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Affinity Purification of *Helicobacter pylori* Urease

RELEVANCE TO GASTRIC MUCIN ADHERENCE BY UREASE PROTEIN*

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A simple, reproducible and high yield method of *Helicobacter pylori* urease enzyme purification was developed using a heparinoid (Cellufine 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)[1] 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.

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[1] 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.

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, Isehara, Kanagawa, Japan). Stock culture of each strain was 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 gyratory 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 prior to incubation. Bacterial cell pellets were collected and stored as stock culture at −80 °C in 20% glycerol or 10% skim milk containing 2% sodium glutamate. For mass urease production, a stock culture of strain 130 was thawed, passaged to 20-ml *Brucella* broth, and incubated for 24 h at 37 °C with gyratory shaking. Five ml of the resulting bacterial broth suspension were inoculated to 1-liter bottles containing 200-ml *Brucella* broth medium with 5% FBS. After overnight incubation with gyratory 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 cells used in adherence assays were propagated once from stock culture by inoculation of *Brucella* broth with 2% FBS and harvested 24 h later. The whole cells were immediately biotinylated as described previously (18).

H. pylori Cell Pellet Collection and Preparation of Crude Urease Extract—The cell biomass pelleted during harvest was collected by scraping with a sterile metal spatula and directly transferring the cell pellet to 10-ml polypropylene tubes. About 1 g or less of *H. pylori* cells (wet weight) was transferred to each tube, and the material was spread as thinly as possible on the tube wall and stocked at −80 °C until use. To extract urease, the cell pellet was thawed at room temperature and then vortexed with about 6.5 ml of sterile distilled water per tube for a total of 20 s with brief stops every 5 s. Cells from the mixture were removed by centrifugation at 15,000 × *g* for 30 min, and the supernatant was filtered using a 0.22 Millex GV Millipore filter. The filtered sample or crude urease extract was added with a 10× concentration of 20 mM phosphate, 1 mM EDTA buffer, 1 mM 2-mercaptoethanol (pH 6.5) at a volume ratio of 1:10 to the total crude urease extract volume. The resulting buffered extract was adjusted to pH 6.5.

Measurement of Urease Enzyme Activity—The enzymatic activity of urease was quantified using a previously described method based on jackbean urease standard (19). Results were expressed in units, with 1 enzyme unit equivalent to 1 μmol of ammonia liberated from urea per min per mg of protein.

Batch Type Affinity Chromatography for Optimization of Gel Adherence by Urease—For preliminary optimization experiments, Cellufine sulfate (Amicon) and heparin-agarose (Sigma) were tested for urease affinity adherence at different pH. Equal volumes of 0.8 ml Cellufine sulfate, cellulose, heparin-agarose, or beaded agarose preadjusted to desired pH in duplicate into glass tubes were equilibrated with the adhesion medium (20 mM phosphate, 0.05% Tween 20). One ml of crude urease extract prediluted 10 times with phosphate buffer at different pH was dispensed to each tube containing gel that had been preequilibrated to the same pH as the urease sample. To determine the effect of physiological salt concentration on binding affinity, another set of tubes similarly prepared contained 0.15 M NaCl in the adhesion medium. The tubes were then shaken in slanted position at room temperature for 15 min using a bidirectional shaker. The gels were centrifuged at low speed and were washed three times with 10 gel volumes of PE buffer (20 mM phosphate, 1 mM EDTA) with pH similar to the gel pH. After aspirating the last wash buffer, bound urease was eluted with 2 ml/tube of 2 M NaCl in PE buffer, pH 7.0. After pelleting the gels, urease enzyme activity in the bound and unbound fractions was measured as described above.

Affinity Column Purification of Native Urease from Crude Urease Extract—Cellufine sulfate was subsequently selected over heparin-agarose based on its generally higher affinity and higher pH optimum. In a typical experiment, the step A gel column (14 mm diameter) containing 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). Urease eluting with the void volume as the first peak was confirmed by enzyme measurement as described above. For analytical purposes, the whole sample input was fractionated, resulting in a total of three major peaks with the urease enzyme fraction coincident with the first peak. For routine urease production purposes, elution was terminated after the first peak and the tail of the second peak were eluted. Fractions were immediately analyzed quantitatively for enzyme activity. Urease-containing fractions were then selected, pooled, adjusted to pH 5.5, and adsorbed to the step B column (14-mm diameter) with about 63 mm of Cellufine sulfate bed height that had been preequilibrated with PE buffer, pH 5.5. The same flow rate as in step A was maintained followed by an extensive wash. For biotin labeling of urease and for immunizations, 20 mM phosphate buffer, pH 5.5, was used (PO55) for washing the step B column. In all experiments, gel-bound urease was eluted using 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PO74). Eluted fractions collected in 2.5-ml volumes were quantitatively analyzed for enzyme activity and protein content before storage at −80 or −135 °C. In some experiments, the peak fractions were immediately labeled with biotin (see procedure below). Some affinity-purified urease samples were quantitatively checked for remaining enzymatic activity before and after a single freeze-thaw cycle. To determine whether a single step 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 samples (step A pooled eluate) was determined by analyzing the integrated densities of protein bands in SDS-PAGE gels using an image analyzer (Aspect, Mitani Corporation, Fukui-ken, Japan) system with software installed on a NEC PC-9801RX 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 based on 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 2 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

