Affinity Purification of Helicobacter pylori Urease

RELEVANCE TO GASTRIC MUCIN ADHERENCE BY UREASE PROTEIN

(Received for publication, December 8, 1997, and in revised form, April 7, 1998)

Faustino C. Icatlo, Jr.‡, Masahiko Kuroki, Chizu Kobayashi, Hideaki Yokoyama, Yutaka Ikemori, Tomomi Hashi, and Yoshikatsu Kodama

From the Immunology Research Institute, Ghen Corporation, 839-1 Sano, Gifu City 501-11, Japan

A simple, reproducible and high yield method of Helicobacter pylori urease enzyme purification was developed using a heparinoid (Celluline sulfate) affinity gel. The purification method involved two sequential steps using the same gel that takes advantage of the differential affinity of urease to the heparinoid at two levels of hydrogen ion concentration. SDS-polyacrylamide gel electrophoresis analysis of affinity-purified urease revealed two major protein bands with about 62- and 30-kDa molecular mass. When whole cell lysates of clinical and laboratory strains of H. pylori were probed by Western blot, anti-urease hyperimmune serum produced by affinity-purified urease in rabbit recognized only the two bands corresponding to the urease A and B subunits. To probe the molecular relevance of affinity gel adherence to mucin adherence, the purified urease was derivatized with N-hydroxysuccinimidobiotin and used in adherence assays. Competitive inhibition tests revealed commonality of urease receptors among gastric mucin, heparin, and heparinoid. Composite data on adherence kinetics modulated by pH, salt, incubation time, and concentration of urease or mucin were indicative of conformation-dependent ligand-receptor interaction.

Underscoring the significance of urease in Helicobacter pylori gastro-duodenal infection are the observations that urease is essential for colonization in animal models (1, 2) and that native (3) or recombinant urease subunits (4–6) could protect animals from experimental challenge when used as a vaccine. To date, urease is the first vaccine candidate to be tested in human clinical trials (7). Although urease is biosynthesized and localized within the cytosolic compartment of H. pylori, it ultimately accumulates on the bacterial surface through a proposed fourth mechanism of protein export to a subcellular compartment (8, 9). According to this model, H. pylori cells undergo spontaneous autolysis, presumably driven by a yet unidentified autolysin gene. The subsequent release of cellular debris brings the urease enzyme to the surface of other bacterial cells with which it gets in contact in vivo or in vitro. A separate study, however, has suggested that autolysis may only be a minor event compared with active secretion of the enzyme (10). Regardless, the surface localization of urease protein potentially confers a central pathogenic role for this cytosolic protein. Urease may influence H. pylori nutrition via utilization of urea-derived ammonia for protein synthesis (11) and induction of proinflammatory cytokines (12) and promote bacterial survival in acidic medium by generating ammonia within its immediate mucinous pericellular space.

Urease is one of the most abundant proteins of H. pylori; therefore, understanding the host-urease interactive events may yet hold the key to a rational and efficacious mode of treatment and prevention of colonization. To date, however, the fundamental mechanism underlying the essential role of urease in the colonization of the gastric mucosa remains unknown (13). A major deterrent in elucidating this essential role is the lack of a simple, reliable, and high yield method of purification of this enzyme. Easy access to highly purified urease proteins by investigators should therefore facilitate advances in therapeutic or preventive approaches based on the abrogation of known or yet undetermined biological activity of this Helicobacter protein. Previous purification methods of native or recombinant urease invariably required two or several steps involving conventional size exclusion and cation exchange (14) or fast performance liquid chromatography (FPLC)† combining cation exchange, size exclusion chromatography, and sometimes the conventional hydrophobic interaction gel (4, 15, 16). FPLC requires sophisticated instrumentation and expensive columns usually inaccessible to small research laboratories, and the multiplicity of chromatographic steps tends to compromise the recovery rate in terms of both protein and catalytically active enzyme. Immunoaffinity purification has been performed using monoclonal antibody (7) that requires a time-consuming antibody-coupling step to the support matrix and with the column so designed not usable over an extended period of time. The simplest method used so far was the procedure involving conventional agarose gel filtration and DEAE-cellulose ion exchange (14). As a first step to facilitate further studies on the pathogenic mechanism of urease, we developed a simpler method of purifying the native enzyme from crude bacterial extract. In this study, we took a step further by using a single type of gel for a two-stage conventional affinity chromatography. The gel, a heparinoid consisting of cellulose matrix with sulfate esters as functional groups, has been shown to be useful for purification of a wide range of viruses, enzymes, and other biomolecules that interact with a heparinoid ligand. Lipopolysaccharide does not bind to the sulfated polymer, making this material suitable for detoxification of biological substances (Amicon product manual). Binding to the heparinoid gel prompted us to use the urease protein thus purified in adherence assays, using other sulfoconjugates as receptor substrate. The observed high affinity binding to heparin and mucin was discussed in relation to the possible adhesin function by H. pylori urease.

