A Proteolytic Enzyme Secreted by *Proteus mirabilis* Degrades Immunoglobulins of the Immunoglobulin A1 (IgA1), IgA2, and IgG Isotypes

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Proteus mirabilis strains associated with human urinary tract infections have previously been shown to secrete an extracellular metalloproteinase which cleaves serum immunoglobulin A (IgA). The enzyme has now been purified to apparent homogeneity from culture supernatants of *P. mirabilis* 64676. The protease activity is associated with a 50-kilodalton (kDa) protein. Unlike that of the classic IgA1 proteases, the substrate specificity of the *P. mirabilis* protease has been found to extend to both subclasses of IgA, IgG, and the nonimmunoglobulin substrates, secretory component and casein. Cleavage of IgA1 and IgA2 by the *P. mirabilis* protease yielded fragments on sodium dodecyl sulfate-polyacrylamide gel electrophoresis whose sizes were consistent with a cleavage site outside the hinge region. Both secretory IgA1 and IgA2 were also cleaved by *P. mirabilis* protease, although the secretory IgA2 molecule was less readily cleaved than secretory IgA1. Free and IgA-bound secretory components were degraded to some extent by *P. mirabilis* protease. Cleavage of IgG, however, occurred at the hinge region as a two-stage process. The first stage was pepsinlike and generated an F(ab')$_2$ fragment of 120 kDa and a small pFc fragment detected on nonreduced polyacrylamide gels. In the second stage, the F(ab')$_2$ product was cleaved to yield papainlike Fab and Fc fragments, visualized as a diffuse band of 40 to 50 kDa.

Immunoglobulin A (IgA), in the form of secretory IgA (sIgA), is the predominant immunoglobulin in mucous secretions, where it protects membranes and underlying tissue from microorganisms and their products. sIgA is a dimer of IgA held together by the J chain and associated with the secretory component, a large fragment of the receptor which is used to transport the immunoglobulin across the mucosal surface (18). sIgA is remarkably resistant to degradation by most proteolytic enzymes. Human serum IgA differs from sIgA by being predominantly monomeric and lacking the secretory component. In serum, the ratio of IgA1 to IgA2 is approximately 9:1, whereas in secretions the ratio of the subclasses varies from 9:1 in nasopharyngeal secretions to approximately equal proportions in others (10). Although the function of serum IgA remains unclear, it is believed to play a role in the regulation of the immune response and in the removal of dietary antigens from the circulation. Recent evidence suggests that it can also be opsonic for yeasts and bacteria (6, 29), acting through a specific IgA receptor which has been purified from human polymorphonuclear leukocytes (1).

Of the vast numbers of microorganisms examined, only a few strains have been found to produce proteolytic enzymes capable of degrading IgA. These include bacterial pathogens of mucosal surfaces such as *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (reviewed in reference 13). Since other related but nonpathogenic species of the same bacterial genera do not produce IgA proteases, the production of these enzymes may be associated with the virulence of the organisms. The IgA proteases of these bacteria are distinguished from other proteases by their extremely narrow substrate specificity, which is restricted to the IgA1 subclass

of immunoglobulins from humans, chimpanzees, and gorillas (14). The enzymes cleave the heavy chain of IgA1 at Pro-Ser or Pro-Thr bonds within a 13-amino-acid, proline-rich segment in the hinge region. This segment is not present in IgA2, which is thereby resistant.

Recently, we reported that *Proteus mirabilis*, a common pathogen of the urinary tract, also produces a proteolytic enzyme able to cleave IgA. The enzyme, which was EDTA sensitive, differed from the other IgA proteases in that it appeared to cleave the IgA heavy chain outside the hinge region (27). In this study, using the purified *P. mirabilis* enzyme, we confirm and extend these results, showing that this novel enzyme cleaves both subclasses of IgA and also IgG. Its potential role as a virulence factor in urinary tract infections is discussed.

MATERIALS AND METHODS

Bacteria. *P. mirabilis* 64676 was isolated from the urine of a patient with a chronic urinary tract infection. The strain was identified by standard biochemical methods and stored on nutrient agar slopes at 4°C. The strains of *H. influenzae* and *N. gonorrhoeae* were originally isolated from clinical specimens but had been maintained for several months by repeated subculture on cooked blood ("chocolate") agar and modified New York City medium, respectively.

Media. Nutrient broth (Oxoid CM 67; Oxoid Ltd., London, United Kingdom), nutrient agar (Oxoid CM 3), and blood agar composed of Columbia agar base (Oxoid CM 331) supplemented with sterile horse blood (Oxoid SR 50; 5% [vol/vol]) were prepared and sterilized according to the instructions of the manufacturer. Cooked blood agar was blood agar heated until the medium turned brown. Modified New York City medium was prepared by the method of Young (30) but with horse blood (Oxoid SR 50).

