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Identification and Characterization of a Metalloprotease Activity from *Helicobacter pylori*

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Helicobacter pylori **produces a metalloprotease with a native molecular size of approximately 200 kDa, as determined by size-exclusion chromatography. Subcellular distribution studies demonstrated that the activity was associated with the outer membrane fraction of the bacterium. In addition, the protease was secreted by the bacterium when grown in liquid culture. The enzyme activity was measured by hydrolysis of azocasein and biotinylated casein and exhibited optimal caseinolytic activity at pH 8.0 (37°C). The activity was inhibited by EDTA, 1,10-phenanthroline, phosphoramidon, pyridine-2,6-dicarboxylic acid, and 8-hydroxyquinoline-5-sulfonic acid (HQSA). Inhibition by HQSA was reversed by zinc, whereas inhibition due to EDTA was reversed by excess calcium, thus indicating that the enzyme was a zinc-dependent, calcium-stabilized endoproteinase. Furthermore, titration with Zn^{2+} of a desalted, active-site zinc-chelated preparation of the protease demonstrated that Zn^{2+} was essential for activity. Leupeptin, phenylmethylsulfonyl fluoride, E-64, pepstatin A, dithiothreitol, and 2-mercaptoethanol had no effect on enzymatic activity. Addition of Ca^{2+} or Mg^{2+} to the incubation medium resulted in approximately a twofold stimulation of the azocaseinolytic activity of the enzyme. The protease was stably expressed since it was active even after repeated subculture of the bacterium. Bovine serum albumin, hide powder azure, and elastin-Congo red remained intact even after prolonged exposure to the enzyme. The surface expression of this metalloprotease activity raises the possibility that this enzyme may be involved in the proteolysis of a variety of host proteins in vivo and thereby contributes to gastric pathology.**

Nineteen species of the genus *Helicobacter* have been identified to date; of these, *Helicobacter pylori* is the most important member. *H. pylori* is a gram-negative multiflagellated unipolar spiral bacterium found almost exclusively on gastric biopsy in approximately 30 to 35% of the population less than 40 years old, with increasing incidence thereafter (20). The organism is responsible for chronic gastritis in humans and is associated with peptic ulcer disease. A causal role has also been proposed for gastric carcinoma in man (23) and primary B-cell mucosa-associated lymphoid tissue lymphoma of the stomach (12).

H. pylori expresses a number of surface-exposed proteins which are thought to be involved in colonization, adhesion, and virulence, including vacuolating cytotoxin (6, 17) and flagellin (7). *H. pylori* gastritis is characterized by protracted inflammatory cell response, abnormal gastric physiology, and gastric mucosal cell injury. The mechanism of pathogenesis has yet to be clarified. However, it is likely that a number of these surface-associated proteins are responsible either directly or indirectly for some of the pathological lesions observed.

Microbial extracellular proteases have been demonstrated or proposed to play important roles as virulence factors in the pathogenesis of a variety of diseases (11). Identification, characterization, and purification of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases. We have identified and partially characterized a zinc-dependent, calcium-stabilized metalloprotease activity from *H. pylori* which is both expressed on the surface of the bacterium and released in a soluble form. The potential role of this protease as a virulence determinant in the pathogenesis of *H. pylori* infection is considered.

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MATERIALS AND METHODS

Materials. All reagents were obtained from Calbiochem-Novabiochem Ltd. (Beeston, Nottingham, United Kingdom), Pierce (Rockford, Ill.), Aldrich Chemical Co. (Gillingham, Dorset, United Kingdom), Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom), and Pharmacia Biotech (Uppsala, Sweden). All buffer reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared in ultrapure deionized water (Elga Prima reverse osmosis; water quality, 1.2 μS/cm).

Western blotting (immunoblotting) and SDS-PAGE. Discontinuous SDS-PAGE was performed essentially as described by Laemmli (15). Proteins from SDS-PAGE gels (12.5% T) were electroblotted (0.8 mA/cm^2 for 1 h) onto a polyvinylidene difluoride membrane (Gelman) by use of a semidry blotting apparatus (LKB/Pharmacia), essentially as described by Towbin et al. (25). Biotinylated proteins were detected by incubating the washed membrane in a solution of ExtrAvidin peroxidase (diluted 1/3,000) in phosphate-buffered saline (PBS; pH 7.5) containing Tween 20 (0.05%, vol/vol) for 1 h at room temperature with gentle mixing. The avidin-biotin complexes were detected on washed membranes following their incubation (1 min) in a solution of iodophenol (400 μM), luminol (1.25 mM), and hydrogen peroxide (0.01%, vol/vol) in 0.1 M Tris-HCl (pH 8.8). Blots were exposed to Kodak X-OMAT S film for 10 to 30 s. Exposed films were developed in Kodak LX-24 developer and fixed in a Kodak dental X-ray fixer.