‡ To whom correspondence should be addressed: Immunology Research Inst., Ghen Corp., 839-1 Sano, Gifu City 501-11, Japan. Tel.: 58-235-7303; Fax: 58-235-7505.

† The abbreviations used are: FPLC, fast protein liquid chromatography; BHI, brain heart infusion; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

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Materials and Methods

Bacterial Strain and Cultivation Conditions—Fresh clinical H. pylori strains 130, 132, and 135, isolated as described previously (17), and NCTC 11637 were used for urease purification experiments (number 130) and for Western blot analysis (all strains). The strains were obtained from Dr. Yuji Aiba (Department of Internal Medicine IV, School of Medicine, Tokai University, Isehashi, Kanagawa, Japan). Stock cultures of each strain were prepared by initial passage in brain heart infusion (BHI, Difco) agar plate containing 5% defibrinated horse blood (4 days, 37 °C) and then passaged to 20-ml Brucella broth (Difco) in 100-ml bottle (plus 0.2% β-cyclodextrin and 10% fetal bovine serum (FBS)). Bottles were incubated overnight with gyroratory shaking at 37 °C. The gas phase inside the bottle for all broth cultures in this study was replaced with a mixture of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide (v/v). Broth cultures were incubated for 24 h at 37 °C with gyroratory shaking. Five ml of the resulting bacterial broth suspension were inoculated to 1-liter bottles containing 200-ml BHI broth with 2–5% FBS. After overnight incubation with gyroratory shaking, 5 ml of the suspension was again passaged to 1-liter bottles containing Brucella broth with 2–5% FBS. The bottles were incubated for 48 h at 37 °C using a bidirectional shaker at 75 rpm (Takasaki Scientific Instruments Corp.). The cell biomass was collected by centrifugation at 12,000 × g for 20 min. To compare urease yield according to cultivation medium composition, BHI broth instead of Brucella broth was used under exactly the same cultivation conditions. H. pylori whole cell antigenic property of 155 mm of Cellufine sulfate bed height was equilibrated with PE buffer, pH 6.5 (PE65). About 5.5 ml of crude urease extract was then applied to the step A column at a flow rate of 9 ml/min. Elution was done with PE65, and the eluate was fractionated with an automatic fraction collector (Atto) (1.25 ml/fraction). Protein concentration in the void volume was measured continuously with a UV monitor (280 nm). Protein concentration of each fraction was determined with the use of the Bradford reagent and after a single freeze-thaw cycle. To determine whether a single step purification method will result in selective binding of urease to the Cellufine sulfate gel, the crude urease extract was adjusted to pH 5.5, immediately adsorbed to the step B gel, and eluted as above.

Protein Analysis—Quantitative protein analysis was performed using the Bradford test reagent (Bio-Rad).

