Secreted Proteases from *Actinobacillus pleuropneumoniae* Serotype 1 Degrade Porcine Gelatin, Hemoglobin and Immunoglobulin A

Erasmo Negrete-Abascal, Victor R. Tenorio, José J. Serrano, Claudia Garcia C. and Mireya de la Garza

ABSTRACT

It was found that 48 hour cultures of *Actinobacillus pleuropneumoniae* secreted proteases into the medium. Electrophoresis in polyacrylamide gels (10%) copolymerized with porcine gelatin (0.1%), of the 70% $(NH_4)_2SO_4$ precipitate from the culture supernatants, displayed protease activities of different molecular weights: >200, 200, 90, 80, 70 and 50 kDa. They had activity over a broad range of pHs (4–8), with an optimal pH of 6–7. All were inhibited by 10 m*M* EDTA, and reactived by 10 m*M* calcium. They were stable at −20°C for more than a month. The proteases also degraded porcine IgA and porcine, human, and bovine hemoglobin, although they appeared to be less active against the hemoglobins. The IgA was totally cleaved in 48 h, using supernatants concentrated with polyvinyl pyrrolidone or the 70% $(NH_4)_2SO_4$. Extracellular proteases could play a role in virulence.

RESUMÉ

Il a été montré que, dans une culture de 48 heures, *Actinobacillus pleuropneumoniae* sécrètent des activités protéases dans le milieu. L'électrophorèse en gel de polyacrylamide (10%), co-polymérisé avec de la gélatine porcine, des surnageants précipités avec $(NH_4)_2SO_4$ à 70%, met en évidence des protéases de différents poids moléculaires: >200, 200, 90, 80, 70, et 50 kDa. Celles-ci ont montré une activité dans un large intervalle de pH (4–8), avec un pH optimal de 6–7. Toutes ont été inhibées par 10 m*M* de EDTA, et réactivées en présence de calcium 10 m*M*. Elles restent stables à −20° C pour plus d'un mois. Les protéases dégradent aussi l'IgA porcine et l'hémoglobine porcine, humaine et bovine, bien qu'elles apparaissent moins actives contre les hémoglobines. L'IgA a été totalement clivée en 48 heures, utilisant des surnageants concentrés avec du polyvinyl pyrrolidone ou précipités avec $(NH_4)_2SO_4$ à 70%. Les protéases extracellulaires peuvent jouer un rôle dans la virulence.

INTRODUCTION

Actinobacillus pleuropneumoniae is the causal agent of porcine contagious pleuropneumonia (PCP). This disease is worldwide, and results in important losses to the pig industry. Animals may become acutely ill, and often die with fibrinohemorrhagic lesions. Survivors grow poorly and can spread the infection to nonimmune herds (21).

Several virulence factors have been reported in *A. pleuropneumoniae*: cytolysins, lipopolysaccharide (LPS), capsule, a permeability factor, and fimbriae (7,9,13,27). There are contradictory reports of porcine IgA-cleaving proteases (14,20). The mechanisms by which this microorganism damages cells or tissues are not completely understood (1,12,26). The secretion of proteases as a virulence factor has been demonstrated in other bacterial species (10,16,22,24,25). Proteases of these microorganisms cleave host protein components facilitating invasion. Proteases could be involved in the severe lesions in pig lungs caused by *A. pleuropneumoniae*.

In this work, we describe proteases in supernatants of cultures of *A. pleuropneumoniae*. They degraded porcine gelatin, hemoglobin and IgA, and bovine and human hemoglobin.

MATERIALS AND METHODS

BACTERIAL CULTURE

Actinobacillus pleuropneumoniae serotype 1 strain 35, was isolated from a lung abscess of a pig with acute PCP, and kindly donated by Dr. V. Pérez-Márquez, México. *Actinobacillus pleuropneumoniae* strain ATCC 27088 was obtained from the collection of Escuela Nacional de Ciencias Biológicas, IPN, México. Cells were maintained on brain heart agar (Difco) with 10% of fresh yeast extract as the NAD source. Proteases were induced in stationary cultures during 48 h in brain heart infusion plus 10 m*M* $CaCl_2$.