Protein measurements. Protein was measured by the method of Markwell et al. (19) with bovine serum albumin as the protein standard.

Bacterial strains and growth conditions. The clinical isolates of *H. pylori* used in this study were isolated from antral biopsies obtained from patients attending the gastroenterology clinic at St. James's Hospital, Dublin, Ireland. *H. pylori* was grown under microaerobic conditions for 4 days on 7% lysed horse blood agar at 37°C. Cells were harvested into ice-cold PBS (pH 7.5) or in 50 mM Tris-HCl (pH 8.0). Details of individual experimental conditions are given in the figure legends where appropriate. The cells were washed twice by centrifugation (10,000 × *g*, 5 min, 4°C) in the appropriate buffer before use. Alternatively, *H. pylori* was grown in broth medium containing brain heart infusion exactly as described previously (27).

Preparation of crude extract of protease. Crude extracts of the protease activity were obtained by three methods. First, agar-grown *H. pylori* (NCTC 11638) was suspended in PBS (pH 7.5) and subjected to sonication on ice with either a Dawe soniprobe (type 7532A) at 100 W for four 30-s bursts or a Branson sonifier 450. After centrifugation to remove intact cells and cellular debris

(12,000 × *g*, 10 min, 4°C), the resulting supernatant was passed through a 0.2-μm-pore-size filter (Gelman Sciences Acrodisc PF). This material was used as the whole-cell-associated protease activity. Second, weakly cell-associated or soluble protease activity was obtained by gently mixing a suspension of freshly harvested agar-grown cells in the appropriate buffer prior to centrifugation (10,000 × *g*, 10 min, 4°C) to remove whole cells. The resulting supernatant was sterile filtered as described above and stored at 4°C. Finally, proteolytic activity was recovered in the broth supernatant obtained from cells grown in liquid culture (see Fig. 3). This membrane vesicular fraction was taken as the soluble protease activity. The extract of proteolytic activity used in individual experiments is given in the figure legends.

Measurement of protease activity. Casein was chosen as a substrate to assay for protease activity since it is essentially a universal substrate for proteinases (2). It has an almost random three-dimensional structure, so there is little steric hindrance, and a wide range of both hydrophobic and hydrophilic sites incorporating most possible types of peptide bond (2). Proteolytic activity was assayed by the increase in trichloroacetic acid-soluble azopeptides produced upon incubation of *H. pylori* protease extracts with azocasein as described below. Protease activity was also determined with biotinylated casein as the substrate. All assays were conducted in either PBS (pH 7.5) or Tris-HCl (50 mM, pH 8.0) as indicated in the figure legends where appropriate. Azocasein (5 mg/ml) was dissolved in Tris-HCl (50 mM, pH 8.0) containing 0.04% NaN_3. Proteolytic activity could be assayed optimally in either PBS or Tris buffer (data not shown). A typical reaction mixture consisted of azocasein (100 μl) and protease extract (100 μl) in the presence or absence of inhibitors or activators. The incubation was performed at 37°C and terminated by the addition of 10% (wt/vol) trichloroacetic acid (400 μl). The precipitated protein was removed by centrifugation (12,000 × *g*, 4 min), and the resulting supernatant was transferred to a clean tube containing 700 μl of NaOH (525 mM). Absorbance was measured at 442 nm on a Perkin-Elmer SP8-100 spectrophotometer. Controls were either substrate and buffer minus enzyme or substrate plus heat-inactivated enzyme. The protease was inactivated completely by heating at 90°C for 10 min (data not shown). The time of incubation and the amount of enzyme varied from experiment to experiment but in all cases were such that the assays were linear with respect to each of these parameters. Routinely, $CaCl_2$ (5 mM), $MgCl_2$ (5 mM), and $ZnCl_2$ (0.1 mM) were included in the assay buffer since they were required for maximal activity.

Inhibition studies were carried out by adding various amounts of inhibitor to a solution of the protease extract and incubating the solution for 20 min. The reaction was initiated by the addition of substrate. Stock solutions of phenylmethylsulfonyl fluoride (100 mM) and 1,10-phenanthroline (100 mM) were prepared by dissolving them in the minimum amount of dimethyl sulfoxide and then diluting them in the appropriate buffer.

Gel filtration chromatography. A sonicate of *H. pylori* (NCTC 11638) was prepared as described above, and 0.5 ml (~10 mg of protein/ml) of the material was applied to a column (diameter, 1.5 cm; height, 29.7 cm) of Sephacryl S-300 superfine (Pharmacia) equilibrated with PBS (pH 7.5) containing NaN_3 (0.02%, wt/vol). The protein was eluted with this same buffer (8.5 cm/h), and the collected fractions were assayed for both protease activity and total protein.