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 unsolubilized cells were removed by centrifugation. For the assay, the solubilized whole cell antigen or affinity-purified urease was diluted to a final concentration of 5 μg/ml using 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 prediluted 100 times 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 dispensed to each well. After 30 min of incubation at 37 °C, plates were washed five times with PBS-Tween. Color reaction was developed with *ortho*-phenylenediamine dihydrochloride and was stopped with 3 N H_2SO_4. Absorbance was read at 490 nm. Two wells/plate not coated with the antigen but treated similarly as the test wells were used as the blank whose OD was subtracted from all test well readings. Titers of test sera were determined based on the regression of a standard calibration curve co-processed with test samples.

Biotinylation of Affinity-purified Urease—To verify the mechanism of adherence by urease to the affinity gels, affinity-purified urease was biotinylated just after elution from the step B column for use in an adherence assay. The purified urease was labeled via amino groups with *N*-hydroxysuccinimide biotin (Sigma) for 180 min (room temperature) at different (1:1 to 128:1) biotin:protein molar ratio based on the monomeric mass of urease. The labeled urease samples were monitored for enzyme activity at different incubation time points for up to 180 min. Labeling was quenched by 20-fold dilution of the sample with PO55 buffer, and the solution was readjusted to pH 5.5 and readsorbed to and eluted from the step B affinity gel as described above. To detect, quantify, and determine the lower detection limit for biotin-labeled urease fractions, samples were coated on ELISA plates and detected using streptavidin-horseradish peroxidase conjugate (Zymed). Urease labeled at a 1:32 biotin:protein molar ratio was found to have a lower limit of detection at about 10 ng/ml. In another instance, biotinylated urease was blotted onto nitrocellulose membrane after SDS-PAGE, and the urease A and B subunits were detected with streptavidin-horseradish peroxidase conjugate as outlined below to confirm specificity of labeling as well as detection of both subunits A and B at the biotin:protein molar ratio used. Peak fractions of labeled urease eluted from the step B column were stored at −135 °C.

SDS-PAGE and Western Blot—SDS-PAGE (10% polyacrylamide gel) (20) under reducing conditions was used to analyze the purification process. To confirm the purity of affinity-purified urease protein, two approaches were followed. First, Western blot was conducted with whole cell lysates of *H. pylori* strains NCTC 11637, numbers 130, 132, and 135 as antigens probed by anti-urease rabbit serum. Second, crude urease extract of *H. pylori* strain 130 was probed in a Western blot with rabbit anti-whole cell serum. In this step, an unfixed duplicate gel was used to transfer the electrophoresed protein to nitrocellulose membrane by the standard procedure of Towbin (21). The blotted proteins were then probed with the appropriate rabbit serum in a suitable dilution, and the rabbit IgG complexed with specific antigen was probed with anti-rabbit IgG-horseradish peroxidase conjugate (Cappel). Blots were developed with 4-chloro-1-naphthol, and the reaction was stopped with distilled water.