Calculation of Percentage Recoveries of Urease Protein and Enzyme from Crude or Partially Purified Urease—The percentage urease content of crude extract or partially purified fractions (step A pooled eluate) was determined by analyzing the integrated densities of protein bands in SDS-PAGE gels using an image analyzer (Aspect, Mitani Corpora- tion, Fukui-ken, Japan) system with software installed on a NEC PC-9801K personal computer. The total urease content was calculated by adding the urease A and B subunit percentage integrated densities. The percentage obtained was used to calculate the total urease protein contained in a crude or partially purified test sample based on the total sample protein obtained by the Bradford test (mg/ml). Step A protein recoveries were based on the urease protein (about 29.5–32%) contained in the original crude extract, while step B recoveries (final product) were calculated for both the urease protein contained in the crude extract and the total protein content of the crude extract. Percentage enzyme recovery calculation was based on the sum total of enzyme activity (units/ml) recovered in all positive fractions just after elution from the step A or B column compared with the original enzyme activity just before purification.

Production of Rabbit Anti-urease and Anti-whole Cell Sera—Anti-urease rabbit serum was produced using 100 mg of affinity-purified urease as antigen mixed with an equal volume of a block copolymer adjuvant CRL89–41 (TiterMax Classic, Cytrx Corp.). The resulting emulsion was administered subcutaneously to a 2.5-kg Japanese White rabbit at four different sites. At 3, 4, and 5 weeks after primer immunization, the same amount of urease protein with the same adjuvant was administered via the same route. The rabbit was exsanguinated 3 weeks after the last booster, and total serum was collected followed by antibody titration (enzyme-linked immunosorbent assay (ELISA)) and storage at −30 °C. Anti-whole cell serum was prepared by immunizing a rabbit using whole cell homogenate of H. pylori number 130. The antigen was prepared by disrupting broth cultivated H. pylori number 130 cells using a glass bead homogenizer (MSK, B. Braun Flexible Biotechnology). A sample of whole cell homogenate was plated onto BHI blood agar plates to confirm cell disruption. The antigen was dispensed in aliquots and stored at −80 °C until use. For primer immunization, about 3 mg of the lysed cell suspension was mixed with an equal volume of complete Freund adjuvant. The emulsified antigen was injected subcutaneously at weeks 2, 3, and 4 postprimer. In the fifth week, the same amount of antigen was injected intravenously via ear vein without adjuvant. The rabbit was exsanguinated 3 weeks after the last booster.

Serum was collected for ELISA titration and stored as hyperimmune anti-whole cell serum at −30 °C until use.

ELISA Titration of Anti-urease and Anti-whole Cell Antibody—The antigenicity of affinity-purified urease was assessed by checking the antibody response of immunized rabbit using the whole cell lysate or purified urease as antigen coating for ELISA antibody titration. Assay plates
Affinity Purification of H. pylori Urease

