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Proteinases of *Proteus* spp.: Purification, Properties, and Detection in Urine of Infected Patients

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The proteinases secreted by pathogenic strains of *Proteus mirabilis*, *P. vulgaris* biotype 2, *P. vulgaris* biotype 3, and *P. penneri* were purified with almost 100% recovery by affinity chromatography on phenyl-Sepharose followed by anion-exchange chromatography. The proteinase purified from the urinary tract pathogen *P. mirabilis*, which we had previously shown to degrade immunoglobulins A and G, appeared as a composite of a single band and a double band (53 and 50 kDa, respectively) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The other *Proteus* proteinases had similar patterns but slightly different mobilities. In each case all proteinase activity in culture supernatants was demonstrated by gelatin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be associated with only the triple-band complex; all three bands were proteolytically active. The *P. mirabilis* proteinase was resistant to inhibitors of both serine and thiol proteinases but strongly inhibited by metal chelators, although it was not affected by phosphoramidon, an inhibitor of the thermolysin group of bacterial metalloproteinases. Active proteinase was detected in urine samples from *P. mirabilis*-infected patients; this is consistent with our detection of immunoglobulin A fragments of a size suggestive of *P. mirabilis* proteinase activity.

Proteus mirabilis is a common cause of urinary tract infection, particularly in young boys (3, 9, 12) and the elderly (26) and is, after *Escherichia coli*, the organism most frequently associated with urinary tract infections. Strains of *Proteus vulgaris* and *P. penneri* can also cause urinary tract infection (15) but are less frequently implicated, probably because of their lower carriage rate in feces (26, 32).

The virulence of *Proteus* spp. for the urinary tract arises through the interplay of several factors. Among these is the ability to grow rapidly in urine (27) and make it alkaline through the formation of potent urease isoenzymes (30), which degrade urea to ammonia. These conditions may result in damage and death to the renal tubular epithelium (5), inactivation of complement (2), and conditions favoring stone formation (8). Most *Proteus* strains form hemolysins (31), some of which are related to the known virulence factor of *E. coli*, alpha-hemolysin (14). *Proteus* hemolysin may permit the organism to invade tissue cells directly (24). Other properties of *P. mirabilis* that may be important in establishing ascending pyelonephritis include motility (23), the formation of certain proticines and/or proticine receptors (26), and the presence of certain fimbriae (35, 36), although the latter may not be an important factor in vivo in humans (25).

Recently we reported that *P. mirabilis* strains of diverse types (28) and some strains of other *Proteus* spp. (29) produced an EDTA-sensitive proteinase activity that cleaved the heavy chain of immunoglobulin A (IgA) outside the hinge region. Subsequently we showed that the enzyme differed from other classic microbial IgA1 proteinases by its ability to degrade the heavy chain of both serum and secretory IgA1, IgA2, and IgG isotypes, the secretory component, and a number of nonimmunological proteins such as gelatin and casein (17). Such a broad range of activity to these immunological defenses of the body suggests that *P. mirabilis* proteinase may be yet another important virulence factor for this organism. This view is supported by the results presented here; we show that these proteinases can be detected in the urine of patients with *P. mirabilis* infection of the urinary tract. A new method for isolating and purifying the proteinases and the properties of the purified enzymes are described.

MATERIALS AND METHODS

Bacteria. *P. mirabilis* 64676 and *P. penneri* 05665V were isolated from the urine of patients with urinary tract infection. *P. vulgaris* biogroup 2 strain 60694/78 was isolated from a groin abscess, and *P. vulgaris* biogroup 3 strain 02987W was isolated from the feces of a patient with diarrhea. The strains were identified by standard biochemical methods (32) and stored in purity on nutrient agar slopes at 4°C. *P. vulgaris* was typed according to fermentation of salicin and degradation of esculin: biogroup 2 strains were salicin positive and esculin positive; biogroup 3 strains were salicin negative and esculin negative.

Media. Nutrient broth (NB) (Oxoid CM 67; Oxoid Ltd., London, United Kingdom) was prepared and sterilized as directed by the manufacturer. Blood agar (BA) was Columbia agar base (Oxoid CM 331) supplemented when molten and cool with sterile horse blood (5%, vol/vol) (Oxoid SR 50).