Crude Mucus Preparation, Purification, and Biotinylation of Gastric Mucin—Crude gastric mucus was obtained from the stomach of a 2-month-old weanling pig. To prepare crude mucus, the lumen of isolated stomach was perfused with PBS, pH 7.4, containing 0.1 M phosphate, 0.15 M NaCl, 5 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. After exposing the luminal wall through a greater curvature incision, the mucosal surface was scraped using a metal spatula with a minimum amount of the perfusion buffer. The scraped material was homogenized with a Polytron homogenizer (Kinematica Gmbh, Switzerland) at 12,000 rpm for 5 min. The sample was centrifuged sequentially at 15,000 × *g* and 25,000 × *g*, both at 4 °C, with the supernatant collected in both instances followed by dialysis in distilled water and freeze-drying. Preparation of purified mucin from crude gastric or duodenal mucus followed a previously described procedure (22) involving two cycles of equilibrium density gradient centrifugation for 48 h. Periodic acid-Schiff-positive fractions from the resulting cesium chloride gradient were pooled and freeze-dried. The sample was then diluted in an appropriate volume of 0.1 M phosphate buffer, 0.1 M NaCl, pH 6.8, and passed through a Sepharose CL-4B column preequilibrated in the same buffer. Fractions showing a single band of glycoprotein (~66 kDa) as revealed in Coomassie Brilliant Blue- and periodic acid-Schiff-stained duplicate SDS-PAGE gels (run under reducing conditions) were pooled, dialyzed in PBS, pH 6.8, and stored in aliquots at −80 °C until use in an adherence assay. Purified swine gastric mucin was labeled with *N*-hydroxysuccinimidobiotin (734:1 biotin:protein molar ratio based on a mucin molecular weight of 2×10^6) with the reaction carried out at room temperature for 1 h. Unbound biotin was removed using a 15-ml capacity Centricon-10 concentrator (Amicon) and stocked at −80 °C until use. Duodenal mucin was purified from the duodenum of the same weanling pig using the same procedure as above but was not labeled.

In Vitro Urease Adherence and Inhibition Assay—For adherence and competitive inhibition assays, the ELISA format was used. Unless specified, gastric mucin was used for all procedures involving mucin. The adhesion medium consisted of 20 mM phosphate, 0.15 M NaCl, and 0.05% Tween 20 preadjusted to the desired pH. All tests were done in duplicate with two wells/sample/test. Wells were coated with substrate or ligand using 50 μl/well. Two blank wells in each plate not coated with receptor substrate but treated similarly as test wells were used to normalize optical density readings to base line. All quantitative analyses of test sample urease concentration that remained bound to the wells were based on the regression of a calibration curve generated by a known standard co-processed with the test samples. For all adherence assays, Immulon 2 ELISA plates were coated overnight at 4 °C with 250 μg/ml substrate (unless stated otherwise) consisting of either mucin or crude mucus in neutral PBS or of heparin (Eastman Kodak Co.) or melted agarose (Seakem) in pH 9.6 carbonate-bicarbonate buffer. Plates were blocked with 3% bovine serum albumin for 1 h and washed three times with PBS-Tween before all assay procedures.

To confirm the effect of pH on adherence, about 2.5 μg/ml biotinylated urease (32:1 biotin:protein molar ratio) in adhesion medium with a pH of 2.0–8.0 was incubated in wells precoated with mucin, heparin, crude gastric mucus, duodenal mucin, or melted agarose for 1 h at 37 °C. Unbound urease was removed by washing five times with 200 μl of the adhesion medium having the same pH as the test well. The remaining bound urease was heat-fixed for 10 min at 65 °C (water bath). Wells were washed three times with PBS-Tween, pH 7.4, probed with streptavidin-horseradish peroxidase, and processed as in ELISA.

To determine saturability of receptors, a 2-fold dilution of labeled urease was allowed to adhere to mucin-coated wells for 1 h at 37 °C (at pH 4.0, since labeled urease exhibited peak adherence at around this pH), and bound urease was probed by ELISA. In another experiment, ELISA wells were precoated overnight with a constant amount (5 μg/ml) 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 over time, biotinylated urease was prediluted at pH 4.0 and 7.0 (control) adhesion medium and incubated in mucin- or heparin-coated wells over a 5-h period. At several time points within this period, wells were washed five times with the adhesion medium having the same pH as the test well. Adherent urease was probed and quantified by ELISA as above.

To probe the molecular make-up of urease receptor, a competitive inhibition assay was performed essentially following the same receptor-based ELISA using plate-immobilized heparin (250 μg/ml). Urease was mixed with 2-fold decreasing concentration of mucin, heparin-agarose, beaded agarose (Sepharose CL-4B, Amersham Pharmacia Biotech), Cellufine sulfate, or cellulose (Takara) at pH 4.0 and incubated for 60 min at 37 °C with gentle shaking. The mixtures were transferred to the heparin-coated wells and incubated with gentle shaking at 37 °C for only 30 min to avoid nonspecific adherence by heparinoid gel. The unbound urease with inhibitor was removed from each well by washing five times with pH 4.0 adhesion medium while the remaining urease was heat-fixed, and plates were processed 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 0.7 OD at 550 nm or about 1.43×10^7 colony-forming units/well) was dispensed to each well and incubated for another hour. Wells were washed 5 times with pH 4.0 adhesion medium and detected by ELISA. The approximate number of adherent cells was calculated based on a decremental response of standard whole cells with known colony-forming unit titer 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.