PREPARATION OF CULTURE SUPERNATANTS AND EXTRACTS

2.5×10^{10} cells were centrifuged at 10,000 rpm (Sorvall, rotor SS34), for 20 min at 4°C. The cell-free supernatant was precipitated with 70% $(NH_4)_2SO_4$ at room temperature, centrifuged, and the precipitate was dissolved in 150 µL of 0.01 *M* phosphate buffer saline (PBS) pH 7.4 (DP). Proteases were also obtained from supernatants concentrated with polyvinyl pyrrolidone-360 (PVP, Sigma) in a dialysis bag, at 4°C (DS). Cell extracts were obtained by four to five cycles of freezing and thawing, or by four cycles of 15 s sonication (Bronson Sonic Power). Protein con-

Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN. Apdo. Postal 14-740 México D.F. 07000 Mexico.

Reprint requests to Dr. M. de la Garza.

Project supported by grant 0038-M9106 from CONACYT.

Submitted February 19, 1993.

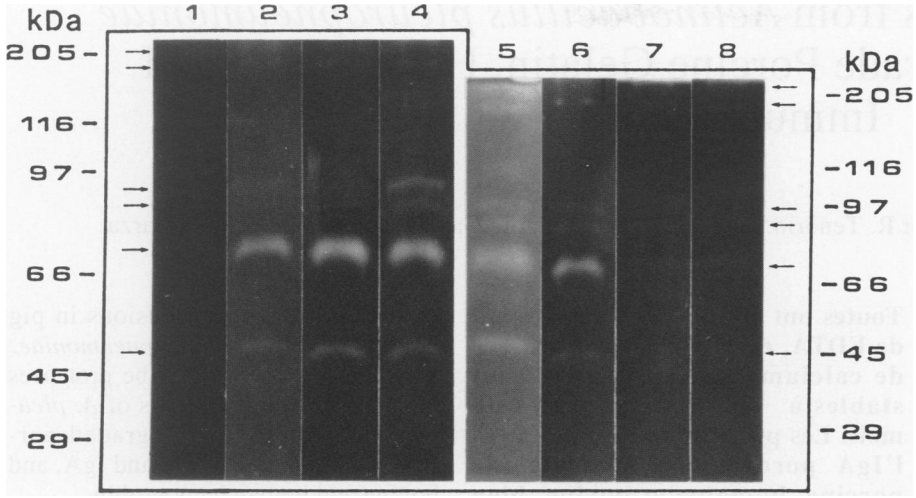

Fig 1. Protease pattern in 10% acrylamide gels copolymerized with 0.1% porcine gelatin. *Actinobacillus pleuropneumoniae* culture supernatants were precipitated with 70% $(NH_4)_2SO_4$ and 25 μg of the dissolved precipitated protein were loaded in each well. Gels were incubated at different pHs: (lane 1) pH 3.0; (2) 4.0; (3) 5.0; (4) 6.0; (5) 7.0; (6) 8.0; (7) 9.0 and (8) 10.0. Major activities are indicated by the arrows.