Analysis of urine by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Urine specimens containing at voiding a significant concentration ($\geq 10^5$ CFU/ml for a midstream urine specimen; a lower number for a catheter specimen of urine was acceptable) of pure growth of a *Proteus* sp. were selected at random from those sent for routine bacteriological examination. Some specimens were received immediately after voiding; others were delayed. Upon receipt, the urine was clarified by centrifugation at 11,600 × *g* for 2 min. The clear supernatant was removed, supplemented with sodium azide to 0.1%, and stored at

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−20°C. It was then analyzed for proteinase activity on SDS-polyacrylamide-gelatin gels.

Kinetics of proteinase production. NB cultures of *P. mirabilis* 64676 and *P. vulgaris* 60694/78 that had been incubated statically overnight at 37°C were diluted 100-fold into NB and incubated with shaking at 37°C. At intervals, 500-μl culture samples were removed and centrifuged at 11,600 × *g* for 2 min at room temperature. A 75-μl sample of the clear supernatant was removed and added to 75 μl of sample buffer (0.125M Tris-HCl [pH 6.8] containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and a trace of bromophenol blue dye). The remainder of the supernatant was discarded, and the cell pellet was washed in saline and then resuspended to volume in saline. The washed cells of 75 μl of the suspension were lysed by adding 75 μl of sample buffer. The samples were stored at −20°C. They were subsequently analyzed for proteolytic activity on SDS-polyacrylamide-gelatin gels.

Preparation of *Proteus* proteinase for purification. Proteinases were prepared from cultures on solid medium and in liquid medium. For cultures on solid medium, a method adapted from that of Higerd et al. (11) was used. Briefly, 20 BA plates were overlaid with a sterile membrane of dialysis tubing and inoculated by using a swab with overnight 37°C nutrient broth cultures of each of the *Proteus* strains. After overnight incubation at 37°C, bacterial growth was scraped from the membranes with a microscope slide and suspended in 50 mM Tris-HCl (pH 8.0) containing 0.04% NaN_3. The membranes were then thoroughly washed in this buffer, and the washings were combined with the bacterial suspension. After centrifugation at 15,000 × *g* for 15 min at 4°C, the clear supernatant (100 ml) containing proteinase was removed and stored at −20°C. For cultures in liquid medium, 1 liter of NB was inoculated with 2 ml of an overnight 37°C NB culture of *P. mirabilis* 64676 and incubated with shaking for 24 h at 37°C. The culture was then centrifuged and stored as described above.

Purification of proteinases by phenyl-Sepharose affinity chromatography. The proteinase-containing supernatants were filtered through 0.45- and 0.22-μm-pore-size filters, and the filtrates were loaded at a rate of 1 ml/min at 4°C onto columns (12.5 by 2.2 cm for 100 ml of crude proteinase from solid medium; XK 50/30 FPLC [25 by 5 cm] for 1 liter of crude proteinase from liquid medium) of phenyl-Sepharose (Pharmacia) equilibrated in 50 mM Tris-HCl (pH 8.0).

Columns were then washed with 10 column volumes of 50 mM Tris-HCl buffer (pH 8.0). Bound proteinase was then eluted with 50 mM Tris buffer (pH 11). The pH of the fractions of eluted proteinase was adjusted to 8.0 with HCl. Purified proteinase, particularly that from NB cultures, contained a nonproteinaceous yellow impurity. This was removed and the proteinase was concentrated by anion-exchange chromatography on an HR5/5FPLC-Mono Q column equilibrated with 50 mM Tris-HCl (pH 8.0) to which a linear gradient of 0 to 0.5 M NaCl was applied. The pure proteinase eluted in the 0.25 to 0.35 M NaCl region of the gradient, whereas the colored impurity eluted with 0.4 to 0.5 M NaCl.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (16) with slab gels consisting of a stacking 3% acrylamide gel over a 5 to 20% acrylamide gradient resolving gel. Samples were boiled for 2 min with an equal volume of reducing sample buffer (0.1 M Tris-HCl [pH 8.0] containing 8 M urea, 2% SDS, 80 mM dithiothreitol, and 0.025% bromophenol blue). After electrophoresis at 35 mA until the dye front reached the bottom of the gel, gels were stained with Coomassie brilliant blue or silver (21). The molecular weights of proteins were estimated from their mobilities relative to those of the following standard proteins: rabbit muscle phosphorylase *b*, 97,400; bovine serum albumin, 66,200; hen egg white ovalbumin, 42,699; bovine carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and hen egg white lysozyme, 14,400 (all from Bio-Rad Laboratories, Richmond, Calif.).