Preparation of crude microbial proteases. The procedure

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for obtaining crude preparations of these enzymes, adapted from the method of Higerd et al. (9), has been described previously (27). In brief, sterile dialysis tubing on the surface of blood agar, cooked blood agar, or modified New York City medium plates was inoculated by a swab with overnight cultures (37°C) of *P. mirabilis* from nutrient broth, *H. influenzae* from cooked blood agar, or *N. gonorrhoeae* from modified New York City medium plates, respectively. The plates were incubated overnight at 37°C in the presence of 8% CO_2. The bacteria were scraped from the membranes with a glass slide and suspended in 50 mM Tris hydrochloride (pH 8.0) containing 0.04% NaN_3. The membranes were then thoroughly washed in this buffer, and the washings were added to the bacterial suspension. After centrifugation to remove the bacteria, the supernatant containing extracellular protease was removed and stored at −20°C.

Purification of *P. mirabilis* protease. Unless stated otherwise, all buffers contained 0.04% sodium azide. Ammonium sulfate was added to a 30% (wt/vol) concentration to the protease-containing supernatants from 150 blood agar plates. After being gently stirred at 4°C overnight, the precipitated proteins were collected by centrifugation at 6,500 × g for 15 min at 4°C and redissolved in 5 ml of 50 mM Tris hydrochloride–100 mM NaCl (pH 8.0). The sample was then applied to a Sephacryl S-200 gel filtration column (80 by 2 cm) and eluted in the same buffer. The broad peak of protease, as detected by its activity on azocasein, was pooled and then diluted 1:5 with 50 mM Tris hydrochloride (pH 8.0). The sample was applied to an HR5/5 fast protein liquid chromatography (FPLC)-Mono Q anion-exchange column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris hydrochloride (pH 8.0), and the proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 50 mM Tris hydrochloride (pH 8.0). The protease, which eluted as a sharp peak in the 0.2 to 0.3 M NaCl region of the gradient, was pooled, concentrated by ultrafiltration, and then diluted with an equal volume of 20 mM Tris phosphate buffer (pH 6.9) containing 30% (wt/vol) sucrose and a trace of bromophenol blue. The sample was then electrophoresed on nondenaturing gels, each comprising a stacking gel of 3% (20% cross-linker) acrylamide in 40 mM Tris phosphate (pH 6.9) over a resolving gel of 10% (5% cross-linker) acrylamide in 0.235 M Tris hydrochloride (pH 8.48). Electrophoresis was carried out at 25 mA with an upper-tank buffer of 37.6 mM Tris–40 mM glycine (pH 8.89) and a lower-tank buffer of 63 mM Tris hydrochloride (pH 7.47).

The gels were cut into 0.5-cm horizontal strips which were then eluted with 1 ml of 50 mM Tris hydrochloride (pH 8.0) at 4°C for 24 h. Those gel slices having protease enzyme activity were eluted with a second sample of buffer. The enzyme preparation was checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and then concentrated 20-fold by ultrafiltration before storage at −20°C.

Preparation of immunoglobulins from serum and colostrum. IgA1, IgA2, and IgG were purified from 250 ml of fresh, pooled normal human serum. After clarification of the serum by filtration through glass wool and centrifugation at 2,500 × g for 5 min, solid ammonium sulfate was added to 50% saturation. After being stirred for 3 h at 4°C, the precipitated proteins were collected by centrifugation at 23,000 × g for 1 h at 4°C. The precipitates were redissolved in distilled water and then applied to a Sepharose 6B gel filtration column (80 by 5 cm) which was eluted with 20 mM Tris hydrochloride (pH 8.0). The fractions containing IgA and IgG, as detected by radial immunodiffusion, were pooled

and further purified by affinity chromatography on a column (7.5 by 1.0 cm) of Jacalin-Sepharose (Vector Laboratories) equilibrated in phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$. The run-through from the Jacalin-Sepharose column which contained IgA2 and IgG was chromatographed on a column of DEAE-Sepharose (10 by 4.4 cm) equilibrated with 20 mM Tris hydrochloride (pH 8.0). IgG was collected from the run-through of this column, whereas IgA2 was eluted from the column in the 0.1 to 0.15 M region of a linear gradient of 0 to 0.5 M NaCl in 20 mM Tris hydrochloride (pH 8.0). The IgA1 which bound to the Jacalin-Sepharose was eluted with 0.8 M D-galactose and further purified on a column of DEAE-Sepharose as described for IgA2. The IgA2 was further purified by passage through protein A-Sepharose to remove contaminating IgG and then by cation-exchange chromatography on an HR5/5 FPLC-Mono S column equilibrated with 50 mM sodium acetate (pH 4.5). Bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl. Most of the IgA2 eluted as a sharp peak between 0.03 and 0.05 M NaCl; the remainder eluted as a broad peak between 0.15 and 0.30 M NaCl.