were prepared by coating 96-well Immulon 2 plates (Dynatech Laboratories, Inc.) with either whole cell lysate of strain 130 H. pylori or affinity-purified urease. The whole cell lysate was prepared by dissolving whole H. pylori cells in 1% SDS, and unadsorbed cells were removed by centrifugation. For the assay, the solubilized whole cell antigen or affinity-purified urease was added to a final concentration of 100 μg/ml in carbonate-bicarbonate buffer, pH 9.6, as diluent. About 100 μl of this preparation was dispensed per well, and plates were incubated overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween (PBS-Tween) and blocked with 3% bovine serum albumin, fraction V (Sigma) for a 1-h incubation at 37 °C. Plates were washed three times with PBS-Tween, and 100 μl of the test rabbit serum (1:100 dilution) was dispensed with PBS-Tween was dispensed to each well. After 1 h at 37 °C, wells were washed three times with PBS-Tween and 100 μl of horseradish peroxidase-goat anti-rabbit IgG conjugate (Cappel) in suitable dilution was used to detect the protein molar ratio used. Peak fractions of labeled urease eluted from the urease A and B subunits were detected with streptavidin-horseradish peroxidase (Zymed). Urease fractions, samples were coated on ELISA plates and detected at a 1:32 biotin:protein molar ratio was found to have a lower peak adherence at around this molar ratio. The labeled urease samples were monitored using streptavidin-horseradish peroxidase conjugate (Zymed) at different (1:1 to 128:1) biotin:protein molar ratio based on the ELISA wells were precoated overnight with a constant amount (5 μg/ml biotinylated whole bacterial cells (10-fold dilution of 9.6 μg/ml) in an ELISA-based assay. A 2-fold dilution series of native urease (using pH 9.6 carbonate-bicarbonate coating buffer), and a 2-fold dilution of labeled mucin was allowed to adhere at pH 4.0 for 1 h at 37 °C followed by ELISA as described above. To determine the kinetics of adherence to mucin and heparin-coated wells over a 5-h period. At several time points within this period, wells were washed five times with PBS-Tween, pH 7.4, and bound urease was probed and quantified by ELISA. In another experiment, ELISA wells were precoated overnight with a constant amount (5 μg/ml) of solubilized whole cell antigen or affinity-purified urease, and plates were processed by ELISA. To determine saturability of receptors, a 2-fold dilution of labeled urease was added to wells coated with native urease (5 μg/ml) in an ELISA-based assay. A 2-fold dilution series of native urease (using pH 9.6 carbonate-bicarbonate coating buffer), and a 2-fold dilution of labeled urease was allowed to adhere at pH 4.0 for 1 h at 37 °C followed by ELISA as described above. To determine the kinetics of adherence to mucin and heparin-coated wells over a 5-h period. At several time points within this period, wells were washed five times with PBS-Tween, pH 7.4, and bound urease was probed and quantified by ELISA. To determine whether urease can prevent adherence of whole H. pylori cells, heparin or mucin was used as substrate (both precoated at 125 μg/ml) in an ELISA-based assay. A 2-fold dilution series of native urease at pH 4.0 with an initial concentration of about 380 mg/ml was allowed to adsorb to immobilized heparin or mucin for 1 h at 37 °C. A constant amount of biotinylated whole bacterial cells (10-fold dilution of 9.6 μg/ml) was added to the coated wells, and plates were processed by ELISA. To determine the approximate number of adherent cells was calculated based on an experimental setup of standard whole cells with known colony-forming unit titers diluted 2-fold, coated on ELISA plates, and detected by streptavidin-horseradish peroxidase by an ELISA method.
RESULTS

The native urease component of crude H. pylori cell extract adhered to the heparinoid and heparin-agarose gels in a pH-dependent manner. Peak detection of gel-bound urease enzyme activity was observed at pH 5.5 and 5.0, respectively, for heparinoid (87%) and heparin-agarose (69%) using adhesion medium without NaCl. Toward the neutral side of the peak, bound urease declined (1–11% at pH 6.0–7.4) in both gels. Toward the more acidic side, bound enzyme gradually declined until it reached zero at pH 4.0 and below. The use of 0.15 M NaCl did not increase but rather decreased affinity (respective peaks of 38 and 22% for heparinoid and heparin-agarose) as well as shifted the pH optimum to a lower level (pH 4.5 in both gels), making this condition less favorable for the preservation of enzyme activity. From these data, subsequent affinity purification experiments were conducted using the heparinoid gel without salt in the adsorption buffer.

The step A column resolved three major protein peaks in the flow-through fractions with the strongest urease enzyme activity and highest protein concentration (SDS-PAGE profile in Fig. 1) localizing entirely within the first peak. Since the second and third peaks were devoid of significant enzyme activity or urease protein bands as determined by SDS-PAGE (data not shown), the present description was delimited to the first peak. SDS-PAGE analysis of proteins that remained adsorbed to the gel at pH 6.5 by elution with 2 M NaCl in PE65 buffer revealed a prominent band of about 25 kDa together with minor bands between 31 and 62 kDa (not shown); visible urease bands were not detected. This adsorbed protein fraction contained about 4.4% of the total protein input and only 0.5% of the enzyme input to the step A column. From the step B column, fractions containing peaks in protein, enzyme activity, and ionic strength coincided with bands seen in SDS-PAGE of the same fractions, indicating that the 62–65- and 30-kDa bands were the urease B and A subunits, respectively, of the urease enzyme. Fig. 1 shows the gel-bound urease after elution from the step B column with the two urease subunits clearly free from contaminating proteins seen in step A eluted fractions. A typical step B elution profile is shown in Fig. 2. Significant stoichiometric variation in subunit urease A:urease B ratio was not observed in gel scan samples of purified urease. Affinity-purified urease generally had no enzyme activity after a single freeze-thaw cycle probably due to liberation of the critical nickel ion by acidity (23) encountered during thawing of phosphate-buffered samples. Direct application of the crude urease extract to the step B gel resulted in high percentage recovery of urease in terms of both protein (75.2%) and enzyme (58.0%). However, some irrelevant proteins were co-purified and appeared as minor bands in SDS-PAGE gel (not shown), indicating that a preparatory purification step was essential. Percentage recovery of urease protein or enzyme from step A or B columns was the same regardless of medium used for cultivation of H. pylori (Table I), although Brucella broth-derived urease tended to be slightly higher. Approximately one-third of the crude urease extract was urease based on gel scan profiles of two batches from each medium used (32.0 ± 6.1% for Brucella broth and 29.5 ± 3.7% for BHI broth). From independent experiments, about 29–44% and 13–26% of the total urease protein and enzyme, respectively, were recovered from the step B column.