SDS-polyacrylamide-gelatin gel electrophoresis. For demonstration of *Proteus* proteinase in culture supernatants, urine supernatants, or column fractions, samples were diluted with an equal volume of reducing sample buffer (0.125 M Tris-HCl [pH 6.8] containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and a trace of bromophenol blue dye) and applied to gels in which the resolving gel was 11% polyacrylamide containing 0.1% gelatin. After electrophoresis at 12 mA for 16 h, the gels were washed twice with 500 ml of 2.5% Triton X-100 in water at 4°C, each time for 1 h, to remove SDS. Gels were incubated in 50 mM Tris HCl (pH 8.0) buffer at 37°C for 4 h and then stained for 2 h in 0.5% Coomassie brilliant blue in 50% methanol–10% acetic acid in water at room temperature. The gels were then destained overnight with 10% methanol–10% acetic acid in water. The location of the proteinase was revealed as an unstained clear area of digested gelatin against a blue background of stained undigested gelatin.

Enzymes. Trypsin type XI from bovine pancreas, papain from papaya latex, and thermolysin proteinase type X were all from Sigma Chemical Co., St. Louis, Mo. These were prepared as 1-mg/ml solutions in, respectively, 50 mM Tris-HCl (pH 7.6), 100 mM sodium phosphate (pH 7.0) containing 10 mM cysteine and 2 mM EDTA, and 50 mM Tris-HCl (pH 7.5). For inhibition studies, 0.1-mg/ml solutions of trypsin, papain, and thermolysin were used.

Enzyme inhibitors. Di-isopropyl fluorophosphate was from Aldrich Chemical Co. Ltd., Gillingham, United Kingdom; phosphoramidon, dithiothreitol (DTT), L-cysteine, and iodoacetamide were from Sigma. The metal chelators EDTA, 1,10-phenanthroline, and 2,2′-dipyridyl were all from Sigma.

In studies on the effect of enzyme inhibitors, 10-μl samples of the above enzyme solutions or *P. mirabilis* proteinase (0.05 to 0.1 U per reaction in 50 mM Tris-HCl [pH 8] buffer) were incubated at 37°C with 65 μl of buffer containing different concentrations of inhibitors for 30 min. The remaining proteinase activity was measured with azocasein as the substrate. In some experiments, ^{125}I-labeled IgG or IgA was used as a substrate. The immunoglobulins were radiolabeled by using chloramine-T (7).

Azocaseinase assay of proteinase activity. Azocasein (50 μl) in water (5 mg/ml) was added to 75 μl of proteinase in 50 mM Tris-HCl (pH 8.0) buffer. After incubation at 37°C for an appropriate time, the reaction was terminated by the addition of 2 volumes (i.e., 250 μl) of 5% (wt/vol) trichloroacetic acid in water. After the sample was left standing for a few minutes, the unhydrolyzed azocasein precipitate was removed by centrifugation at 11,600 × *g* for 2 min. The clear supernatant was removed and added to 3 volumes (i.e., 375 μl) of 0.5 M NaOH, and the A_{440} was determined relative to that of a buffer control. One unit of proteinase was defined as the activity hydrolyzing 1 mg of azocasein in 1 h at a given pH and was calculated from the following equation: units of proteinase = $(A_{440} \times 60)/[1.6 \times \text{incubation time (minutes)}]$.

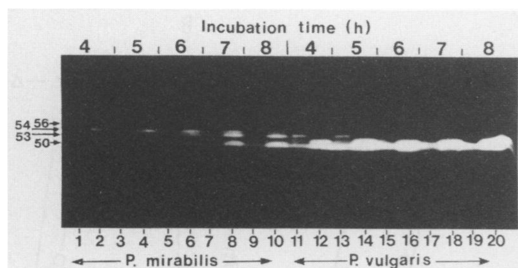

FIG. 1. Gelatin-SDS-PAGE analysis of proteinase production in NB cultures of *P. mirabilis* 64676 and *P. vulgaris* biogroup 2 strain 60694/78 growing at 37°C and sampled after 4, 5, 6, 7, and 8 h of incubation. Cell lysates of *P. mirabilis* (lanes 1, 3, 5, 7, and 9) or *P. vulgaris* (lanes 11, 13, 15, 17, and 19) and cell-free culture supernatants of *P. mirabilis* (lanes 2, 4, 6, 8, and 10) or *P. vulgaris* (lanes 12, 14, 16, 18, and 20) are shown. The relative molecular masses of the proteinase bands are indicated on the left in kilodaltons.

