDTA and 10 mg of lysozyme, and the suspension was sonicated for 5 min at 50% of the maximum energy output using a sonicator, model W-385 (Heat Systems-Ultrasonics, Inc., Plainville, N.Y.). The suspension was then incubated for 4 h at 37°C, phenylmethyl-

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FIG. 1. Molecular size estimate of the microbial urease obtained by Sephadex G-200 chromatography. The column was calibrated with hemoglobin, IgG, and amylase, and the urease activity peak was eluted just ahead of amylase. OD, optical density.

sulfonyl fluoride and nickel sulfate were added to it at final concentrations of 0.5 and 0.1 mM respectively, and the suspension was sonicated again for 5 min as described above. The sonicated cell suspension was centrifuged for 10 min at 12,000 rpm with an SS34 rotor and a Sorval centrifuge, and the supernatant was collected. The pellets, consisting mainly of unbroken cells, were suspended in 80 ml of 20 mM Tris-HCl buffer (pH 7.8) and sonicated and centrifuged as described above, and the supernatant was collected. This process of sonication and centrifugation was repeated three or four more times, and the pooled supernatant was used as a starting material for urease purification.

Enzyme purification. The pooled supernatant (375 ml) was applied to a DEAE-cellulose column (7 by 2 cm) equilibrated with 30 mM Tris-HCl buffer (pH 7.8), and the turbid eluant containing unadsorbed materials was saved. This turbid eluant contained cell wall fragments and over half of the urease activity of the supernatant. The column was then washed with 0.25 M KCl in 30 mM Tris-HCl buffer (pH 7.8) and the eluant, which did not contain urease activity, was discarded. The column was then washed with 0.4 M KCl in 30 mM Tris-HCl buffer (pH 7.8), and a clear eluant containing urease activity was saved. The turbid eluant containing urease activity (which initially passed through the column) was rechromatographed as described above, and the urease that partitioned into the 0.4 M KCl eluant was saved, while the urease that was eluted with unbound materials was rechromatographed again. The combined eluants in 0.4 M KCl were concentrated to 10 ml by means of ultrafiltration and applied to a Sephadex G-200 column (60 by 3 cm) equilibrated with 0.1 M KCl in 30 mM Tris-HCl buffer, pH 7.8, and the column was eluted with the same buffer. The fractions with urease activity (Fig. 1) were pooled and applied to a DEAE-cellulose column (6 by 1.5 cm). This column was washed with 0.25 M KCl in 30 mM Tris-HCl buffer (pH 7.8), and the urease was eluted with 300 ml of 0.25 to 0.4 M KCl gradient in the same buffer. The fractions with urease activity that was eluted between 0.29 and 0.34 M KCl were pooled and applied to a hydroxylapatite column (5 by 1.5 cm) equilibrated with 30 mM Tris-HCl buffer (pH 7.2). This column was washed with Tris-HCl buffer, and the urease was eluted with a 200-ml gradient from 30 mM Tris-HCl (pH 7.2) to 0.15 M phosphate buffer (pH 7.2). The fractions with urease activity that was eluted at 0.03 M phosphate were pooled and concentrated to 3 ml by means of ultrafiltration. This urease preparation was loaded onto two preparative 7.5% polyacrylamide slab gels (14 cm by 14 cm by 1 mm; Hoefer Scientific Instruments) and the buffer system of Laemmli (22) was used, with the omission of sodium dodecyl sulfate (SDS). After polyacrylamide gel electrophoresis (PAGE) at 65 V for 22 h at room temperature, a section of the gel with urease activity was removed, and urease was electroeluted from the gel section.

Enzyme assays. During enzyme purification urease activity was monitored by observing the color change of a phenol red pH indicator from yellow to red, which results from a pH increase due to urea hydrolysis. Enzyme solution (10 μ l) was mixed with 100 μ l of a yellow test solution, pH 3.0, containing a 1 mM concentration of the free-acid form of phenol red and 100 mM urea, and red color development was observed. To determine the urease activity in a polyacryl-

amide gel, 5-mm-thick gel sections were introduced into test tubes containing 1 ml of the test solution, and color development was observed. Quantitative measurements of urea hydrolysis were determined spectrophotometrically by assaying glutamate dehydrogenase-mediated NADPH oxidation which was coupled to ammonia utilization (21). This assay was carried out at room temperature in a 1-ml volume in a cuvette containing 240 μ M NADPH, 810 μ M α -ketoglutarate, 15 U of glutamate dehydrogenase (Sigma type II), 10 mM urea, 30 mM Tris-HCl (pH 8.0), and 10 μ l of urease solution. Urea hydrolysis was determined by measuring NADPH oxidation at 340 nm.