Biotinylation of native urease at different biotin:protein molar ratios based on the monomeric mass of the enzyme (1:1, 4:1, 8:1, or 32:1) did not significantly alter the enzymatic activity of affinity-purified urease over a 180-min period. This indicates that the putative catalytic site involving 8 histidine and 1 cysteine residue (24) was not involved in the nucleophilic displacement reaction involving unprotonated urease amine and the biotin ester. Alternatively, biotinylation at the biotin:protein molar ratio used may not have been enough to affect the catalytic site. Gel scan densitometry of labeled urease in SDS-PAGE gel showed a biotin dose-related broadening of bands.
pH-dependent manner as gastric mucin, although duodenal mucin peaked at a slightly more neutral pH at 4.5, 5.0, and 5.5 (not shown). The level of adherence at pH 8.0 and 9.0 was similar to that at pH 7.0 in both duodenal and gastric mucin samples. Saturability of mucin receptors was demonstrated at pH 4.0 based on a linear response with increasing urease or mucin concentration against a constant concentration of mucin or urease, respectively (Fig. 5). In some of our preliminary trials, adherence to heparin-agarose and heparin-agarose conjugate (Fig. 3) served as a positive control for adherent cells. Only the two bands of monomeric urease were recognized among the whole cell lysate proteins of several \textit{H. pylori} strains, indicating the high degree of purity of the antigen used to raise the antiserum. In the first Western blot experiment, anti-urease rabbit serum specifically recognized the urease B and A subunits of several \textit{H. pylori} strains (NCTC 11637, numbers 130, 132, and 135) when each of their whole cell lysates were probed in nitrocellulose membrane blots (Fig. 3A). The recognition of only two urease-specific protein bands indicates a high degree of homogeneity of the affinity-purified urease antigen used to raise the antiserum. In the second Western blot conducted (Fig. 3B), anti-whole cell hyperimmune serum specifically recognized only the two subunits of affinity-purified urease in contrast to the crude urease extract, where 10 protein bands were recognized. The discrepancy further indicates a high degree of purification of the urease protein. In another instance, the labeled urease (8:1) electroblotted onto nitrocellulose membrane from SDS-PAGE gel was detected with a streptavidin-horseradish peroxidase conjugate, which revealed that only two bands in the purified urease sample in contrast to the recognition of several bands for the crude urease extract, having about 80% of total bound urease detected within a 15-min period versus 60 min for mucin to attain a similar level of binding (Fig. 6). At pH 7.0, mucin was saturated at the same rate as the pH 4.0 incubated heparin but the total amount of urease bound was much lower even when incubation was prolonged to 5 h at 37 °C.