RESULTS

Kinetics of production of proteinase by *P. mirabilis*. Analysis of cell lysates and culture supernatants of *P. mirabilis* 64676 for proteinase activity after electrophoresis on SDS-polyacrylamide-gelatin gels is presented in Fig. 1. Proteinase activity was first detected after 4 h of growth at 37°C, when a 56-kDa proteinase was detected only in cell lysates and a 54-kDa proteinase was detected only in culture supernatants. Over the next 4 h, the internal proteinase continued to be detected only in cell lysates, whereas the secreted proteinase was eventually replaced by two bands of proteinase of 53 and 50 kDa. These were formed in increasing amounts over the next 40 h. A similar process appeared to occur during the synthesis of *P. vulgaris* 60694/78 proteinase (Fig. 1). Further prolonged incubation resulted in autodigestion of the secreted proteinase to smaller forms.

Purification of *Proteus* proteinases by affinity chromatography on phenyl-Sepharose. When proteinase preparations from overnight cultures of the *Proteus* strains were applied to columns of phenyl-Sepharose equilibrated in 50 mM Tris-HCl (pH 8.0), proteinase activity bound to the column. After thorough washing of the column, the bound proteinase was eluted with 50 mM Tris-HCl (pH 11.0). The pH of the eluted fractions was adjusted to pH 8; the fractions were analyzed by SDS-PAGE, and their proteinase activities were determined by the azocasein assay and by assay with ^{125}I-labeled IgA1 and IgG.

A typical result is presented in Fig. 2, which shows the phenyl-Sepharose affinity chromatography of a culture supernatant of *P. vulgaris* biotype 3. Most of the nonproteinase proteins were removed from the column at pH 8. Subsequent application of buffer at pH 11 eluted two peaks of UV-absorbing material. Both peaks had azocaseinase activity and IgA1- and IgG-degrading activity. The first eluting peak also contained a nonprotein yellow pigment devoid of proteinase activity. PAGE analysis of the peaks showed each to contain a double band of protein of about 50 kDa and a faint band of 53 kDa. The yellow pigment was not visible on SDS-PAGE. The proteinase activity was concentrated and freed from the contaminating nonprotein pigment by subsequent anion-exchange chromatography on an FPLC Mono Q column. When a salt gradient was applied, proteinase eluted at 0.25 to 0.35 M NaCl and the pigment eluted at 0.4 to 0.5 M NaCl.

Application of this method to proteinase preparations of all four *Proteus* strains enabled us to isolate each proteinase