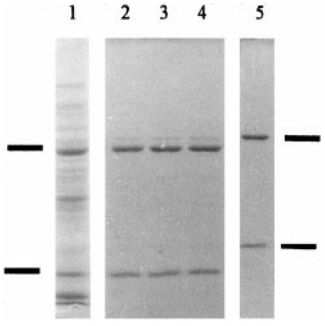

FIG. 1. **SDS-PAGE (reducing condition) of step A and B peak fractions.** *Lane 1*, a typical sample of crude urease extract prior to purification. *Lanes 2–4*, enzyme and protein peak fractions eluted from step A column at pH 6.5 showing two major bands at the 30- and 62-kDa loci (urease A and B subunits) with minor contaminants seen as a faint band of about 70 kDa and several indistinct bands with molecular mass between 31 and 60 kDa. *Lane 5*, enzyme and protein peak fraction eluted from step B column (fraction 62 of Fig. 2) showing the urease protein purified to homogeneity as a paired band of A and B subunits at the 30- and 62-kDa loci. *Upper* and *lower bars* on both sides correspond to 66- and 31-kDa molecular mass markers, respectively. *Bars* on the *left* are for *lanes 1–4*, while those on the *right* are for *lane 5*.

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

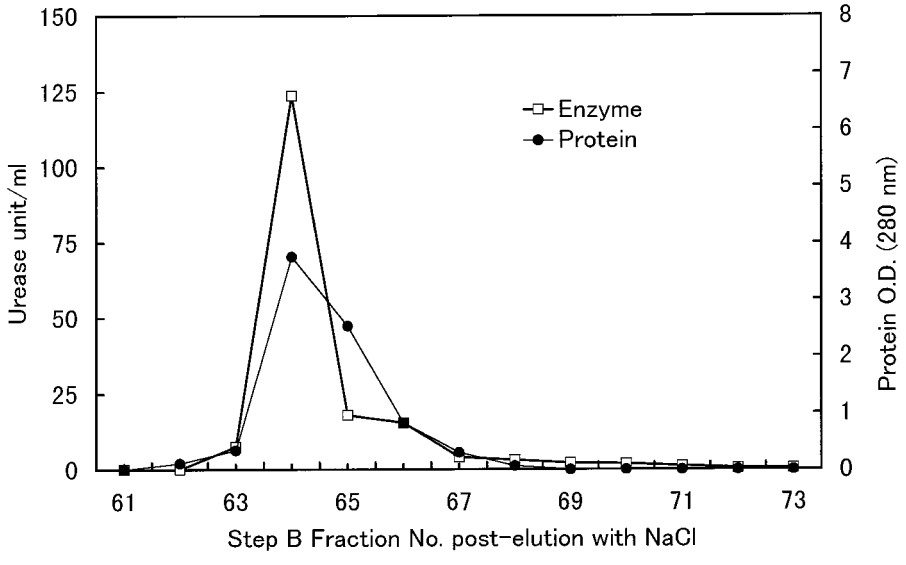

FIG. 2. **Desorption of urease from step B column.** The pooled step A enzyme fractions preadjusted to pH 5.5 were selectively adsorbed to the heparinoid gel at the same pH. After an extensive wash, urease appeared in the eluate as a sharp peak in enzyme activity and protein absorbance coinciding with the rise in ionic strength to 0.15 M NaCl, pH 7.4.

TABLE I
Percentage recovery of H. pylori urease protein and enzyme by affinity chromatography

Culture medium	Cell pellet weight (extract volume)	Crude urease extract input			Recovery rate as percentage of urease input (specific activity)				Percentage recovery from total protein input (final volume)
		Total protein	Urease protein	Total enzyme	Step A protein	Step A enzyme	Step B protein	Step B enzyme	
	g (ml)	*mg*	*mg*	*units (sp. act.)*[a]	*%*	*units (sp. act.)*	*%*	*units (sp. act.)*	*% (ml)*
Brucella broth									
A	1.0 (5.5)	26.048	7.215	2863 (109.9)	86.2	52.8 (243.0)	44.4	25.7 (229.8)	15.1 (12.5)
B	0.498 (3.3)	17.398	6.315	2415 (138.8)	92.6	43.0 (177.5)	35.1	20.3 (221.1)	12.7 (10.0)
Mean ± S.D.					89.4 ± 4.5	47.9 ± 6.9	39.8 ± 6.6	23.0 ± 3.8	13.9 ± 1.7
BHI broth									
A	0.94 (6.3)	37.699	12.101	3865 (102.5)	74.5	41.9 (179.7)	29.2	19.7 (215.5)	9.4 (12.5)
B	0.827 (5.3)	14.331	3.855	1027 (71.7)	85.7	25.0 (77.8)	31.8	13.2 (110.6)	8.6 (10.0)
Mean ± S.D.					80.1 ± 7.9	33.5 ± 12.0	30.5 ± 1.8	16.5 ± 4.6	9.0 ± 0.6