### Table I

**Percentage recovery of \textit{H. pylori} urease protein and enzyme by affinity chromatography**

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Cell pellet weight (extract volume)</th>
<th>Crude urease extract input</th>
<th>Recovery rate as percentage of urease input (specific activity)</th>
<th>Percentage recovery from total protein input (final volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI broth</td>
<td>mg</td>
<td>mg</td>
<td>% units (sp. act.)</td>
<td>% units (sp. act.)</td>
</tr>
<tr>
<td>A</td>
<td>0.94 (6.3)</td>
<td>37.699</td>
<td>7.9 ± 0.6</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td>B</td>
<td>0.827 (5.3)</td>
<td>14.331</td>
<td>3.8 ± 0.3</td>
<td>1.7 ± 0.0</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td></td>
<td>6.6 ± 0.5</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Brucella broth</td>
<td>g (ml)</td>
<td>mg</td>
<td>% units (sp. act.)</td>
<td>% units (sp. act.)</td>
</tr>
<tr>
<td>A</td>
<td>1.0 (5.5)</td>
<td>26.048</td>
<td>86.2 ± 4.5</td>
<td>25.7 (229.8)</td>
</tr>
<tr>
<td>B</td>
<td>0.498 (3.3)</td>
<td>17.398</td>
<td>92.6 ± 1.6</td>
<td>20.3 (221.1)</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td></td>
<td>91.3 ± 1.5</td>
<td>23.8 (228.1)</td>
</tr>
<tr>
<td>BHI broth</td>
<td>mg</td>
<td>mg</td>
<td>% units (sp. act.)</td>
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</tr>
</tbody>
</table>

* sp. act., specific activity.
The adherence of biotinylated urease at pH 4.0 to heparin was inhibited in a linear fashion by decreasing concentration of mucin, heparin-agarose, and heparinoid gel but not by agarose (Fig. 7). Starting from an inhibitor concentration of about 6.25 μg/ml (mucin) or 6.25 mg/ml (heparin-agarose or heparinoid gel), there was a dose-dependent inhibition of adherence by urease. Above 0.78 mg/ml concentration, cellulose had some weak inhibitory activity, but this was abolished at 0.78 mg/ml and below.

When native urease at different concentration was preincubated in heparin- or mucin-coated wells at pH 4.0, there was an initial promotion of adherence at lower urease concentration (5.63 and 11.3 μg/ml) (Fig. 8). Inhibition started to take effect with further increase in urease concentration until reaching a leveled off region (a plateau in terms of percentage of inhibition) at the highest urease concentration used (180 μg/ml), which suggested that no significant further inhibition would be possible beyond this level. Inhibition of absorbance in mucin- or heparin-coated wells at the maximum urease inhibitor concentration used was translated to about 59 or 53% reduction in whole cell numbers, respectively, when compared with the control wells.

**DISCUSSION**

The present study describes a simplified dual step process of urease purification that takes advantage of the differential interaction between urease and a sulfated polymer at two levels of hydrogen-ion concentration. Step A at pH 6.5 was essentially a bifunctional process involving size exclusion and some degree of subtractive affinity chromatography due to retention of some extraneous bacterial proteins in the column. In a Superose 12 FPLC gel filtration of concentrated water extract of *H. pylori* urease conducted by another group (15), urease eluted with a 54-kDa protein, probably the HspB. In contrast,
step A peak fractions in the present method contained only the urease protein and enzyme as the major component along with some proteins of negligible amount. The band just below the urease B subunit, although a major band in the crude extract, was prominently absent in the step A purified fractions. At pH 6.5, it appears that urease had a lower density of net positive charges relative to other proteins in the crude aqueous extract and may perhaps explain the absence of a major 54-kDa contaminating protein in the void volume. Step B at pH 5.5 (elution profile in Fig. 2), which selectively bound urease to the heparinoid ligand, effectively excluded residual adventitious proteins, as could be seen in SDS-PAGE gels (Fig. 1) and the confirmatory Western blot (Fig. 3B). Anti-whole cell H. pylori serum failed to recognize in Western blot all other proteins including the HspB. This indicates that HspB, although a sulfatide-binding protein specifically recognizing ligands on acidic glycosphingolipids of the gastric mucosa (25), had an elution pattern in a heparinoid column distinct from that of...
urease. The net charge density of the urease protein appeared to increase dramatically with the shift in pH from 6.5 to 5.5 retaining about 29–44% of the urease protein in the column. Omitting step A may result in contamination of urease with other proteins such as flagella and outer membrane proteins visible by SDS-PAGE, although the non-urease proteins copurified was possibly a result of mostly nonspecific interaction, since Cellufine sulfate is a relatively strong cationic exchanger. The higher enzyme recovery rate in the batch than in the column method at pH 5.5 may have been due to a slight change in urease affinity upon exposure to pH 6.5 and 5.5 over a prolonged period at room temperature during column affinity purification. This is supported by the observation that in a single step purification, there was a higher percentage recovery of both protein and enzyme (75.2 and 58% respectively) than in the double step procedure despite similarity in elution conditions in the step B column. Despite some degree of inactivation, it was possible that inactivated enzymes could have been selectively co-purified with active urease molecules in the present purification method based on the generally higher percentage of protein recovery compared with enzyme (Table I). In retrospect, the use of pH lower than 5.5 in the step B column may increase protein recovery but will certainly decrease the percentage of recoverable enzyme activity. This may be suitable for experiments where only the urease protein is of interest. However, a pH below 3.0 is not advisable, since it may hydrolyze the sulfate esters on the affinity matrix. The percentage recovery of both protein and enzyme were high (Table I) relative to existing FPLC procedures (15, 16) but similar to that obtained by the conventional size exclusion plus ion exchange method (14). It must be understood that the present study focused on a small scale setup. We have tried scaling up by simultaneously running two step A columns for a single Step B column with recovery rates similar to figures shown in Table I.