The identity of both serum and secretory IgA1 and IgA2 isotypes was confirmed by immunodot blotting with the following human isotype-specific mouse monoclonal antibodies: 2D7 (anti-A1 and -A2), NIF2 (anti-A1), and 2E2 (anti-A2), kindly provided by R. Jefferis, University of Birmingham, Birmingham, United Kingdom. The identity of the serum IgA2 was further confirmed by an anti-IgA2 radial immunodiffusion (RID) assay kit (The Binding Site, Birmingham, United Kingdom) and also by its resistance to cleavage by the IgA1 protease from *H. influenzae*. Serum IgM was a human polyclonal commercial preparation (Calbiochem-Behring, La Jolla, Calif.).

sIgA1 and sIgA2 and free secretory component (SC) were purified from colostrum. Twenty milliliters of colostrum from a single donor was mixed with 10 ml of isotonic saline (0.9% [wt/vol] NaCl) and centrifuged at 100,000 × g for 1 h at 4°C. The clarified colostrum between the surface layer of fat and the pellet of cell debris was removed, and the pH was lowered to 4.0 with HCl to precipitate casein. After centrifugation at 30,000 × g for 30 min at 4°C, the supernatant was neutralized to pH 7.0 with 2 M Tris, recentrifuged, and passed through a 0.22-µm-pore-size filter (Millipore Corp., Bedford, Mass.). The sample was then subjected to gel filtration on a column (100 by 5 cm) of Sepharose 6B equilibrated in 50 mM Tris hydrochloride (pH 8.0). The IgA- and secretory component-containing fractions were subsequently chromatographed to purity by anion-exchange chromatography on an HR5/5 FPLC-Mono Q column eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM Tris hydrochloride (pH 8.0). The sIgA1 and sIgA2 were resolved by affinity chromatography on Jacalin-Sepharose and characterized as described above. The identity of the secretory component was confirmed by double radial immunodiffusion with a goat antiserum (ICN Biomedicals, High Wycombe, United Kingdom).

The pure immunoglobulins were radiolabeled with ^{125}I by the chloramine T method of Greenwood et al. (7).

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (15) with slab gels consisting of a 5 to 20% acrylamide gradient resolving gel and a 3% acrylamide stacking gel. Reduced samples were boiled with an equal volume of 0.1 M Tris–8 M urea–2% SDS–80 mM dithiothreitol–0.025% (wt/vol) bromophenol blue for 1 min, and then iodoacetamide was added to a final concentration of 80 mM. Unreduced samples were boiled in 0.1 M Tris–8 M urea–2%

SDS–40 mM iodoacetamide–0.025% (wt/vol) bromophenol blue for 1 min. After electrophoresis at 35 mA until the dye front reached the bottom of the gel, the gels were stained with Coomassie brilliant blue or silver (21). The molecular weights of proteins were estimated from their mobilities relative to the 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 (Bio-Rad Laboratories, Richmond, Calif.).

SDS-polyacrylamide gels for autoradiography were dried under a vacuum onto filter paper and exposed at −40°C to Cronex 7 film (Du Pont Co., Wilmington, Del.) in a cassette with double intensifying screens.

Protease enzyme assays. (i) Azocaseinase assay. Seventy-five microliters of enzyme in 50 mM Tris hydrochloride (pH 8.0) was incubated with 50 μl of azocasein (5 mg/ml) for an appropriate period (4 to 48 h) at 37°C. The reaction was then terminated by the addition of 250 μl of 5% trichloroacetic acid, and after a few minutes the unhydrolyzed azocasein precipitate was removed by centrifugation at 11,600 × *g* for 2 min. The supernatant was removed and added to 375 μl of 0.5 M NaOH, and the amount of hydrolyzed azocasein was determined from the A_{440}.

(ii) IgA protease assay. Ten microliters of ^{125}I-IgA1 (10 μg of IgA1 per ml of PBS–0.3 M D-galactose; 10,000 cpm) was incubated with 50 μl of protease in 50 mM Tris hydrochloride (pH 8.0) or 50 mM Tris hydrochloride–5 mM EDTA (pH 8.0) as a control for an appropriate time (4 to 48 h) at 37°C. The reaction was then terminated by the addition of 60 μl of SDS gel sample buffer and by boiling the mixture for 1 min. Digests were analyzed by SDS-PAGE and autoradiography.