[a] sp. act., specific activity.

particularly evident in the urease B subunit. In the removal of unbound biotin from the step B column, there was an initial peak in biotin without increase in protein concentration just after sample application, indicating the washing down of unbound biotin. This was followed by another peak coinciding with an increase in ionic strength and protein absorbance during elution with 2 M NaCl. This finding further indicated that urease was specifically labeled.

Rabbit IgG raised against *H. pylori* had a titer of 243 and 3,847 when equal concentrations of whole cell lysate and affinity-purified urease, respectively, were used for antibody capture in an indirect ELISA for antibody titration. This indicates that the urease so purified was highly antigenic in the lepine host. In the first Western blot experiment, anti-urease rabbit serum specifically recognized the urease B and A subunits of several *H. pylori* strains (NCTC 11637, numbers 130, 132, and 135) when each of their whole cell lysates were probed in nitrocellulose membrane blots (Fig. 3*A*). 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. 3*B*), 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, and the urease B and A subunits were visualized (Fig. 3*B*), indicating that both subunits were labeled at this and higher biotin:protein ratios.

Binding of native urease to heparinoid and heparin-agarose gels prompted us to examine the adherence of biotinylated urease to soluble heparin and mucin (another sulfated glycoconjugate) in ELISA-based adherence assays. Adherence to the well immobilized sulfated glycoconjugates was found to be pH-sensitive (Fig. 4). Peak affinity was observed at pH 3.5–4.0 (heparin) or pH 3.5–4.5 (mucin), whereas control agarose had only a slight increase in adherence at pH 3.0. Crude gastric mucus preparation bound about 25% less urease at the pH optimum than purified gastric mucin but otherwise faithfully mirrored its pH gradient curve. Duodenal mucin bound urease in a similar 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 heparinoid gels was likewise dose-dependent (not shown). When tested for adherence over time, heparin was more rapidly saturated with urease, 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.

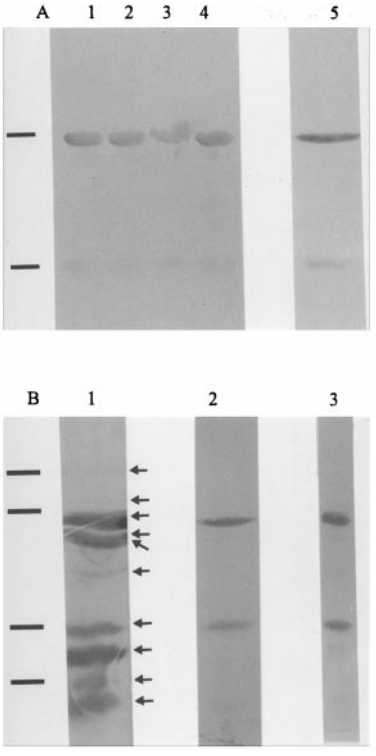

FIG. 3. **Western blot analysis.** *A*, *upper* and *lower bars* on the *left* correspond to 66- and 31-kDa molecular mass markers, respectively. *Lanes 1–4*, anti-urease hyperimmune serum was used to probe whole cell lysates of *H. pylori* strains NCTC 11637, numbers 130, 132, and 135, respectively. *Lane 5*, affinity-purified urease probed with the same serum as positive control. Only the two bands of monomeric urease were recognized among the whole cell lysate proteins of several *H. pylori* strains, indicating the high degree of purity of the antigen used to raise the antiserum. *B*, *bars* on the *left* from *top* to *bottom* correspond to 97-, 66-, 31-, and 22-kDa molecular mass markers, respectively. *Lanes 1* and *2*, crude urease extract and affinity-purified urease, respectively, when probed with anti-whole cell hyperimmune serum. Note the presence of only two bands in the purified urease sample in contrast to the recognition of about 10 bands in the crude urease extract (position indicated by *small arrows* in *lane 1*). *Lane 3*, biotinylated urease, prelabeled at 8:1 biotin:protein ratio based on monomeric urease mass, was probed with streptavidin-horseradish peroxidase conjugate, which revealed that both urease subunits (A and B) were labeled with biotin.