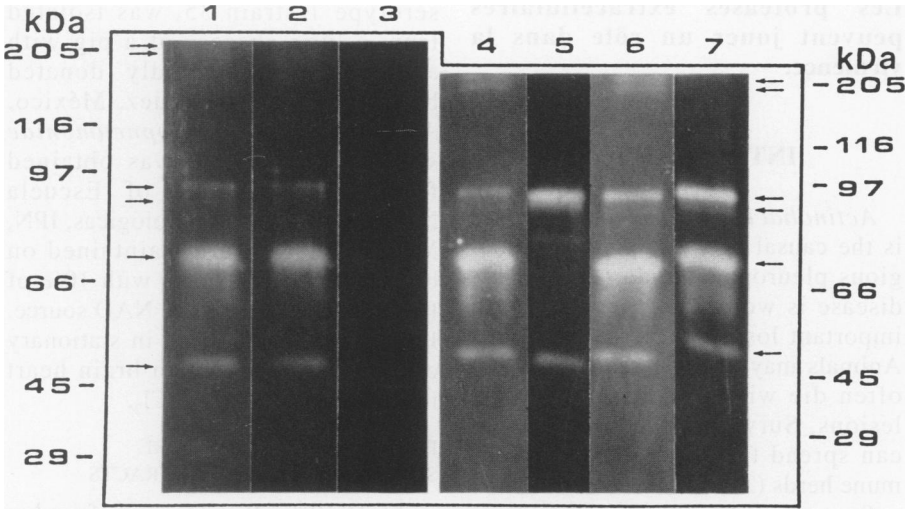

Fig 2. Protease pattern in 10% acrylamide gels copolymerized with 0.1% porcine gelatin. *Actinobacillus pleuropneumoniae* supernatants were precipitated with 70% $(NH_4)_2SO_4$ and 25 μg of the dissolved protein were loaded in each well. Gels were incubated at pH 7.0 with different protease inhibitors: (1) 5 m*M* DIFP; (2) 2 m*M* PMSF; (3) 10 m*M* EDTA; (4) 1 m*M* TLCK; (5) 10 m*M* pHMB; (6) control without inhibitor; (7) 5 m*M* NEM. Major activities are indicated by the arrows.

centration was determined by the method of Bradford (4).

SUBSTRATE GELS

To test for proteolytic activity, the method of Heussen *et al* was followed (11). Briefly, 10% polyacrylamide (Serva) (15) was copolymerized with 0.1% porcine gelatin or 0.05% bovine, human or porcine hemoglobin (Sigma). Twenty-five μg protein of the DP were solubilized in sample buffer without reducing agents or boiling and loaded in the wells. Electrophoresis was carried out at 17 mA for 17 h, at 4°C. SDS was removed from the gels by incubation with 2.5% Triton ×100 (Sigma) for 1 h. Gels were incubated with 0.1 *M* Tris-HCl (Sigma) pH 7–10 and 0.1 *M* sodium acetate acetic acid (Sigma) buffer pH 3–6 for 12 h. Ten mM $CaCl_2$ were added to both activation buffers. Substrate degradation could be seen after staining with Coomassie blue R250 (Sigma) and destaining with acetic acid-methanol-water (10:40:50).

EFFECT OF INHIBITORS ON PROTEOLYTIC ACTIVITY

Prior to the addition of sample buffer, the following inhibitors were added to the samples, and incubated 20 min at room temperature: 5m*M* diisopropyl fluorophosphate (DIFP), 5 m*M* N-ethylmaleimide (NEM), 0.1 m*M* tosyl-L-lysine chloromethyl ketone (TLCK), 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 m*M* ethylendiamine tetraacetic acid (EDTA) and 10 m*M* p-hydroxymercuribenzoate (pHMB), all from Sigma. The inhibitors at the same concentration were also added to the incubation mixture (pH 7.0) after electrophoresis. Gels were stained and destained as was described above.

DEGRADATION OF PORCINE IgA

Porcine IgA was purified from colostrum (17), and identified immunologically using a mouse monoclonal antiporcine IgA (Serotech). Two hundred micrograms of a) the DP or b) the DS, were incubated with 200 μg of IgA, for 0, 3, 6, 12, 24, 48 and 96 h at 37°C, according to the method of Mulks, without β-mercaptoethanol (18). Immunoglobulin A alone, or concentrated supernatants without IgA, were incubated for 96 h as controls. Samples of 50 μg protein were taken at each time period and Laemmli's buffer (15) was added. Samples were separated by electrophoresis in polyacrylamide gradient gels (7–12%).

Degradation of IgA was also assayed using overlay gels (2). Briefly, samples of 20 μg of protein from the DP were separated in 10% polyacrylamide gels. After electrophoresis, the proteins were transferred by capillary activity to a polyacrylamide gel copolymerized with 0.1% porcine IgA, for 24 h at 37°C. Gels with IgA were stained as the other substrate gels.

RESULTS

SUBSTRATE SDS–PAGE

Degradation of porcine gelatin by *A. pleuropneumoniae* 35 DP is shown in Fig. 1. The substrate was hydrolyzed by several proteases of approximate: >200, 200, 90, 80, 70 and 50 kDa. This hydrolysis occurred over a wide pH range (4 to 8), with optimal activity detected between 6–7 and