For assays with other immunoglobulin isotypes as substrates, the equivalent volumes of radioiodinated protein giving 10,000 cpm were 15 μl of sIgA1 (30 μg/ml of PBS), 20 μl of sIgA2 (40 μg/ml of PBS), 25 μl of secretory component (20 μg/ml of PBS), 3 μl of IgG (20 μg/ml of PBS), and 25 μl of IgM (100 μg/ml of PBS).

Protease digestion of IgG. Pepsin from hog stomach (Koch-Light Laboratories) and papain (Sigma Chemical Co., St. Louis, Mo.) were incubated as 1-mg/ml solutions at 37°C for 30 min in the appropriate buffers (0.1 M sodium acetate [pH 4.5] for pepsin and 0.1 M sodium phosphate [pH 7.0] containing 0.01 M cysteine and 2 mM EDTA for papain); *P. mirabilis* protease in 50 mM Tris hydrochloride (pH 8.0) was purified from culture supernatants as described above. For IgG digestion studies, 3.5 μl of radioiodinated IgG (10^5 cpm) was added to 50-μl volumes of pepsin (1 μg/ml), papain (10 μg/ml), and *P. mirabilis* protease and incubated for 24, 16, or 48 h, respectively, at 37°C. The IgG cleavage products were resolved by electrophoresis on SDS-polyacrylamide gels and visualized by autoradiography.

RESULTS

Purification of the *P. mirabilis* 64676 protease. During the purification of the enzyme, protease activity was monitored both by measuring azocaseinase activity and by the hydrolysis of ^{125}I-IgA. Secreted proteins from *P. mirabilis* were first precipitated with ammonium sulfate. The precipitate was then applied to a Sephacryl S-200 gel filtration column. The enzyme eluted as a broad peak followed by a second, smaller peak. Both enzyme peaks eluted after the bulk of the A_{280} absorbing material (Fig. 1). From a comparison on SDS-PAGE of the molecular masses of the proteins in the active fractions with those of the standards, it appeared that
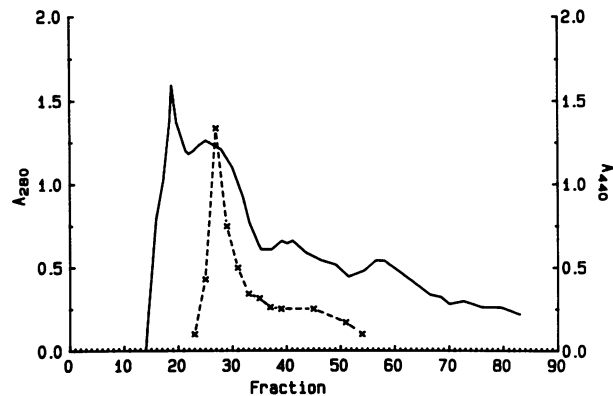

FIG. 1. Sephacryl S-200 gel filtration of proteins precipitated from a *P. mirabilis* culture supernatant with 30% (wt/vol) ammonium sulfate. ——, Protein elution profile (A_{280}); – – –, azocaseinase activity (A_{440}).

the two protease peaks on gel filtration corresponded to proteins of around 50 and 40 kilodaltons (kDa).

The active fractions of the peaks were combined into two pools, and each was chromatographed on an FPLC-Mono Q column. In each case, protease eluted as a sharp peak in the middle of the salt gradient from 0 to 0.5 M NaCl (Fig. 2). SDS-PAGE analysis of the most active fractions at this stage of the purification procedure revealed several bands. The peak activity in each pool was associated with a protein
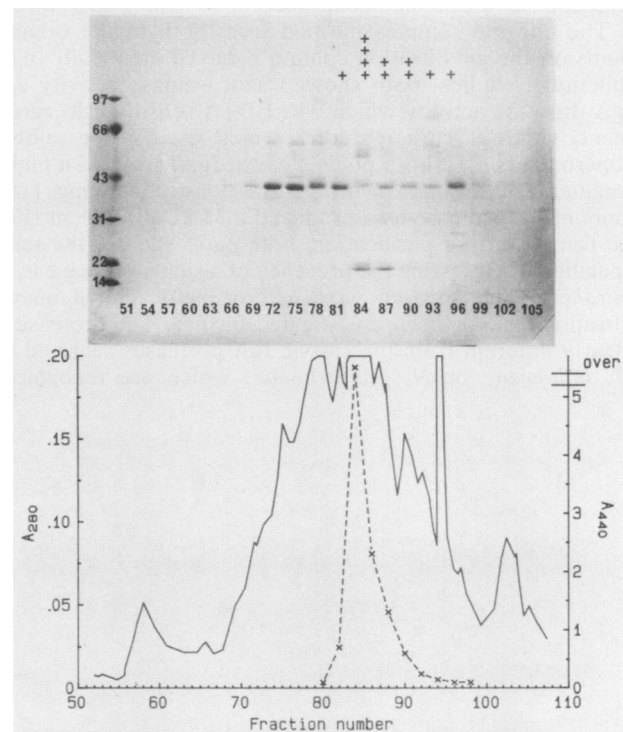