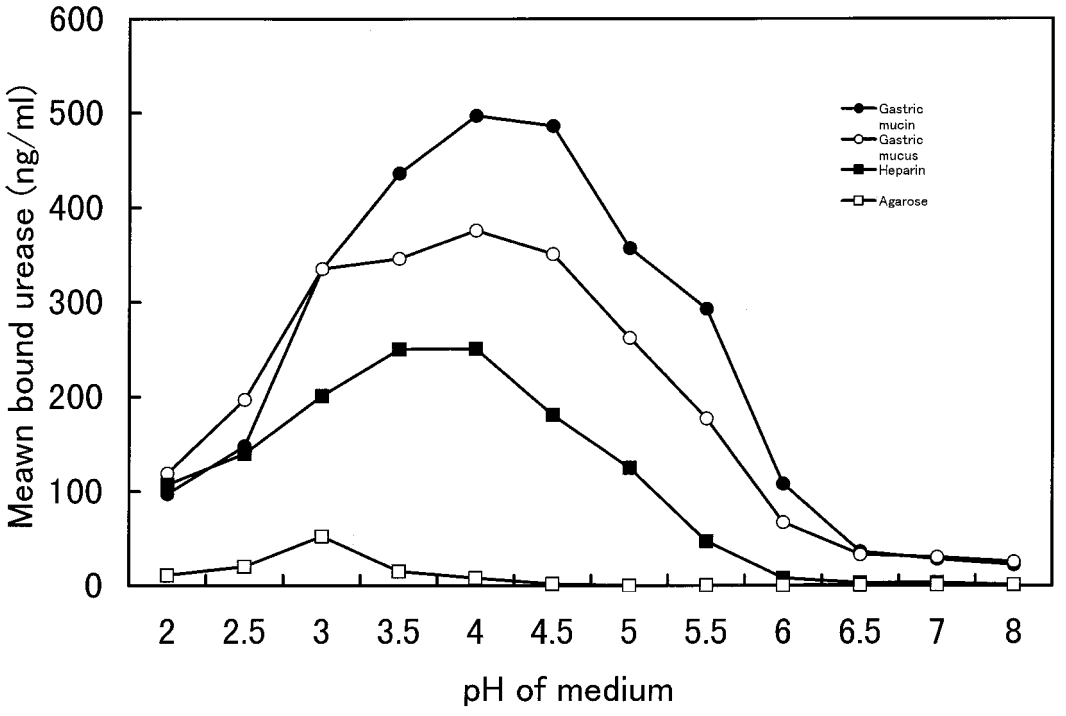

FIG. 4. **pH-dependent adherence of urease to sulfated glycoconjugates.** Similar curves were generated when plate-immobilized mucin and heparin were used as substrates for biotinylated urease adherence at different pH levels with a peak at pH 4.0. Crude mucus mirrored the mucin curve but with lower affinity. Control melted agarose showed only a slight increase at pH 3.0 and no appreciable increase at other pH levels.

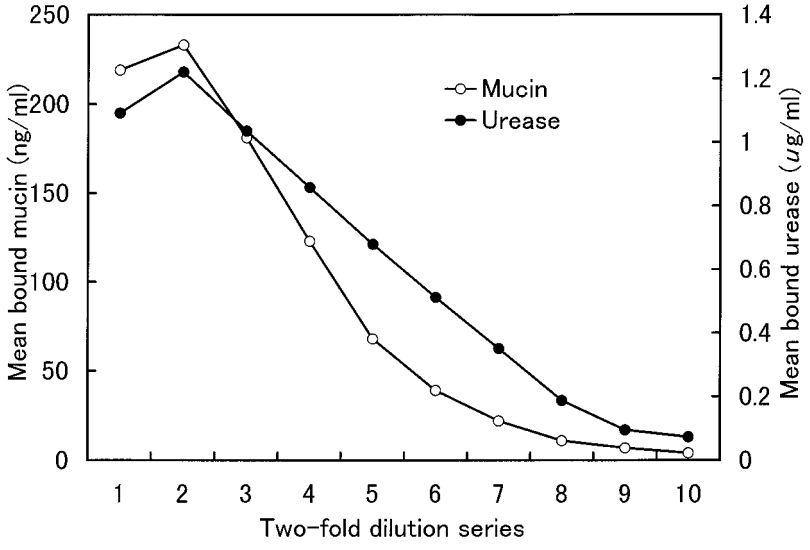

FIG. 5. **Dose-related adherence of urease to mucin or heparin.** Shown is the dose-related adherence of biotinylated urease to heparin when urease with an initial concentration of 34.1 μg/ml (concentration of first wells in the series) was diluted 2-fold and allowed to bind to heparin. A similar dose-related curve was generated when 13.8 μg/ml (concentration of first wells in the series) of biotinylated mucin was diluted 2-fold and allowed to bind to immobilized native urease.