To date, the critical role of urease in colonization is virtually unknown, and its adhesion function, although frequently speculated upon, has not been directly proven. Based on our initial observation that urease bound to a heparinoid gel and on the assumption that the purified urease was in the main physically intact after inactivation (as could be seen in Coomassie Brilliant Blue-stained SDS-PAGE bands), we decided to test the adherence of urease to other sulfo-conjugates. Since ammonium-based detection of urease is unreliable under moderate to severe acidity due to enzyme inactivation, affinity-purified urease was derivatized by biotinylation and then allowed to bind to heparin or mucin within a pH gradient similar to that of the gastric mucus. While completely devoid of enzymatic activity, urease adhered to both substrates in a similarly pH-dependent manner (Fig. 4). Thus, biotinylation essentially retained the structural integrity of urease. The pH-dependent binding and the observed shift in pH optimum for adherence with change in salt concentration implies a three-dimensional change in molecular folding that exposes or ensconces clusters of positively charged sites according to ionic strength or availability of hydrogen ion. The findings also suggest that the catalytic site in the urease molecule was structurally and functionally distinct from the adhesive domain and that urease may function both as an adhesive and enzyme at the same time. The inverted cup-shaped curve over a pH 2.0–7.0 gradient reflects a unique requirement for and adaptation to a well defined range of acidic milieu not previously seen but may also be present in other gastric microbial inhabitants of humans and animals. The data also suggest a possible complementary role by glycosaminoglycans, mucin, and other sulfated proteoglycans in mediating adherence by H. pylori in the stomach.

The saturability of mucin or heparin receptors was demonstrated by controlled ligand or receptor extinction experiments (Fig. 5). This was further demonstrated by the time-controlled incubation of constant urease concentration at two pH levels (high and low binding pH) (Fig. 6). Altogether, the above data argue for a specific mode of affinity rather than stochastic macromolecular interaction. The more rapid saturation of heparin compared with mucin may reflect a less exposed binding site in mucin that necessitates some time to equilibrate at pH 4.0. Preincubation of the mucin-coated wells in the adhesion medium (pH 4.0) might shed some light on this difference in saturation rate.

The striking similarity in pH gradient adherence curves by urease in mucin or heparin substrates (Fig. 4) was strongly suggestive of a common receptor molecule for urease and was probed in a competitive inhibition assay. Since immobilized mucin (at the concentration used for well coating) had the tendency to nonspecifically bind soluble heparin, we therefore used immobilized heparin as solid substrate with mucin (at a much lower concentration of 6.25 μg/ml), heparin-agarose, or heparinoid gel as test receptor blockers. The dose-related inhibition of urease when allowed to bind to heparin in the presence of decreasing concentration of mucin, heparin-agarose, or heparinoid (Fig. 7) suggests a binding site common to the inhibitory polymers tested. Weak inhibition by cellulose and noninhibition by agarose suggest that the sulfate side chain residues in Cellufine sulfate, mucin, and heparin were the likely major target of urease adherence. The data do not, however, rule out some weak affinity to some polysaccharide forms as demonstrated by the finding on cellulose.