Subcellular fractionation of *H. pylori*. Outer membrane protein fractions were prepared essentially as described by Blaser et al. (5). Briefly, cells were harvested in 50 mM Tris-HCl (pH 8.0) at 4°C and washed twice by centrifugation (10,000 × *g*, 10 min, 4°C). The pellet was resuspended in 50 mM Tris-HCl (pH 8.0) and sonicated on ice. Following centrifugation (5,000 × *g*, 20 min, 4°C), the supernatant was centrifuged for 1 h (100,000 × *g*, 4°C). The pellet was resuspended in water (2 ml) and added to 1% (wt/vol) sodium lauryl sarcosine (20 ml) in 7 mM EDTA (SLS-EDTA). After a 20-min incubation at 37°C, the mixture was centrifuged (100,000 × *g*, 2 h, 4°C) before the pellet was suspended in SLS-EDTA and recentrifuged as before. The insoluble pellet was used as the outer membrane protein preparation.

Biotinylation of casein. Casein (1 mg/ml) was biotinylated with sulfosuccinimidyl-6(biotinamido)-hexanoate (Calbiochem) in PBS (pH 7.5). Biotin ester was added to a final concentration of 1 mM and prepared immediately before use. After this combination was mixed for 1 h at room temperature, the labelling reaction was terminated by the addition of 1.5 M Tris-HCl (pH 8.0) to a final concentration of 10 mM. Unreacted biotin ester was removed from the reaction mixture by desalting on a 1-ml Sephadex G-25 column by use of the spin-chromatography technique described by Penefsky (24).

Determination of pH optimum. Azocasein was used as the substrate in assays to determine the pH optimum of the proteolytic activity. The pH was varied from 6.6 to 7.5 [0.1 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (TES buffer)] and from 7.5 to 8.5 (0.1 M Tris-HCl buffer).

RESULTS

Identification of protease activity. Preliminary experiments revealed the presence of EDTA-sensitive proteolytic activity in crude extracts of *H. pylori* which was capable of degrading azocasein (Fig. 1). The time course of azocasein hydrolysis remained linear for several hours and typically did not become markedly nonlinear until approximately 10 to 12 h of incubation had elapsed (data not shown). Subcellular distribution studies indicated that the protease activity was associated with the outer membrane fraction of the bacterium (Fig. 2). Furthermore, protease activity loosely associated with whole cells was recovered readily by gently resuspending the cells in either Tris buffer (Fig. 2) or PBS (data not shown). In addition, extracellular soluble caseinolytic activity was found in the culture fluid of *H. pylori* NCTC 11638 grown in broth medium

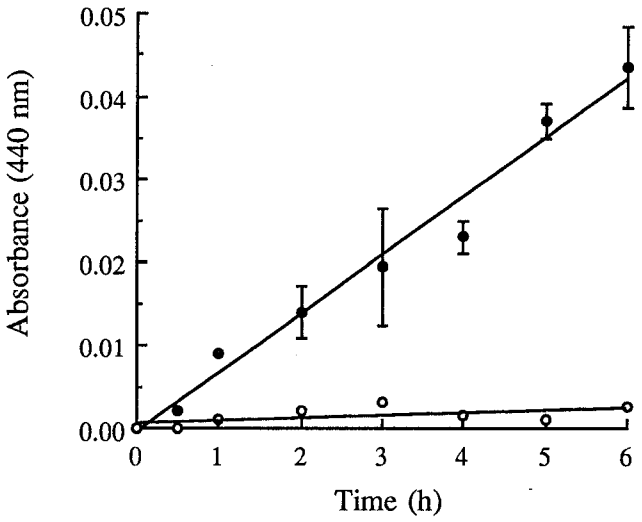

FIG. 1. Time course of protease activity. Protease activity was determined with azocasein as the substrate. The assay was performed exactly as described in Materials and Methods in Tris-HCl buffer (pH 7.5) supplemented with 5 mM EDTA (○) or in Tris-HCl buffer alone (●). The results are presented as the increase in absorbance at 442 nm due to azocaseinolysis versus time. Each time point represents the mean ± standard error (n = 3).