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 coeluted with a 54-kDa protein, probably the HspB. In contrast,

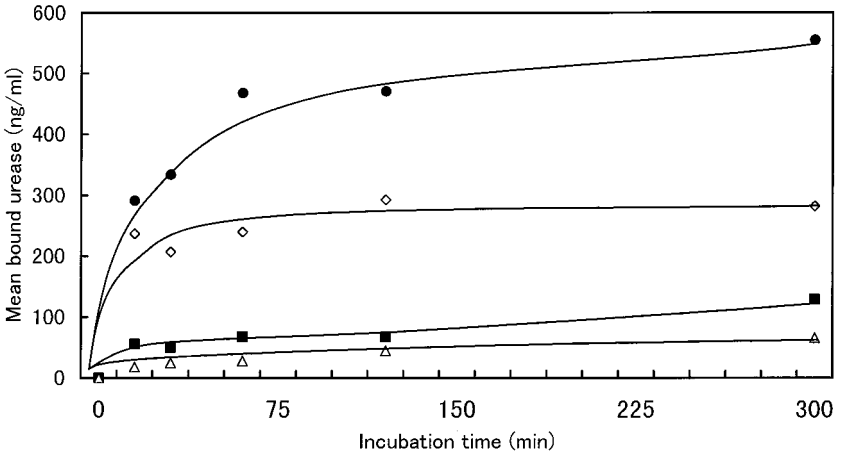

FIG. 6. **Time-dependent adherence of urease to mucin or heparin.** About 80% saturation of heparin binding sites was achieved by biotinylated urease within 15 min *versus* 60 min in mucin-coated wells at pH 4.0. From these respective time periods, there was no significant increase in binding until 5 h of incubation at 37 °C. The pH 7.0 controls had much less binding by labeled urease even with prolonged incubation of up to 5 h. , mucin, pH 4.0; ■, mucin, pH 7.0; ◇, heparin, pH 4.0; △, heparin, pH 7.0.

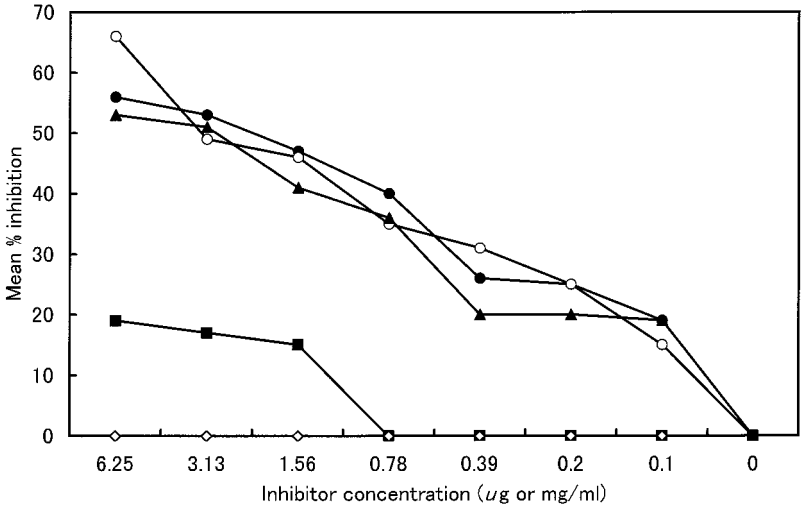

FIG. 7. **Competitive inhibition of urease adherence to heparinized wells at pH 4.0.** Biotinylated urease was inhibited at pH 4.0 in a dose-related pattern using gastric mucin, heparin-agarose, or heparinoid (Cellufine sulfate) gel. The beaded agarose control did not inhibit at the concentrations used, while cellulose (control for the heparinoid gel) inhibited at a much lower degree when used at a concentration of 1.56 mg/ml or more. Units are in μg/ml for mucin and mg/ml for all other polymers. , Cellufine sulfate (mg/ml); ■, cellulose (mg/ml); ○, heparin-agarose (mg/ml); ◇, beaded agarose (mg/ml); ▲, gastric mucin (μl/ml).