FIG. 2. FPLC-Mono Q anion-exchange column of Sephacryl S-200 column fractions 23 to 35. ——, Protein elution profile (A_{280}); – – –, azocaseinase activity (A_{440}). The photograph shows a Coomassie blue-stained SDS-PAGE gel of the column fractions numbered across the bottom of the gel; azocaseinase-active fractions are denoted on a scale of 1+ to 4+. Molecular mass markers are given on the left in kilodaltons.
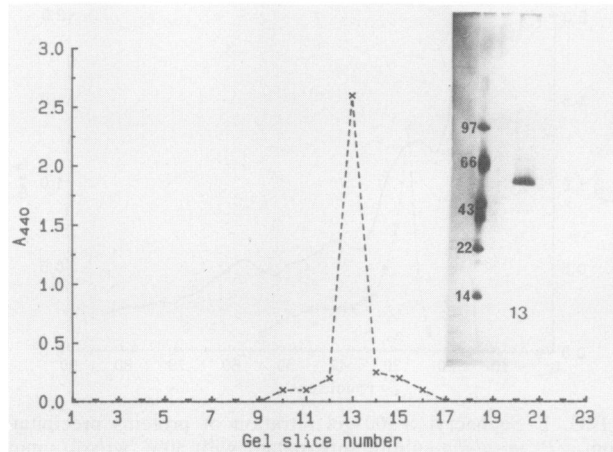
FIG. 3. Nondenaturing PAGE of proteins found in a pool of active fractions from a Mono Q column. The azocaseinase activity profile (A_{440}; – – –) of 0.5-cm gel slices in descending order towards the dye front is shown. The photograph shows silver-stained SDS-PAGE of gel slice 13, the slice containing the highest level of azocaseinase activity, together with molecular mass markers (in kilodaltons).

FIG. 5. Autoradiograph of an SDS-PAGE gel run under reducing conditions, showing ^{125}I-IgA hydrolyzed by crude protease preparations from *H. influenzae* (HI), *P. mirabilis* (PM), and *N. gonorrhoeae* (NG). From the left: lane 1, IgA standard; lanes 2 to 7, protease-digested IgA (duplicate samples). H, Heavy chain; L, light chain. Molecular mass markers are given on the left in kilodaltons.

appearing as a doublet corresponding to a protein of 50 kDa. Further purification by nondenaturing PAGE confirmed the 50-kDa doublet to be the protease, with enzyme activity being detected only in gel slices containing this protein (Fig. 3 and 4). The final enzyme preparation eluted from these gels was seen on SDS gels stained with silver or Coomassie blue as a doublet with a molecular mass of 50 kDa (Fig. 3, inset).

The enzyme samples purified from both of the original pools off the gel filtration column behaved identically in all functional studies; both showed azocaseinase activity and IgA protease activity which was EDTA sensitive. In agreement with our earlier studies which used crude culture supernatants (27), both pools cleaved IgA1 to yield a major fragment of around 47 kDa. Fractions containing large amounts of protease also produced a 34-kDa fragment (Fig. 4). During further purification, both pools showed the same specificity, suggesting the presence of a single protease in *P. mirabilis*. The apparent size heterogeneity shown on gel filtration has not been investigated further. The protease is clearly different from the classic IgA proteases secreted by *H. influenzae* or *N. gonorrhoeae*, which are recognized
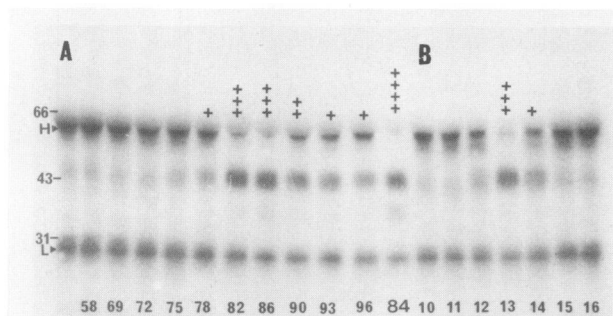
hinge-cleaving enzymes. Our results suggest that the *Proteus* enzyme cleaves outside the hinge region (Fig. 5).