Amylase from sweet potatoes (Sigma) and immunoglobulin G (IgG) were used as molecular weight markers for Sephadex G200 chromatography (Fig. 1). To determine amylase activity, 5 g of corn starch was suspended in 100 ml of 50 mM acetate buffer (pH 5.0) and the suspension was boiled for 5 min. The suspension was centrifuged for 10 min at 3,000 rpm with a Beckman microcentrifuge, and the turbid supernatant was collected. Aliquots (2 ml) of the starch solution were mixed in test tubes with 100- μ l aliquots of the fractions from the Sephadex G200 column and the mixtures were incubated overnight at 37°C. The amount of free sugar with a reducing end was determined by Fehling's reaction. For this procedure 2 ml of 0.1% CuSO_4 and 0.1% sodium tartrate in 0.1 N NaOH was added to each reaction tube, the tubes were placed in boiling water, and the amount of reddish metallic Cu_2O deposited on the bottom of the tubes was recorded. To detect IgG, 20- μ l aliquots of the fractions from the Sephadex G200 column were spotted on nitrocellulose, blocked with 2% skim milk, and then incubated with a solution of covalently protein A-linked horseradish peroxidase. The peroxidase activity bound to the spots was determined with 0.05% diaminobenzidine and 0.03% H_2O_2 .

Acid stability of urease. The phosphate concentration of the urease preparation from the hydroxylapatite step was reduced 10-fold to 3 mM by means of ultrafiltration. Aliquots (20 μ l) of this enzyme solution were mixed in microcentrifuge tubes with 20 μ l each of buffers at pH 1.3 (100 mM HCl), 3.0 (100 mM citrate), 4.0 (100 mM formate), 5.0 (100 mM acetate), 6.0 (100 mM succinate), and 7.0 (100 mM phosphate). After incubation for 30 min at room temperature, 1 μ l of phenol red pH indicator solution was added to each tube, and 1 M Tris-HCl (pH 9.0) was added dropwise until the indicator turned red. Volume adjustments were made by adding water to the enzyme solutions at or near neutral pH. The neutralized enzyme solutions were allowed to stand at room temperature for 30 min, after which 10- μ l aliquots of the enzyme solutions were assayed for urease activity by measuring glutamate dehydrogenase-mediated NADPH oxidation.

RESULTS

Cell disruption. Initial attempts to break open cells revealed that the external integument of this strain has a structure that makes it resistant to routine cell disruption by sonication, ly-

TABLE 1. Purification of urease of strain SL100

Purification step	Amt of total protein (mg)	Total activity (10^3 μ mol/min)	Sp act (μ mol/min/mg)	Yield (%)	Purification (fold)
Crude extract	288	2.32	8.10	100	1.0
DEAE cellulose (0.4 M eluate)	118	1.35	11.4	58	1.4
Sephadex G-200	26.0	0.74	28.5	32	3.5
DEAE-cellulose (0.2 to 0.4 M gradient)	7.30	0.49	67.1	21	8.3
Hydroxylapatite	2.96	0.26	88.0	11	11
Nondenaturing acrylamide gel (electroelution)	0.17	0.20	1,120	8.6	138

sozyme, or the use of a French press. However, a combination of sonication at maximum energy output and prolonged incubation with lysozyme was effective in cell disruption. Cells suspended in buffer containing lysozyme were sonicated in an effort to expose peptidoglycan sites to digestion. Five successive sonications of 5 min each followed by 4 h of incubation with lysozyme resulted in the disruption of over two thirds of the cells as judged by the amount of protein in the supernatant after centrifugation. The relative number of cells lysed by this procedure increased when the cells were harvested several days after reaching confluence.

Enzyme purification. In a typical purification procedure (Table 1), approximately 375 ml of turbid cell extract was passed through a DEAE-cellulose column. The first 220 ml of eluant was clear in appearance and was devoid of urease activity, but the following 150 ml was turbid and contained 42% of the urease activity. The turbid fraction eluted from the DEAE column was clarified by centrifugation and all of the urease activity was found in the pellet. The remaining portion (58%) of the urease activity was tightly bound to DEAE-cellulose, and it was eluted with 0.4 M KCl. When the 0.4 M KCl eluant from the DEAE-cellulose column was chromatographed on a Sephadex G-200 column, urease activity was eluted at approximately 260 kDa (Fig. 1). The peak urease fractions from the G-200 column were applied to a DEAE-cellulose column and then eluted with a 0.25 to 0.4 M KCl gradient, and the urease activity was eluted between 0.29 and 0.34 M KCl. The urease fraction from this step was applied to a hydroxylapatite column and was eluted with a potassium phosphate gradient. The major urease peak was eluted at 0.03 M and a minor peak was eluted at 0.08 M potassium phosphate. It is not clear how the activities in the major and minor peaks differ. The hydroxylapatite step did not increase specific activity appreciably, because the same two major protein species eluted with the urease in both the DEAE-cellulose and the hydroxylapatite steps (Fig. 2). The major urease fraction that was eluted at 0.03 M phosphate was purified by preparative PAGE in the absence of SDS. The urease activity migrated as a broad band to a position between the two major protein species. All enzyme preparations (Fig. 2) yielded an identical broad band when stained with Coomassie blue, and this band corresponded to the urease activity in a parallel gel. The urease preparation from the gel electrophoresis step showed this band only (Fig. 2, lane 6), indicating that these urease fractions were homogeneous by these criteria.