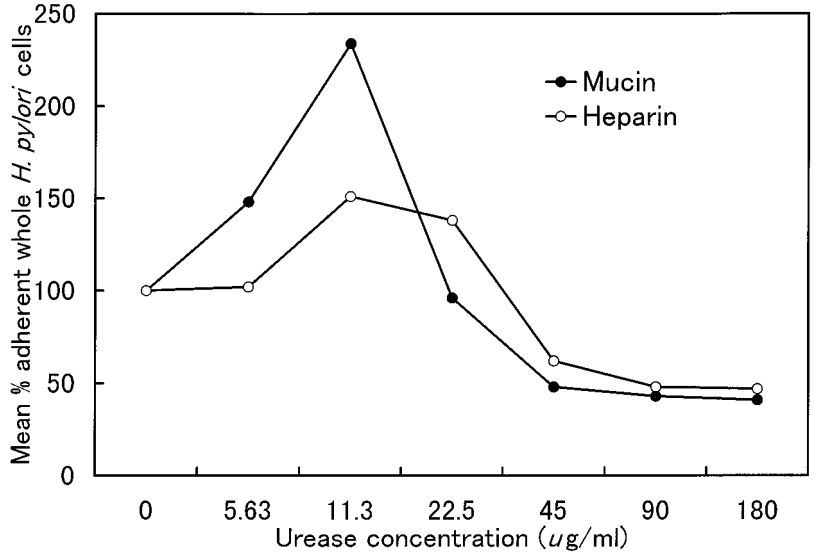

FIG. 8. **Whole cell *H. pylori* adherence to mucin or heparin in the presence of purified native urease at pH 4.0.** *H. pylori* number 130 whole cells were allowed to bind to immobilized mucin or heparin at pH 4.0 in the presence of increasing concentrations of native urease. Facilitated adherence was observed in the initial region of the curve followed by a dose-dependent inhibition that ended in a plateau at the two highest urease concentrations used. At 180 μg/ml urease, the number of adherent cells was reduced by close to 60% in mucinized wells or about 53% in heparinized wells.

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. 3*B*). 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 co-purified 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 adhesin 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 ammonia-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 adhesin 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 metaplastic 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*

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 pathobiological role that cannot be underestimated.

The present results expand previous independent observation 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 vitro* 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 the HspB described elsewhere (25). Thus, sulfate-binding by *H. pylori* cells may possibly involve more than one ligand including HspB (25), neutrophil-activating protein (35), some three or four species of outer membrane proteins (36), and urease, with each ligand operating within a distinct pH optimum. The present observations may be particularly related to the earlier findings that *H. pylori* cells bound to heparan sulfate (37) and to cultured human intestinal cells (38) in a pH-dependent manner. One recent study failed to implicate cell surface glycosaminoglycans in the adherence of *H. pylori* to human gastric cells, gastric adenocarcinoma cells, and HeLa cells (39). Such a conclusion, however, was based on an assay condition with a presumably neutral pH. The observations that urease-deficient *H. pylori* colonizes gastric explants *in vitro* (40) but not gastric mucosa *in vivo* (1, 2) only indicate that urease-mediated colonization depends on an intact gastric microenvironment (40) and that this relates most probably to the existence of a mucus blanket with an acidic mucoluminal interphase.

A recent study to specifically uncover the adhesin function of urease utilized a recombinant approach (41), where the *in vitro* adherence of urease-negative isogenic mutant was compared against that of the parent urease-positive strain. Significant binding was observed in both instances, and the conclusion was that the urease was not an adhesin. The study, however, failed to exclude other confounding adhesins in the assay, and the role of hydrogen ion concentration in adherence was not considered. In the present study, we used a biochemical approach by initially purifying urease and taking into account the pH optimum for adherence, since the organism thrives in an environment with a generally acidic but widely fluctuating pH. For the first time, our group provides compelling evidence that interaction between *H. pylori* urease and heparin or gastric mucin results in a specific ligand-receptor complex responding predictably to pH, salt, time, and dose modulation. The increased binding affinity with corresponding decrease in pH suggests adaptation to an acidic milieu that is required by the bacteria for survival in the presence of urea (42) and to which the bacteria is persistently exposed due to the state of flux in the gastric mucosa (43). This mode of affinity by urease to mucin may also be a major, if not a decisive, factor in initial bacterium-mucus contact before colonization and during maintenance of colonization against physical constraints such as the normal mucus turnover mechanism, peristalsis, and passage of luminal contents.

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