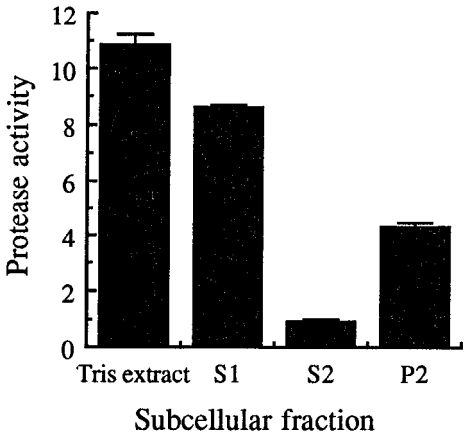

FIG. 2. Subcellular distribution of protease activity. Agar-grown *H. pylori* was fractionated as described in Materials and Methods into the following fractions: Tris extract, supernatant from cells harvested in 50 mM Tris-HCl (pH 8.0) buffer; S1, supernatant from sonicated cells; S2, sodium lauryl sarcosine-soluble material; P2, sodium lauryl sarcosine-insoluble material (outer membrane protein fraction). Assay of protease activity in the P2 fraction was performed as described in Materials and Methods with the exception that the buffer was supplemented with $CaCl_2$ (28 mM) to abrogate the inhibitory effect of EDTA on the protease activity present in this fraction. The results are expressed as the total amount of protease activity present in each of the four fractions. Protease activity was determined after 6 h of incubation at 37°C. The results are presented as the mean ± standard error of triplicate determinations.

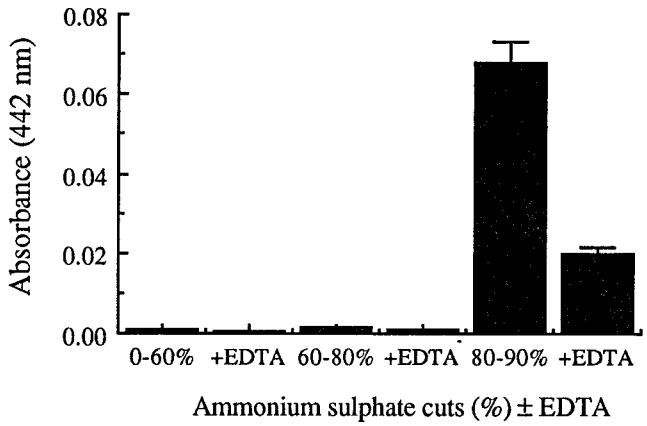

FIG. 3. Detection of protease activity in culture supernatant of *H. pylori*. *H. pylori* was grown in broth medium as described in Materials and Methods. The cells were harvested by centrifugation (10,000 × *g*, 15 min, 4°C), and the supernatant was carefully decanted and subjected to a second round of centrifugation before the addition of solid $(NH_4)_2SO_4$ to the supernatant to 60% saturation. After gentle stirring at 4°C for 1 h, the solution was centrifuged (3,000 × *g*, 40 min). Solid ammonium sulfate was added to the resulting supernatant to 80% saturation and stirred on ice for 2 h. The precipitated protein was collected by centrifugation (3,000 × *g*, 40 min). The resulting pellet was dissolved in 50 mM Tris-HCl (pH 8.0). Any residual insoluble material was removed by centrifugation (10,000 × *g*, 10 min; 4°C). The precipitated protein from each round of $(NH_4)_2SO_4$ additions was assayed for protease activity in the presence and absence of EDTA (5 mM). Results are shown as the mean ± standard error of triplicate determinations. The experiment was repeated three times; the results of one representative experiment are shown. The *x*-axis labels 0 to 60%, 60 to 80%, and 80 to 90% represent the percentages of $(NH_4)_2SO_4$ used.

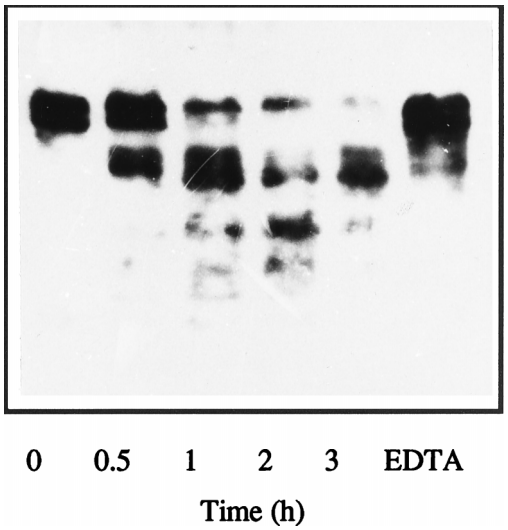

FIG. 4. Time course of protease-catalyzed degradation of biotinylated casein. Protease extract was incubated (37°C) for the times indicated with biotinylated casein in 50 mM Tris-HCl (pH 8.0) containing $MgCl_2$ (5 mM), $CaCl_2$ (5 mM), and $ZnCl_2$ (0.1 mM). The EDTA (5 mM)-treated sample was incubated for the duration of the experiment (3 h). The reaction was terminated by the addition of an equal volume of double-strength sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4.1% SDS, 2% 2-mercaptoethanol, and 0.001% bromophenol blue). Samples were then boiled (4 min) and subjected to SDS-PAGE (12.5%) followed by Western blotting, as described in Materials and Methods.