Analysis of the cleavage of immunoglobulins of different isotypes by the purified *P. mirabilis* protease. Radioiodinated immunoglobulins of different isotypes were digested with the purified *P. mirabilis* protease for 48 h at 37°C, and their cleavage products were analyzed by SDS-PAGE under reducing conditions (Fig. 6). When the products were visualized by autoradiography, the protease was seen to cleave the heavy chains of serum and secretory IgA1, IgA2, and IgG but not their light chains (Fig. 6, lanes 2, 5, 8, 11, and 17). Wherever cleavage occurred, it could be inhibited substantially by the addition of 5 mM EDTA. sIgA1 and sIgA2 were cleaved to similar fragments. The heavy chain of serum IgA1 was cleaved to yield a major fragment of about 47 kDa and a minor fragment of about 34 kDa, while the heavy chain of IgA2 yielded fragments of 47 and about 31 kDa. sIgA1 was cleaved to yield fragments of sizes similar to those observed for serum IgA1. Although sIgA2 was more resistant to proteolysis than either serum IgA2 or sIgA1 (Fig. 6, lanes 7 to 12), the protease caused a clear diminution of the amount of heavy chain and produced fragments of 47 and 31 kDa which were faintly visible. The yield of the detected fragments from sIgA was consistently lower than that from

FIG. 4. Autoradiograph of an SDS-PAGE gel run under reducing conditions, showing ^{125}I-IgA hydrolysis by FPLC-Mono Q column fractions (A) and nondenaturing PAGE slices derived from Mono Q column fraction 84 (B). Azocaseinase-active fractions are denoted on a scale of 1+ to 4+. H, Heavy chain of IgA; L, light chain of IgA. Molecular mass markers are given on the left in kilodaltons.
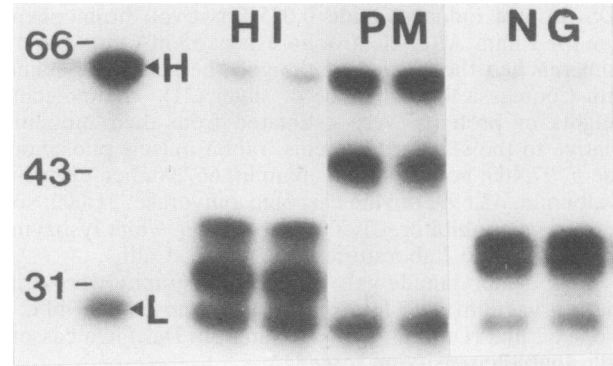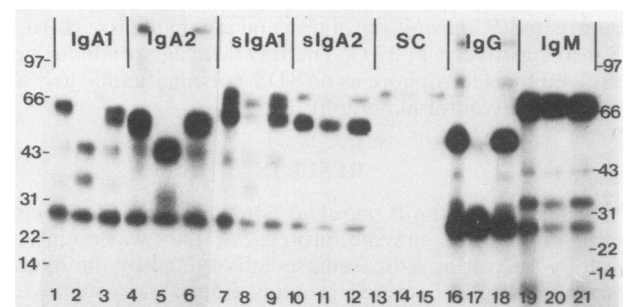

FIG. 6. Autoradiograph of an SDS-PAGE gel run under reducing conditions, showing ^{125}I-labeled immunoglobulins after incubation at 37°C in the presence of buffer alone (lanes 1, 4, 7, 10, 13, 16, and 19), purified protease from *P. mirabilis* in buffer (lanes 2, 5, 8, 11, 14, 17, and 20), or purified protease in buffer containing 5 mM EDTA (lanes 3, 6, 9, 12, 15, 18, and 21). SC, Secretory component. Molecular mass markers are indicated in kilodaltons.
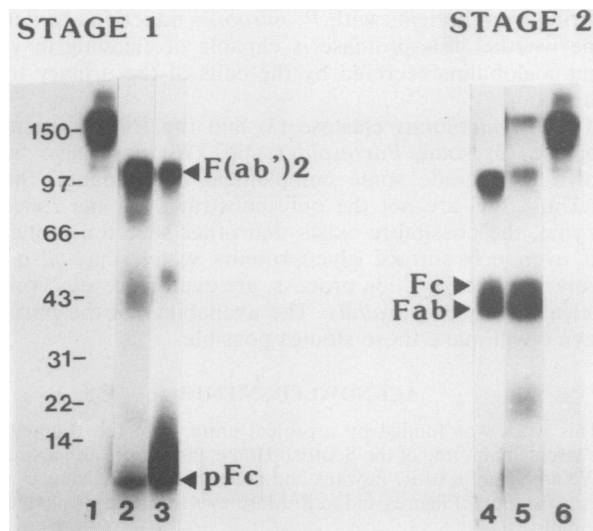
FIG. 7. Autoradiograph of an SDS-PAGE gel run under nonreducing conditions, showing ^{125}I-IgG digested with pepsin, papain, or P. mirabilis protease. Lanes 1 and 6, IgG standard; lanes 2 to 5, IgG digested with pepsin (lane 2), P. mirabilis protease diluted 1:20 (lane 3), undiluted P. mirabilis protease (lane 4), or papain (lane 5). Molecular mass markers are indicated on the left in kilodaltons.

the corresponding serum IgA. Free secretory component (lanes 13 to 15) and bound secretory component in sIgA1 or sIgA2 (lanes 7 to 12) appeared to be degraded slowly by the protease, but the breakdown products were not seen on SDS-PAGE. The purified protease cleaved the heavy chain of IgG to yield a 31-kDa fragment which was visible on SDS-PAGE directly above the light chain (lanes 16 to 18); IgM was not cleaved (lanes 19 to 21).