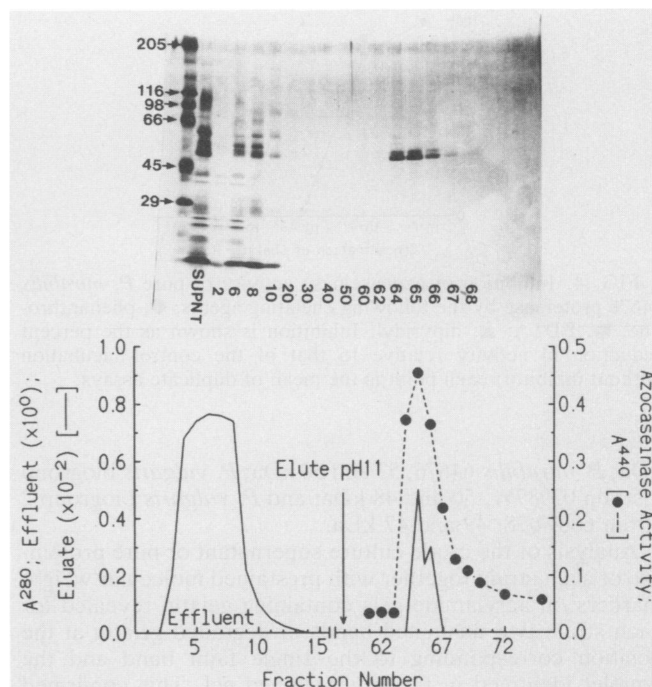

FIG. 2. Purification of *P. vulgaris* biogroup 3 strain 02987W proteinase from solid medium culture supernatants (supnt.) by affinity chromatography on a phenyl-Sepharose column (12.5 by 2.2 cm) equilibrated in 50 mM Tris-HCl (pH 8) and eluted with buffer at pH 11. The fraction volume was 15 ml. The protein elution profile, A_{280} (——), azocaseinase activity, A_{440} (- - -), and protein composition on SDS-PAGE (stained with silver) of the fractions across the column are shown. The positions of standard molecular mass markers (kilodaltons) are shown to the left of the gel.

in a pure form. Coomassie blue staining of the purified proteinases on SDS-PAGE showed each as a double band; those from *P. vulgaris* had a third, fainter, higher-molecular-weight band (Fig. 3A). With silver staining this higher-molecular-weight band was also visible in proteinases from *P. mirabilis* and *P. penneri*. The apparent molecular masses of the proteins were as follows: *P. penneri* 05665V, 49 and 48

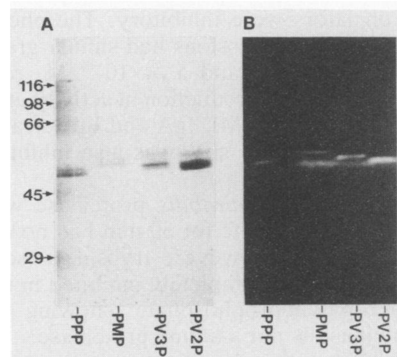

FIG. 3. Analysis of phenyl-Sepharose purified *Proteus* proteinases on SDS-PAGE gels stained with Coomassie blue (A) and proteinase activity in *Proteus* culture supernatants on gelatin-SDS-PAGE (B). PPP, *P. penneri* 05665V; PMP, *P. mirabilis* 64676; PV3P, *P. vulgaris* biogroup 3 strain 02987W; PV2P, *P. vulgaris* biogroup 2 strain 60694/78. The positions and masses (kilodaltons) of standard protein molecular weight markers are given on the left.

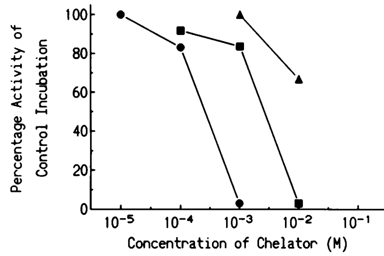

FIG. 4. Inhibition of azocaseinase activity of pure *P. mirabilis* 64676 proteinase by the following chelating agents: ●, phenanthroline; ■, EDTA; ▲, dipyridyl. Inhibition is shown as the percent reduction in activity relative to that of the control incubation without inhibitor; each point is the mean of duplicate assays.

kDa; *P. mirabilis* 64676, 53 and 50 kDa; *P. vulgaris* biogroup 3 strain 02987W, 50 and 48 kDa; and *P. vulgaris* biogroup 2 strain 60694/78, 49 and 47 kDa.

Analysis of the crude culture supernatant or pure proteinase of each strain together with prestained molecular weight markers on acrylamide gels containing gelatin revealed for each strain two unstained bands of degraded gelatin at the position corresponding to the single faint band and the doublet identified in the silver-stained gel. This confirmed that all proteolytic activity was confined to the proteins purified on phenyl-Sepharose (Fig. 3B).

Physicochemical properties of *Proteus* proteinases. (i) Stability. Both crude and pure *Proteus* proteinases were stable for long periods at 4°C in 50 mM Tris-HCl (pH 8.0) buffer containing 0.04% sodium azide. When stored at −20°C they lost a little activity over several months. The proteinases were unaffected by heating at 60°C for 5 min.

(ii) Effect of pH on activity. The azocaseinase activity of pure *P. mirabilis* proteinase was assayed in buffers over a range of pH 3 to 10. Enzyme activity was detectable over a broad range of from pH 6 to 10. The pH for optimum activity was 8. Enzymes purified from each strain showed similar kinetics of cleavage of azocasein with similar final values for release of azotyrosine. This suggested that the enzymes have similar specificities, consistent with our earlier studies on their cleavage of IgA (29).