(Fig. 3). Control experiments demonstrated that there was no endogenous proteolytic activity in the broth medium (data not shown). In addition, any endogenous broth proteases would most likely have been rendered inactive since the medium was autoclaved prior to use. Furthermore, the protease activity appeared to be stably expressed since it was detectable in two reference strains (NCTC 11638 and CCUG 17874) after repeated subculture in addition to two clinical isolates (data not shown). The protease activity was of the same order of magnitude in all four strains.

In addition to the hydrolysis of azocasein, the protease activity degraded biotinylated casein. Complete hydrolysis of biotinylated casein was visualised by SDS-PAGE and Western blotting. Casein proteolysis resulted in the appearance of lower-molecular-weight biotinylated peptides with time (Fig. 4). This protease-catalyzed hydrolysis of casein was inhibited by EDTA. In control experiments, no degradation of biotinylated casein was observed when the substrate was incubated in buffer alone or in the presence of heat-inactivated enzyme throughout the duration of the experiment (data not shown). Biotinylated bovine serum albumin, hide powder azure, and elastin-Congo red were resistant to hydrolysis by this activity (data not shown).

Characterization of protease activity. The apparent native molecular mass of the *H. pylori* metalloprotease was estimated by size exclusion chromatography. The protease migrated as a single uniform peak, with an approximate molecular size of 200 kDa, when subjected to gel filtration over Sephacryl S-300 (Fig. 5). Similar results were found when gel filtration was performed with Sephadex G 200 (data not shown). These data indicate that *H. pylori* produces a single high-molecular-weight metalloprotease.

The optimum pH for activity of the protease was measured against azocasein in a series of buffers of different pH values from 6.6 to 8.5. Maximum protease activity was observed at pH 7.5 to 8.0. Protease activity declined rapidly below pH 7.5 and above pH 8.0 (Fig. 6). The minimum pH value tested was pH 6.6 since azocasein precipitates at a pH of <6.0. The reduced activity observed at high and low values of pH is not reversed by supplementing the enzyme preparation with additional Zn^{2+}, indicating that the decreased activity was unlikely to be due to dissociation of catalytically essential zinc from the enzyme.

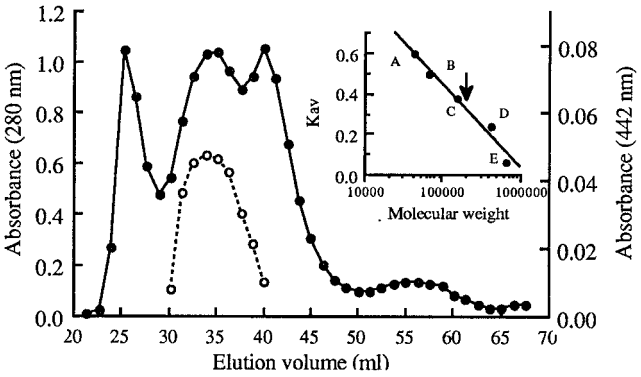

FIG. 5. Molecular weight estimation of protease by gel filtration chromatography. Gel filtration chromatography of the *H. pylori* sonicate was performed with a Sephacryl S-300 superfine column as described in Materials and Methods. Protease activity was assayed with azocasein as the substrate (○), and total protein was measured at 280 nm (●). The column was calibrated with the following protein standards (Pharmacia Biotech calibration kit): A, ovalbumin (M_r = 43,000); B, albumin (M_r = 67,000); C, aldolase (M_r = 158,000); D, ferritin (M_r = 440,000); E, thyroglobulin (M_r = 660,000). The inset shows the semilogarithmic calibration curve used to determine the molecular weight of the protease (arrow). K_{av} values for each protein were calculated by the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$. V_e, elution volume; V_o, void/exclusion volume; V_t, total bed volume.