Molecular size and subunit composition of urease. Initial experiments revealed that treatment of the urease in 0.2% SDS at room temperature for 30 min resulted in partial denaturation of the enzyme, with some fraction of the enzyme retaining catalytic activity detected *in situ* after SDS gel electrophoresis. Prior to PAGE, aliquots of the crude extract, hydroxylapatite eluate, and final purified enzyme preparation were incubated in 0.2% SDS at room temperature for 30 min

and duplicate samples were boiled in 0.4% SDS containing 2 mM dithiothreitol to completely denature the enzyme (Fig. 3). One section of the gel was stained with Coomassie blue (Fig. 3, lanes 1 to 8), and another gel section (Fig. 3, lanes 10 to 15) was immersed in a solution containing phenol red and urea to detect urease activity. The activity of the urease was detected in the purified enzyme, hydroxylapatite eluate, and crude extract incubated at room temperature prior to electrophoresis (Fig. 3, lanes 10 to 12, respectively). The Coomassie blue-stained section of the gel revealed that the purified enzyme (Fig. 3, lane 3) contained dissociated subunits of 21 and 65 kDa, as well as detectable levels of native urease that migrated above the 200-kDa size marker (Fig. 3, lane 8). This result was consistent with the molecular mass estimate of approximately 260 kDa for the native enzyme (Fig. 1) and indicated the presence of a hexameric holoenzyme containing three subunits each of the 65- and 21-kDa monomers. When these samples were boiled prior to being loaded on the gel, all catalytic activity of the urease was lost (Fig. 3, lanes 13 to 15) and the purified urease showed only the subunits of 65 and 21 kDa (Fig. 3, lane 6). In order to test for the possible presence of smaller subunits in the purified enzyme preparation that may

FIG. 2. Electrophoresis of various urease preparations on a nondenaturing polyacrylamide gel. One gel section (lanes 1 to 7) was stained with Coomassie blue and the other section (lanes 8 to 13) was immersed in a solution containing 100 mM urea and 1 mM phenol red pH indicator to measure urease activity. The urease preparations analyzed were the crude extract (lanes 2 and 9), the fractions from Sephadex G-200 (lanes 3 and 10), the DEAE-cellulose gradient (lanes 4 and 11), the hydroxylapatite (lanes 5 and 12), and the preparation from electroelution from a nondenaturing gel (lanes 6 and 13). Lanes 1, 7, and 8 were not loaded with samples.

FIG. 3. Identification of urease catalytic form and subunits by nondenaturing SDS-PAGE. One set of urease preparations (lanes 1 through 3 and 10 through 12) was incubated with 0.2% SDS at room temperature, and a second set of the same preparations (lanes 4 through 6 and 13 through 15) was boiled with 0.5% SDS and 2 mM dithiothreitol. The urease preparations analyzed were the crude extract (lanes 1, 4, 10, and 13), the hydroxylapatite eluate (lanes 2, 5, 11, and 14), and the electroeluted sample (lanes 3, 6, 12, and 15), with Coomassie blue and urease assays as described in the legend to Fig. 2.

not be detectable with the quantities of protein loaded in Fig. 3 (lanes 3 and 6), 10 times more purified enzyme was analyzed by SDS-PAGE using a 16% acrylamide gel. Additional minor contaminants were detected between the 31- and 65-kDa subunits, but no protein bands migrating faster than the 31-kDa subunit were detected.

Stability of urease in cell extracts. In the initial attempts at urease purification, nickel was not added at any stage. Under these conditions urease activity decayed rapidly during purification. Subsequently, nickel sulfate (5 μ M) was added to the culture medium and to the cell suspension (100 μ M) after incubation with lysozyme and prior to sonication. When these techniques were used, urease activity remained completely stable at room temperature throughout the purification steps.