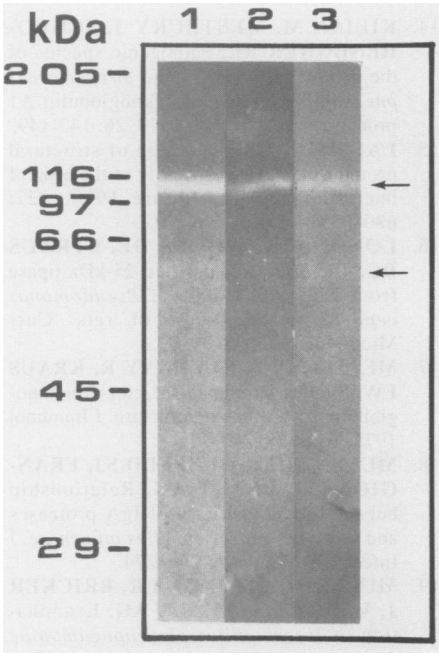

Fig 3. Protease pattern in 10% acrylamide gels copolymerized with 0.05% hemoglobin: (1) porcine, (2) bovine and (3) human. The same conditions for protein and electrophoresis as in Fig. 2. Major activities are indicated by the arrows.

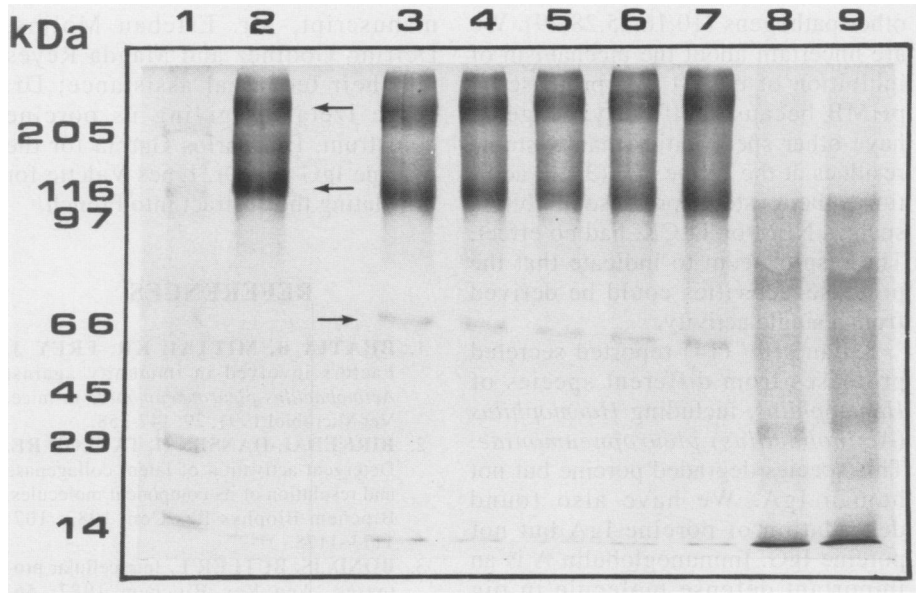

Fig 4. Porcine IgA degradation. Culture supernatants concentrated with PVP (200 μg) were mixed with 200 μg of porcine IgA, and incubated at 37°C for 96 h. At different times, samples of 50 μg were separated by 7–12% SDS–PAGE. (Lane 1) molecular weight markers; (2) control of IgA (96 h); (3) 0 h; (4) 3.0; (5) 6.0; (6) 12; (7) 24; (8) 48, and (9) 96 h incubation. Left arrows indicate the bands of IgA, and right arrow, the major protein of supernatant culture.

minimal activity at pHs 3, 9 and 10 (Fig. 1).

Of the several different inhibitors tested (Fig. 2), it was found that only 10 m*M* EDTA inhibited all proteases (Fig. 2, lane 3) and pHMB diminished the activity of the 70 kDa protease (Fig. 2, lane 5); the activity in samples with EDTA was restored in presence of 10 m*M* calcium (data not shown). The rest of the inhibitors did not affect the proteolytic activities (Fig. 2).

Less degradation was found when porcine, human or bovine hemoglobins were used as substrates (Fig 3). The best definition of proteolytic bands was seen when a small concentration of each hemoglobin was used (0.05%). The proteolytic pattern was similar for the three hemoglobins. *Actinobacillus pleuropneumoniae* strain ATCC 27088 showed a similar proteolytic activity in both gelatin and hemoglobin.

All these results were reproducible and proteases maintained their activity at least for one month at −20°C. These activities were present in supernatants from early log phase to at least four days. For practical reasons, 48 h cultures were chosen for the protease determination. No proteolytic activity could be detected when culture extracts, or culture medium without inoculum, were tested in gelatin or hemoglobin gels (data not shown). The profile of degradation could depend on the strain and the culture conditions, since other lots of BHI gave less proteolytic bands. Fedorka-Cray *et al* (8) have observed that growth conditions and medium composition can play an important role in the expression of secretion factors by *A. pleuropneumoniae*. Furthermore, less proteolytic activity was observed when the strain was subcultured for several months.