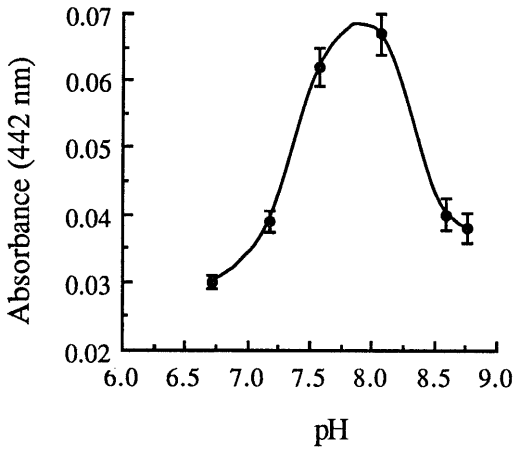

FIG. 6. Effect of pH on protease activity. Proteolysis of azocasein was assayed at different concentrations of H^+ as described in Materials and Methods. Each value represents the relative rate of hydrolysis of substrate averaged over the 5-h incubation period at 37°C. Rates were corrected for any nonenzymatic hydrolysis of the substrate at all values of pH. The results are shown as the mean value of quadruplicate determinations ± standard error.

The divalent metal ion requirements of the protease were examined. Desalting the crude protease after pretreatment of the enzyme with 5 mM EDTA to remove Zn^{2+} resulted in almost complete inactivation of the azocaseinolytic activity. This activity could be restored to near-control levels by titration with Zn^{2+} (Fig. 7A). Addition of high concentrations of Zn^{2+} (>1 mM) resulted in complete inhibition of the activity (data not shown). Protease activity was also stimulated by the addition of calcium and magnesium to the incubation buffer (Fig. 7B). Supplementing the assay buffer with Ca^{2+} did not enhance the thermostability of the activity (Fig. 7C).

The effects of several protease inhibitors and group-specific reagents on the protease-catalyzed hydrolysis of azocasein were tested by incubating each reagent with the enzyme preparation for 20 min prior to initiation of the assay. Inhibitors of serine (phenylmethylsulfonyl fluoride and leupeptin), cysteine (E-64 and leupeptin), and aspartic (pepstatin A) proteases did not inhibit the hydrolysis of azocasein (Table 1). In addition, the protease activity was insensitive to the reducing agents dithiothreitol and 2-mercaptoethanol, thus indicating that the protease did not require sulfhydryl residues for activity. However, the metal-chelating agents EDTA, 1,10-phenanthroline, pyridine-2,6-dicarboxylic acid, and phosphoramidon (Table 1) almost completely inhibited hydrolysis. Moreover, inhibition of the activity by 8-hydroxyquinoline-5-sulphonic acid (HQSA) was reversed by the addition of zinc (Fig. 8A), whereas the inhibition by EDTA was reversed by excess Ca^{2+} (Fig. 8B), thus indicating that the azocaseinolytic activity was due to a zinc metalloenzyme that requires calcium for full activity.

DISCUSSION

We have described the identification and preliminary characterization of protease activity from *H. pylori*. Protease activity was found to be associated with the outer membrane fraction of the cell. In addition, protease activity was recovered from agar-grown cells by washing the cells in buffer alone, thus indicating that the protease may be loosely bound to the surface of the cell. This observation was supported by the finding that the bacteria appear to secrete the protease when grown in