In another competitive inhibition experiment, H. pylori whole cell adherence was facilitated at lower but blocked at higher urease concentration (from 22.5 μg/ml) in a dose-related manner using heparin- or mucin-coated wells as substrates (Fig. 8). This experiment directly brings to the fore the dominant adhesive function by urease in H. pylori-mucus adherence under acidic condition. The facilitated adherence in the initial region of the curve provided a classical picture of how this protein, when present at the optimal ratio with whole bacterial cells, creates maximum contact points between mucin (or heparin) and H. pylori. At the maximum urease level used (180 μg/ml), the inhibition percentage reached a plateau (around 60%), suggesting that about this percentage of adherence inhibition at the most could be accounted for by urease alone. The presence of residual 40% adherent whole cells in mucin (~53% in heparin) reflects the plurality of proadhesive entities at work in whole H. pylori cells. Heparin does not contain sialic acid such that the remaining uninhibited percentage in heparin (and perhaps in mucin) can be explained by whole cell adherence to the polysaccharide backbone that has also been identified previously in another study (26) as a receptor for H. pylori whole cells.

The similarity in pH-dependent urease adherence curves between gastric and duodenal mucin together with our unpublished observations of similar curves using rat and rabbit gastric, duodenal, jejunal, or ileal crude mucus samples suggest that the putative receptor is a ubiquitous molecule common to the stomach and small intestinal mucosa. Considering the preferential colonization of the stomach but not the intestines (except for metaphastic areas in the duodenum thought to be due to gastric acid insults (27)), acidity may therefore be implicated as the gastric factor that favors H. pylori colonization of the mucosa. The pattern of urease adherence described above interestingly coincides with the preferred localization of H. pylori in vivo.

Our densitometric analysis of crude extract of broth-cultivated bacterial cells showed that about a third of the autolytic bacterial debris adsorbed onto the surface of intact H. pylori...
Affinity Purification of H. pylori Urease

cells consisted of urease protein. This is paralleled by the observation that sulfated proteoglycans including heparin and other glycosaminoglycans are ubiquitous among animal cells (28). Gut mucin contains sulfate residues (29), while sulfatides are highly enriched in the human gastric and duodenal mucosa (30) and are thought to act as nonspecific barriers to infection (31). Thus, the constitutive and high level expression of urease by *H. pylori* as well as the natural enrichment of the gastric mucosa with sulfatides together suggest that such binding may be biologically relevant and point to a pathological role that cannot be underestimated.

The present results expand previous independent observations on *H. pylori* adherence and fit well into a unified model of multifactorial adherence by urease-producing *Helicobacter* spp. The observed adherence of *H. pylori* cells in *vivo* under neutral or probably near neutral pH to human gastric mucin (26), sulfated but not desulfated mucin (29), human gastric cells (32), and even sulfatides (33–35) can only be understood in terms of largely non-urease-mediated adherence. Six of these adhesins (13, 35) have been described, although much remains to be elucidated in terms of the relationship of each adhesin to a putative receptor. The heparan sulfate-binding protein under near neutral pH as described previously (36) may actually be near neutral pH as described previously (36) may actually be synonymous with sulfatides together suggest that such binding may be biologically relevant and point to a pathological role that cannot be underestimated.

REFERENCES


Affinity Purification of *Helicobacter pylori*Urease: RELEVANCE TO GASTRIC MUCIN ADHERENCE BY UREASE PROTEIN
Faustino C. Icatlo, Jr., Masahiko Kuroki, Chizu Kobayashi, Hideaki Yokoyama, Yutaka Ikemori, Tomomi Hashi and Yoshikatsu Kodama

doi: 10.1074/jbc.273.29.18130

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