Stability of the purified urease at low pH. Because the coccus isolate from which the urease was purified would be exposed to gastric acid during the course of an infection, the stability of the urease at an acidic pH was examined. The urease was incubated for 30 min at room temperature in buffers with pHs ranging from 1.3 to 7. The pH was then adjusted to a neutral level and enzyme activity was determined quantitatively by measuring NADPH oxidation in a glutamate dehydrogenase-coupled reaction. The urease exposed to pHs as low as 5.0 retained full activity, and even the urease exposed to the lowest pH tested (1.3) retained most of the activity (Fig. 4). The purified urease was also completely stable when treated with 5% SDS for 30 min at room temperature.

Enzyme kinetics. The kinetics of urea hydrolysis was determined from Lineweaver-Burk plots for urease activity detectable in the whole cell and for the purified enzyme (Fig. 5). The calculated K_m values for the whole cell and the purified enzyme were 0.56 and 1.7 mM, respectively, and the maximum velocities of urea hydrolysis were 9.3 for the whole cell and 1,350 μ mol/min/mg of protein for purified urease, respectively. The approximately threefold-higher affinity for urea when the ure-

FIG. 4. Effect of pH on stability of the microbial urease. The urease preparation from the hydroxylapatite step was incubated for 30 min in buffers at pH 1.3 to 7.0, the solutions were neutralized, and enzyme activities were determined as described in Materials and Methods.

ase is present in intact cells apparently is due to some association of the enzyme with accessory proteins or cellular components. The activity of the urease in intact cells is consistent with a role of this enzyme as a virulence factor during initial colonization of the stomach.

DISCUSSION

With the exception of those of *H. pylori*, most microbial ureases appear to be located in the cytoplasmic fraction, although conflicting reports have appeared in this regard (31). The urease of *H. pylori* was shown to be readily released from the cell upon exposure to water, 0.15 M NaCl, or nondenaturing detergents (8). In contrast, a prolonged exposure of strain SL100 to these agents did not release urease.

Ureases of *Helicobacter* species provide protection from gastric acid, as demonstrated by the observation that *H. pylori* survived at pH 1.5 only if urea was present in the medium (26). Since the purified urease of strain SL100 retained most of its enzyme activity after exposure to a pH of 1.3 (Fig. 3), it should be active during colonization of stomach mucin. However, strain SL100 survived after overnight exposure to pH 1.3 even in the absence of urea (unpublished data). The ability to withstand an acid pH would be expected to be important for the survival of this organism because it appears to colonize the surface of the mucus layer (24) and would thus be fully exposed to gastric acid. In contrast, *Helicobacter* species reside within and under the mucus attached to stomach epithelial cells in a location more protected from gastric acid.

The urease of strain SL100 is composed of subunits of 21 and 65 kDa (Fig. 4). The ureases of *Helicobacter* species are composed of two subunits, whereas the ureases of all other non-*Helicobacter* bacterial species, including *Staphylococcus xylosum*, are composed of three distinct subunits (2, 4, 5, 7, 19, 20, 23, 25, 33, 38, 41). Initial taxonomic classification of the coccus based on fatty acid composition and metabolic characterizations showed relationships to *Staphylococcus cohnii* and *S. xylosum*, respectively (24). Thus, the coccus resembles *S. xylosum*

FIG. 5. Lineweaver-Burk plots showing urease activity in whole cells and purified enzyme. Urease activity was determined by measuring glutamate dehydrogenase-mediated NADPH oxidation as described in Materials and Methods.

and *S. cohnii* if classified by standard typing approaches, but its urease is more similar to that of the gram-negative gastric pathogen *Helicobacter*.

The urease of *H. pylori* has one of the lowest K_m values (0.17 mM) for urea (18), in contrast to the higher K_m values (13 to 130 mM) for enzymes in microorganisms that are found in the urinary tract or soil (31). This has been interpreted to suggest a correlation between the K_m and an organism's ecological niche. *H. pylori* colonizes the gastric mucosal lining and depends upon the low concentrations of urea supplied from the serum. The K_m values for strain SL100 (0.56 mM for the whole cell and 1.7 mM for the purified enzyme) are more similar to the K_m values of 0.2 to 0.5 mM reported for the ureases of various *Helicobacter* species (8, 11, 16, 18) than to the values for other *Staphylococcus* species (e.g., 9.5 mM for *S. saprophyticus* [38]) or other bacteria that are urinary tract pathogens or soil microorganisms.

Thus, the properties of the urease of strain SL100, including its subunit composition, K_m for urea, and relative abundance as a percentage of total cell protein, are not typical of ureases of previously described *Staphylococcus* species. These properties are more similar to those of the urease of the known stomach pathogen *H. pylori* and provide further support for the pathogenic potential of these coccoid isolates.

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