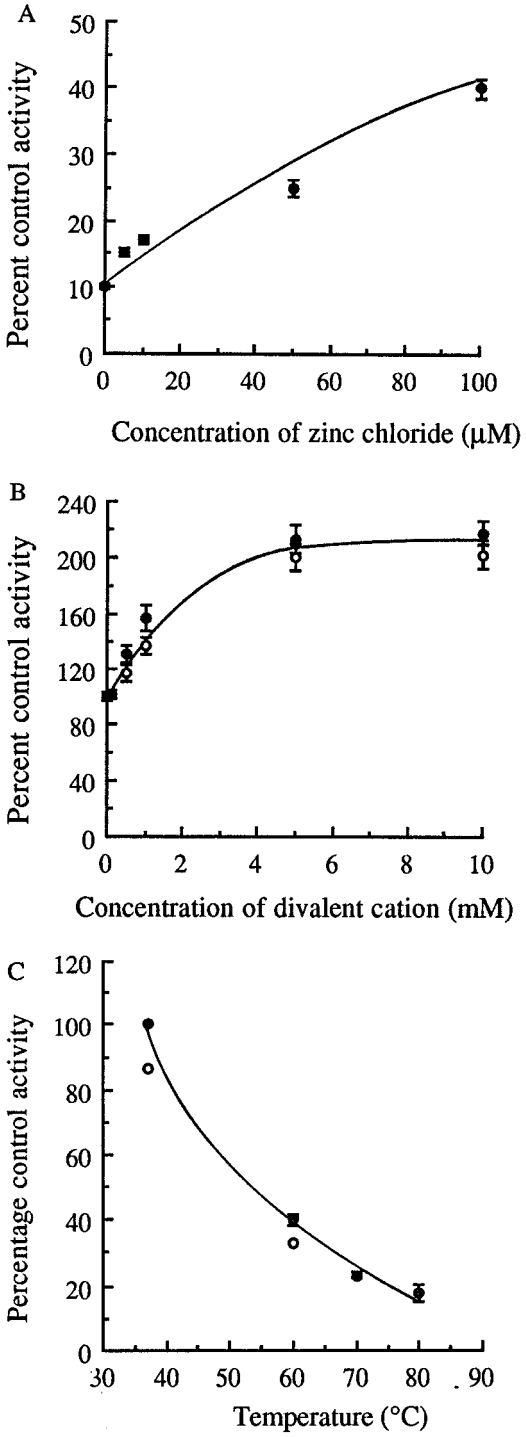

FIG. 7. Effect of divalent metal cations on protease activity. (A) Soluble protease activity was treated with EDTA (5 mM) and desalted on a 1-ml Sephadex G-25 column as described in Materials and Methods. The residual activity was titrated with zinc chloride (0 to 100 μM), and the protease activity was assayed as described in Materials and Methods. (B) Desalted soluble protease activity in Tris-HCl buffer was titrated with increasing concentrations of either $CaCl_2$ (●) or $MgCl_2$ (○) in the presence of $ZnCl_2$ (0.1 mM). (C) Crude extracts of protease activity were incubated in 50 mM Tris-HCl (pH 8.0) for 5 min at the indicated temperatures in either the absence (●) or presence (○) of Ca^{2+} (10 mM). Residual protease activity was assayed as described in Materials and Methods. The results are expressed as a percentage of control, untreated protease activity.

TABLE 1. Effects of protease inhibitors and group-specific reagents on protease activity

Addition[a]	Concn	Inhibition (%)[b]
PMSF	1.0 mM	1.40 ± 0.05
Pepstatin A	10.0 μM	0
E-64	1.0 μM	0
	10 μM	0
Leupeptin	10 μM	1.30 ± 0.06
	100 μM	1.60 ± 0.06
EDTA	0.1 mM	72.0 ± 2.64
	1.0 mM	97.0 ± 5.04
1,10-Phenanthroline	0.5 mM	84.1 ± 3.91
	10 mM	82.0 ± 3.18
Phosphoramidon	10 μM	76.0 ± 4.65
PDA	1 mM	82.0 ± 3.82
DTT	1 mM	0
	10 mM	0
2-Mercaptoethanol	1 mM	0
	10 mM	0

[a] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; PDA, pyridine-2,6-dicarboxylic acid; DTT, dithiothreitol.
[b] Values are means ± standard deviations ($n = 3$).

liquid culture as demonstrated by the appearance of activity in the broth supernatant.

Characterization of the protease with respect to inhibitor specificity revealed the protease to be a metalloenzyme. Chelators inhibit metalloenzyme catalysis either by removing the metal from the enzyme or by directly binding to it. The *H. pylori* metalloprotease activity was inhibited by both planar aromatic bidentate chelators (1,10-phenanthroline and HQSA) and polydentate carboxylic acid chelators (EDTA and pyridine-2,6-dicarboxylic acid), indicating that this activity is a metalloprotease with zinc as a cofactor. This was confirmed by removal of zinc from the enzyme followed by titration with zinc to restore activity. The protease was also susceptible to inhibition by phosphoramidon (Table 1). It is noteworthy that not all microbial zinc metalloproteases are inhibited by phosphoramidon (for examples, see references 1, 9, 18, and 26). 1,10-Phenanthroline displays a high binding constant for zinc and a weak one for calcium (13). For calcium-activated zinc peptidases, HQSA is useful since there is approximately a 10^{10} difference in the affinity of Ca^{2+} and Zn^{2+} towards HQSA. Chelation of the active-site zinc is most likely responsible for the inhibition by HQSA, since it is reversed by the addition of excess zinc but not by the addition of excess calcium ions. EDTA cannot distinguish between nonmetalloenzymes and metalloenzymes that are activated by calcium or magnesium since it binds tightly to Ca^{2+}, Mg^{2+}, and Zn^{2+}. Given the functional roles that have been described for Ca^{2+}, EDTA likely removes enzyme-bound Ca^{2+} ions that are critical to the tertiary structure of the protein. Addition of calcium and magnesium to the assay medium stimulated proteolytic activity in addition to stabilizing the activity during storage. However, the presence of calcium did not protect the activity from denaturation by heat treatment, as has been reported for several metalloenzymes.