Comparison of the products of IgG cleaved with P. mirabilis protease with those generated by pepsin and papain. The products of digestion of purified human IgG by P. mirabilis protease were compared with those generated by pepsin and papain by using SDS-PAGE under nonreducing conditions (Fig. 7). As expected, pepsin cleaved IgG behind the hinge region, generating a 100-kDa F(ab')$_2$ fragment and a small pFc fragment (Fig. 7, lane 2), whereas papain (lane 5) cleaved the IgG in front of the hinge region, producing two Fab fragments and an Fc fragment of similar size (50 kDa). Digestion of IgG with the P. mirabilis protease generated both an F(ab')$_2$ fragment of 100 kDa and Fab fragments visualized as single diffuse bands of 40 to 50 kDa (lane 4).

Repeat experiments with dilutions of the enzyme indicated that these cleavage products were produced by a two-stage process; the first stage was a pepsinlike cleavage which produced the larger F(ab')$_2$ fragment and a small pFc fragment (represented in Fig. 7, lane 3, by the 1:20 dilution of the enzyme), and the second stage was a papainlike cleavage during which the F(ab')$_2$ fragment was further broken down to two Fab fragments (represented by the undiluted enzyme; Fig. 7, lane 4). These observations strongly suggest that, in contrast to its action on IgA, the Proteus protease cleaves IgG in the hinge region on either side of the disulfide bond. When analyzed on nonreducing SDS-PAGE, IgA was seen to be cleaved by the protease to a single fragment of approximately 130 kDa, reminiscent of the F(abc')$_2$ fragment produced by pepsin cleavage (17).

DISCUSSION

Previous studies from our laboratories have shown that many strains of P. mirabilis and other Proteus species from a variety of clinical sources secrete an EDTA-sensitive protease which cleaves IgA (27, 28). We have now purified an extracellular proteolytic enzyme from culture supernatants of a strain of P. mirabilis isolated from a patient with a chronic urinary tract infection. The enzyme, which has a polypeptide chain with an apparent molecular mass of 50 kDa, appears to account for all of the IgA-cleaving activity previously identified in crude extracts of the same species. We have demonstrated the enzyme to have broad substrate specificity, cleaving not only the two isotypes of IgA but also IgG and a limited number of nonimmunoglobulin proteins such as secretory component, casein, and bovine serum albumin (results not shown). The enzyme also accounts for all of the azocaseinase activity detectable in secretions from the same bacterium, although the properties of this enzyme appear to be different from those of the protease of P. mirabilis reported by Milazzo and Delisle (19), which hydrolyzed myeloma IgA to small fragments yet was unable to hydrolyze colostral IgA, IgG, or albumin.

Although the classical IgA proteases produced by mucosal pathogens such as N. gonorrhoeae, H. influenzae, and S. pneumoniae also belong to the metal chelator-sensitive class of microbial proteinases, these enzymes are characterized by their exquisite substrate specificity for IgA1 of humans and higher primates, which they cleave to yield intact Fab and Fc fragments. The Proteus enzyme also appears to cleave IgA in a limited manner; however, the much broader substrate specificity suggests that the enzyme should be classified separately from the IgA proteases.

The classic IgA proteases cleave IgA at specific sites in a 13-amino-acid proline-rich sequence in the hinge region of the α-1 heavy chain at peptide bonds at which proline contributes the carboxyl group and serine or threonine contributes the amino group (24). This amino acid sequence is absent in IgA2, which is therefore resistant to these enzymes. The present results, which used purified P. mirabilis enzyme, confirm and extend our previous observations (obtained with crude enzyme) that the products of cleavage of IgA with the protease from P. mirabilis 64676 were of different sizes from those produced by the classic IgA proteases and of sizes indicative of cleavage outside the hinge region (27). From our studies with radiolabeled IgA, we believe that the P. mirabilis protease probably cleaves the α chain mainly between the C_H2 and C_H3 domains to produce the 47-kDa fragment with extensive breakdown of the released C_H3 domain. The cleavage of both IgA1 and IgA2 by the enzyme to yield fragments of similar sizes is consistent with this interpretation.