(iii) Effect of enzyme inhibitors. The effects of the chelating agents EDTA, 1-10 phenanthroline, and dipyridyl on the activity of pure *P. mirabilis* proteinase are presented in Fig. 4. All three chelators were inhibitory. The phenanthroline and EDTA inhibition regressions had similar gradients with pK_I values of 5×10^{-4} and 5×10^{-3} M, respectively. Dipyridyl gave only a 33% reduction in activity at the highest concentration tested (10 mM). IgA and IgG cleavage by the proteinases of the *Proteus* spp. was also inhibited with 10 mM EDTA (data not shown).

Incubation of pure *P. mirabilis* proteinase with 10 mM di-isopropyl fluorophosphate for 30 min had no effect on its azocaseinase activity. However, trypsin (a serine group proteinase) activity was completely inhibited in this time by 1 mM di-isopropyl fluorophosphate, showing that the *P. mirabilis* proteinase is not a serine proteinase.

The activity of pure *P. mirabilis* proteinase was unaffected over 30 min by iodoacetamide (100 mM), an inhibitor of thiol enzymes. However, papain, a typical thiol proteinase, was completely inhibited within 30 min with 100 μM iodoacetamide.

The effects of the thiol compounds DTT and cysteine on the activity of pure *P. mirabilis* proteinase are presented in

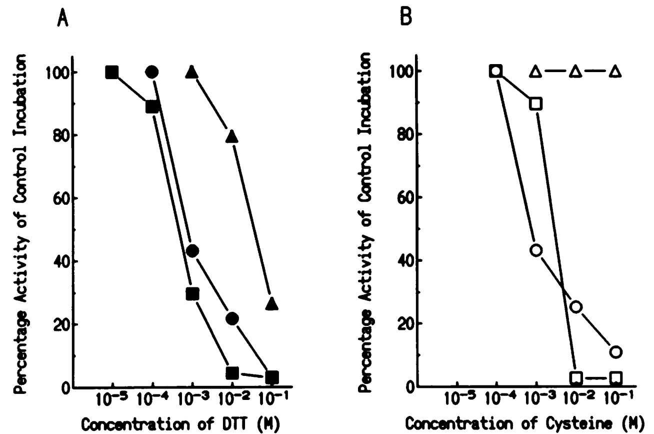

FIG. 5. Comparison of inhibition of azocaseinase activity by thiol compounds DTT (A) and cysteine (B) of pure *P. mirabilis* 64676 proteinase (○), thermolysin (□), and papain (△). Inhibition is shown as the percent reduction in activity relative to that of the control incubation; each point is the mean of duplicate assays.

Fig. 5. Both were strong inhibitors. DTT at 1 mM gave 50% inhibition of the activities of *P. mirabilis* proteinase and thermolysin, whereas 50% inhibition of papain required 100 mM DTT. Cysteine gave pK_I values for *P. mirabilis* proteinase and thermolysin of 10^{-3} and 10^{-2} M, respectively; papain was not inhibited by cysteine at 100 mM. It seems probable that the inhibition of activity of both the *Proteus* enzyme and thermolysin by these thiol compounds is the effect of their chelating properties rather than their ability to reduce protein disulfide bonds.

Phosphoramidon, an inhibitor of some metalloproteinases, had no affect at 1 mM for 30 min on the activity of pure *P. mirabilis* proteinase. However, thermolysin activity was completely inhibited in this time by 100 μM phosphoramidon.

Taken together, these results indicate that *P. mirabilis* proteinase belongs to a novel metalloproteinase class in that it is inhibited by chelating agents but not by phosphoramidon.

Analysis of urine samples. Examination of 17 urine samples from *Proteus*-infected individuals of both sexes (6 to 86 years old) revealed that all were infected by strains of *P. mirabilis*. Analysis of these cell-free urine samples on polyacrylamide-gelatin gels along with diluted purified *P. mirabilis* 64676 proteinase is presented in Fig. 6. With the excep-

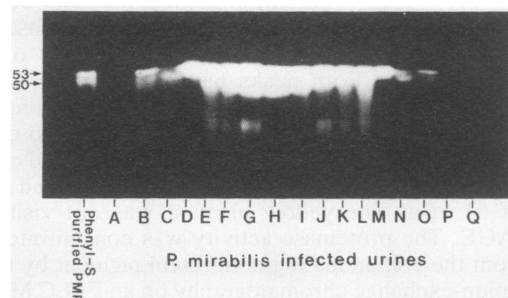

FIG. 6. Comparison of proteinase activity in urine samples (A through Q) from patients with *P. mirabilis* infections of the urinary tract with that of proteinase purified from *P. mirabilis* culture supernatants (Phenyl-S purified PMP). The receipt of urine samples E through L was delayed.