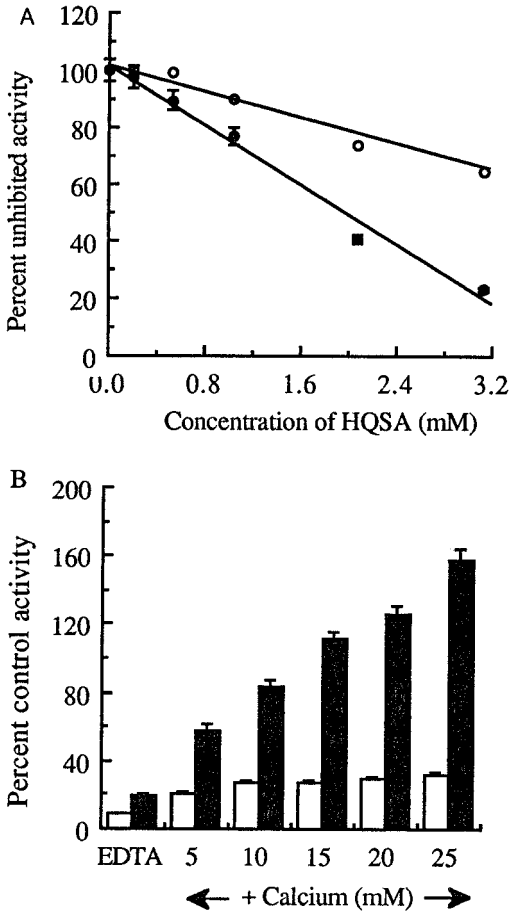

FIG. 8. Effect of various inhibitors on protease activity. Crude protease was obtained by gently washing freshly harvested cells in 50 mM Tris-HCl (pH 8.0) and then centrifugating them (12,000 × *g*, 10 min, 4°C). The resulting supernatant was used as the source of protease activity. (A) Inhibition of protease activity by HQSA in 50 mM Tris-HCl buffer (pH 8) containing 1 mM Ca^{2+} (●) or 1 mM Ca^{2+} plus 0.1 mM Zn^{2+} (○) is shown. (B) Soluble protease activity was treated with EDTA (5 mM) for 20 min prior to initiation of the protease assay in the presence of increasing concentrations of calcium and magnesium (open bars) or in the presence of increasing concentrations of Ca^{2+} and 100 μM zinc chloride (closed bars). The results are shown as the mean ± standard error of triplicate determinations. Where no error bars are shown, the error was smaller than the representation of the point.

Typically, the *H. pylori* protease activity was inhibited by elevated concentrations of zinc (>1 mM). Several zinc peptidases are known to be inhibited by excess zinc (16). The inhibition is competitive and occurs due to formation of zinc monohydroxide multidentate complexes which bridge the catalytic Zn^{2+} to a side chain in the active site. Most of the zinc-dependent metalloenzymes studied to date contain 1 g-atom of zinc per molecule as an essential component. The noncompetitive inhibition observed by heavy metal ions has been attributed to binding of the ion at a site distinct from the active site. The *H. pylori* protease activity was also inhibited by Cu^{2+} but not by Al^{3+} (data not shown).

An interesting and unusual feature of the *H. pylori* metalloprotease was its large apparent molecular size of approximately 200 kDa. The enzyme appears to be unique with respect to size when compared with other well-characterized metalloproteases from several species of gram-negative bacteria. Typically, the native molecular sizes of these enzymes are smaller by a factor of four to six times as shown by the following

examples: *Pseudomonas aeruginosa* elastase, 33 kDa (3); *Vibrio cholerae* hemagglutinin/protease, 32 kDa (10); *Vibrio anguillarum*, 36 kDa (21); *Legionella pneumophila*, 38 kDa (4); *Serratia* sp., 50 kDa (22); *Erwinia carotovora*, 38 kDa (14). In view of the fact that the *H. pylori* protease appears to be secreted in vitro, it is worth speculating that the large molecular mass of this protease may provide an element of protection for the active site of the enzyme from the acidic environment to which it would otherwise be exposed in vivo.

The conserved and ubiquitous nature of these metallopeptidases suggest that they are indispensable to the bacteria, some of which are clearly associated with virulence (11). Furthermore, a number of workers have demonstrated that vaccination with extracellular proteases elicits protection against challenge (for an example, see reference 8). It has been shown, too, that protease-deficient mutants are less virulent than wild-type strains. However, protease function can be correctly documented only by transformation of a vector with a protease gene or knockout of the same gene in an animal model of infection.

The role of the *H. pylori* metalloenzyme described in this present study and the identity of its substrates in vivo are not known. Whether the proteolytic activity performs a nutritional role by providing heme, amino acids, and small peptides to transporters of the bacterium or whether it plays a defensive role against host proteinases or immunoglobulins remains to be demonstrated. Further investigations are needed to identify and fully characterize the protease produced by *H. pylori*. Such information should prove useful for identifying pathogenic processes initiated by this protein. Current experiments are focused on purifying this metalloprotease and further characterizing its substrate specificity.

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