DEGRADATION OF PORCINE IgA

Secretory and monomeric IgA were cleaved by concentrated supernatants of *A. pleuropneumoniae* 35, after 24 h of incubation. Several bands of degradation were shown from IgA (Fig. 4, lanes 8 and 9). When the experiment was performed with the DP of a strain of lower passage, degradation of IgA was seen after three hours of incubation (data not shown). This difference in activity could be due to less protease content, in respect to the total protein, in the concentrated supernatants. Proteases were not able to cleave IgG obtained from pig serum.

Immunoglobulin A cleavage was also observed in overlay gels, but this method was less sensitive (data not shown). Cellular extracts did not have activity against the porcine IgA.

DISCUSSION

In order to test whether *A. pleuropneumoniae* can secrete proteolytic enzymes *in vitro*, the proteins present in the $(NH_4)_2SO_4$ precipitate of culture supernatants or in the PVP concentrated supernatants, were separated by electrophoresis in substrate gels. This method preserves the enzymatic activity and the molecular weight of proteases to be determined.

We found that *A. pleuropneumoniae* can secrete several proteases cleaving porcine gelatin (Fig. 1), but cell extracts were inactive. The proteases are probably inactive when inside the cell and activated when secreted, as in other species (5,28). They were not likely inactivated during freeze–thawing or sonication, since these methods have been successfully used in the preparation of many other enzymes.

This is the first report of proteolytic activity against porcine gelatin and hemoglobin for this bacterium. Since these secreted proteases were all inhibited with EDTA, they are most likely metalloproteases. This notion was supported by the fact that proteolytic activity was restored when calcium was added to the incubation buffer and because the activity increased when calcium was added to the culture medium. Secretion of metalloproteases has been reported in

other pathogens (10,16,25,28,29). We are uncertain about the mechanism of inhibition of the 70 kDa protease by pHMB because sulfhydryl reagents have other specificities than cysteine residues at the active site (3). In addition, other cysteine protease inhibitors such as NEM or TLCK, had no effect. The results seem to indicate that the protease-activities could be derived from a single activity.

Kilian *et al* (14) reported secreted proteases from different species of *Haemophilus*, including *Haemophilus (Actinobacillus) pleuropneumoniae*. This species degraded porcine but not human IgA. We have also found degradation of porcine IgA but not porcine IgG. Immunoglobulin A is an important defense molecule in pig nasal and tracheal secretions, and IgG has been predominantly found in deep respiratory tissues (23). The IgA-cleaving proteases could be facilitating the mucosal spread of this microorganism.

Mulks *et al* (19) did not find IgA-cleaving proteases when they purified the proteins of culture supernatants by 60% $(NH_4)_2SO_4$ precipitation dialysis and chromatography. We have observed decrease of activity when precipitates were dialyzed in the absence of calcium or when they were obtained with less than 70% $(NH_4)_2SO_4$; these factors could explain the different results.

Actinobacillus pleuropneumoniae has three cytolysins, two of which have hemolytic activity. In pathogenic microorganisms, hemolysis and proteolytic cleavage of hemoglobin could be a mechanism for iron acquisition. Such a mechanism has been described in *Vibrio vulnificus*, which utilizes different heme proteins as sole iron source after using an extracellular protease (20). Protease-deficient mutants of *V. vulnificus* did not utilize any heme proteins as iron source. Furthermore, in *A. pleuropneumoniae* it has been reported that porcine hemoglobin can be used as the sole iron source (6).

The capacity of *A. pleuropneumoniae* proteases to degrade porcine gelatin, IgA and hemoglobin, could function as a virulence factor in this pathogen bacterium.

ACKNOWLEDGMENTS

We are grateful to Drs. J. Calderón, J. L. Rosales, M. V. Ortega and P. Harrison for their comments on the manuscript, Mr. Esteban Molina, Delfino Godinez and Magda Reyes for their technical assistance; Dr. Jorge Izeta for giving us porcine colostrum, Dr. Carlos García for the porcine IgG and Dr. Ignes Valette for translating the abstract into French.

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