Only one other IgA protease has been shown to cleave both IgA1 and IgA2. This protease is secreted by a strain of Clostridium ramosum associated with ulcerative colitis (4, 5). This enzyme cleaves a Pro-Val bond found in the α chains of IgA1 and the IgA2m(1) allotype but not in IgA2m(2).

Among the human immunoglobulin isotypes tested so far, both serum and secretory IgA1, IgA2, and IgG and free and IgA-bound secretory component have been found to be susceptible substrates for the Proteus enzyme; IgM was apparently resistant. Other bacterial pathogens shown to produce proteolytic enzymes capable of degrading IgG as well as IgA include some species of the periodontal pathogens Bacteroides and Capnocytophaga (11, 22). Although certain strains of Bacteroides gingivalis and Bacteroides

intermedius show extensive degradation of these immuno-globulins, the remaining *Bacteroides* and *Capnocytophaga* species cause specific cleavage of IgA1 but not IgA2 while cleaving IgG in the hinge region. *Pseudomonas aeruginosa* has been shown to produce two proteolytic enzymes: an EDTA-resistant alkaline protease with a molecular mass of 57 kDa and a pH optimum of 9 to 10 and an EDTA-sensitive elastase of 39 kDa with a pH optimum of 8 to 9 (23). The alkaline protease degrades serum IgA slowly, yielding products of 50 and 40 kDa, whereas sIgA and sIgG are resistant. The elastase rapidly degrades serum IgA, sIgA, its associated secretory component, and IgG (3). Although the 50-kDa *Proteus* enzyme appears to share properties of both *Pseudomonas* enzymes, it more closely resembles the elastase enzyme in its cleavage properties.

In contrast to that seen with IgA (27), cleavage of the IgG molecule by the *Proteus* protease was a two-stage process. The first cleavage yielded an F(ab')$_2$ product similar to that produced by pepsin, whereas the second cleavage generated a papainlike Fab fragment. This suggests that the cleavage sites for the *Proteus* enzyme lie on either side of the disulfide bond in the hinge region of the IgG molecule. Since only the pepsinlike cleavage product was observed with diluted *Proteus* enzyme, it is likely that the first stage of the cleavage process occurs quickly while the second stage takes much longer, being dependent on the generation of the first cleavage product to unfold the molecule in some way to reveal the second cleavage site. A similar process has been reported for the cleavage of IgG by *Pseudomonas* elastase (3).

The *Proteus* protease we have isolated appears to be the same as that reported briefly in earlier work by Hampson et al. (8). This enzyme cleaved oxidized B chain of insulin between amino acids with acidic or large hydrophobic side chains in a manner similar to that of the *P. aeruginosa* enzymes (20). Insulin B chain is not cleaved by the classic microbial IgA proteases from *Streptococcus sanguis* or *N. gonorrhoeae* (14).

The highly specific cleavage of both IgG and IgA molecules by the *Proteus* enzyme could make it a valuable immunochemical reagent for investigation of the functions of individual domains of the different immunoglobulin isotypes.

The role of the *P. mirabilis* protease as a virulence factor in urinary tract infections is uncertain. Clearly, the wide range of activity of the enzyme on a number of different immunoglobulin types could be to the advantage of the organism. The relative amounts of specific antibody isotypes in urinary tract secretions are unknown. However, it is likely that for both IgA and IgG, Fab fragments which bind to the epitopes on the bacterial surface could protect the bacterial cell from the immune system by blocking access of intact antibody molecules, resulting in the loss of such effector functions as complement fixation, opsonization, and cell binding.

The presence of Fab and Fc fragments and detectable IgA protease activity in secretions of patients infected with IgA protease-producing bacteria (2, 11, 16, 24) suggests that these proteases are produced and are active in vivo. Although the *Proteus* enzyme appears to be less active on serum IgA1 than the classical IgA proteases of *H. influenzae* and *N. gonorrhoeae* are, it appears to be more active on sIgA. Senda et al. (*in* T. T. MacDonald and S. J. Challacombe, ed., *Advances in Mucosal Immunology*, in press) have shown that fragments of IgA of similar sizes to those generated by the *Proteus* enzyme are produced in the gut, where *Proteus* organisms are regularly present. We intend to search for similar signs of immunoglobulin protease activity

in urine from patients with *P. mirabilis* infections to determine whether this protease is capable of cleaving in vivo immunoglobulins secreted by the cells of the urinary tract epithelium.

The *Pseudomonas* elastase (3) and the EDTA-sensitive proteases of some *Bacteroides* spp. (26) have also been shown to degrade some complement components. Since IgA1 and IgG are not the only substrates for the *Proteus* enzyme, the possibility exists that other secreted proteins, and even cell surface glycoproteins which may also be involved in the infection process, are even more susceptible to cleavage by *P. mirabilis*. The availability of the purified enzyme will make these studies possible.

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