tion of one specimen (a catheter specimen) that contained little or no proteinase activity, all contained detectable proteinase activity that electrophoresed at the same position as that of pure *P. mirabilis* proteinase. This indicated that proteinase was produced and was active in vivo. Some specimens whose receipt was delayed showed evidence of proteinase autodigestion. Lower-molecular-weight bands with proteinase activity were detected on SDS-PAGE. These bands comigrated on immunoblots with a monospecific antibody raised against the enzyme purified from strain 64676 (data not shown).

DISCUSSION

A few bacteria like *Neisseria meningitidis*, *N. gonorrhoeae*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, which are associated with diseases at mucosal surfaces, and some periodontal pathogens are thought to be pathogenic because, unlike most other microorganisms and the nonpathogenic species of these genera, they are able to produce proteinases that cleave the heavy chain of IgA1 (for reviews, see references 13 and 34). *P. mirabilis* strains of diverse types (28) and some strains of other *Proteus* spp. (29) also produce a proteinase that degrades the heavy chain of both serum and secretory forms of IgA1, IgA2, and IgG (17). Since the enzymes have this wide range of activity against immunoglobulins, it is possible that they are important virulence factors in *Proteus* spp.

Attempts by earlier workers (10) to purify *P. mirabilis* proteinase gave only low yields of the enzyme. Although our previously described methods (17) of purifying *P. mirabilis* proteinase gave substantially higher yields, the method was time-consuming and laborious. We have exploited our discovery of the affinity of *Proteus* proteinases for phenyl groups by using chromatography on phenyl-Sepharose to purify the proteinases of strains of different *Proteus* species with speed and ease to give yields amounting to a virtual total recovery. The fractions from the affinity column with proteolytic activity contained almost pure proteinase. The contaminant, a nonproteinaceous pigment, was readily removed by ion-exchange chromatography, although its presence appeared to have no influence on the activity of the proteinase. Phenyl-Sepharose is usually used with buffers of high ionic strength (e.g., 2 M ammonium sulfate) for hydrophobic interaction chromatography. The remarkable selectivity of this resin for the proteinase in buffers of low ionic strength and the dependence on pH but not on ionic strength suggest that the resin is acting as an affinity column rather than a conventional hydrophobic resin. Preliminary results suggest that this technique will be applicable to the proteinases secreted by a number of other bacterial species with similar specificities, such as those from *Bacillus thermoproteolyticus* (thermolysin) and *Aeromonas* spp.

The pure proteinases of the different *Proteus* spp. appeared to constitute a family of related but distinct molecules, each having a double and a single protein band of about 50 kDa as determined by SDS-PAGE and polyacrylamide-gelatin gel electrophoresis. All bands showed the same inhibition with EDTA, and all inhibitor studies were consistent with the presence of only one type of proteolytic activity. Attempts to resolve the individual proteins from each species by a number of chromatographic procedures were unsuccessful. No separation was ever observed in fractions eluted from the phenyl-Sepharose column. Furthermore, preliminary studies suggest that proteins are synthesized and secreted simultaneously and are antigenically related (27a). The nature of the difference between the isoforms of the enzyme remains to be elucidated. Gel filtration of the preparation showed that each isoform ran with an apparent molecular weight lower than that suggested from SDS-PAGE. For example, estimation of the size of *P. mirabilis* pure proteinase by gel filtration on an FPLC-Sepharose 6 column calibrated with proteins of known molecular weight gave the proteinase a size of only 35 kDa (data not shown). This anomaly is difficult to explain. It might be that the enzyme has a weak affinity for the column resin and that this retards its elution from the column. However, the data do suggest strongly that the different bands represent independent proteins and not the subunits of a dimeric protein.

Study of the effect of inhibitors shows quite conclusively that the proteinases are metalloenzymes. In their sensitivity to metal chelators and reducing agents, the *Proteus* enzymes behave in a manner similar to that of the archetypal bacterial metalloproteinase, thermolysin, from *B. thermoproteolyticus*. However, unlike thermolysin, they were unaffected by phosphoramidon, an inhibitor of most documented bacterial metalloproteinases, and thus they appear to be novel metalloproteinases.

The *Proteus* proteinases seem to be similar in some respects to the metalloproteinases of *Pseudomonas aeruginosa* and *Serratia marcescens*, both of which degrade IgA and IgG (6, 19, 22), although that of *P. aeruginosa* is phosphoramidon sensitive (20). However, these enzymes cleave IgA and IgG at the hinge region, whereas *P. mirabilis* proteinase cleaves IgG at either side of the disulfide band in the hinge region of IgG but outside the hinge region in IgA (17). The *P. mirabilis* proteinase therefore appears to be unique among this group of metalloproteinases in having a more restricted specificity. It appears to cleave IgA in a manner similar to that of pepsin. The affinity of *P. mirabilis* proteinase for phenyl groups suggests that its cleavage of IgA might involve phenylalanine residues, possibly Phe-443 at the end of the CH_3 domain and Phe-345 at the junction between the CH_2 and CH_3 domains. Both of these phenylalanines are exposed on the surface of the molecule; Phe-345 is unique to IgA, being replaced by Arg and His in IgG and IgM, respectively.

The finding in the urine of patients with *P. mirabilis* infection of the urinary tract of fragments of the heavy chain of IgA of a size indistinguishable from those produced when pure *P. mirabilis* proteinase cleaves pure IgA indicates indirectly that the proteinase is both formed and active in vivo (33). The proteinases that make up the characteristic electrophoretic pattern recognized in the purified enzyme were found in all urine samples that had proteolytic activity. The activity of the enzyme in urine is not surprising, because urine from patients infected with *P. mirabilis* is usually alkaline through the action of urease. This condition would permit the proteinase, whose pH optimum was 8 (Fig. 7), to act efficiently.

Both IgA and IgG, substrates for *P. mirabilis* proteinase, are known to be present in urine in a ratio of about 1:3 (4), although their relative amounts in urinary tract secretions are not known. Complement is not present in secretions in large amounts, and therefore it is probable that the major defense mechanism against infection of the urinary tract is antibody-mediated opsonization of bacteria and their eventual disposal by the binding of the Fc part of antibody to receptors on phagocytic cells. It is now known that both IgA and IgG can be opsonic (1), and the Fc receptor for IgA has been purified from human polymorphs (18). Moreover, se-

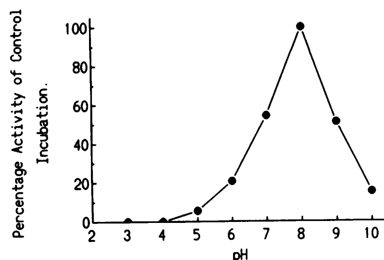

FIG. 7. The effect of pH on the azocaseinase activity of the purified proteinase. Azocaseinase activity was determined as described in Materials and Methods, except that the following buffers were used: pH 3 and 4, 50 mM sodium acetate; pH 5 and 6, 50 mM morpholineethanesulfonic acid; pH 7, 8, and 9, 50 mM Tris-HCl; and pH 10, 50 mM sodium bicarbonate. Optimum activity of *P. mirabilis* proteinase at pH 8 indicates that the alkaline conditions in infected urine would permit the proteinase to act efficiently.

rum and secretory forms of IgA have been found to be as efficient as IgG in opsonization of antigen-antibody complexes (37). The production and activity of *P. mirabilis* proteinase in vivo in the urinary tract might lead to the generation of ineffective fragments of opsonic antibodies. Preliminary studies suggest that these $F(abc')_2$, $F(ab')_2$, or Fab fragments, although capable of binding epitopes on the bacterial surface, are unable, through loss of their Fc portion, to trigger phagocytosis. It is likely that they will block binding of antibody with intact Fc regions. Phenyl-Sepharose affinity chromatography now provides a convenient method of preparing large amounts of pure *P. mirabilis* proteinase with which to examine its properties. Studies are under way to examine the effect of the proteinase on complement proteins, host proteinase inhibitors, and cell surface and secreted glycoproteins of the urinary tract and to assess the effect of proteinase on complement activation and opsonization effector functions of specific antibodies of IgA and IgG isotypes.

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