Purification and Characterization of a Protease from *Actinobacillus pleuropneumoniae* Serotype 1, An Antigen Common to All the Serotypes

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ABSTRACT

A high molecular-mass proteolytic enzyme of *Actinobacillus pleuropneumoniae* serotype 1, was purified from culture supernatants (CSN) by using DEAE-cellulose and sepharose-4B-gelatin chromatography. In 10% SDS-polyacrylamide gels copolymerized with porcine gelatin, the protease showed a single band of activity of > 200 kDa. However, minor molecular-mass proteolytic bands were observed when the protease was electrophoresed in the presence of either 5% ß-mercaptoethanol, 50 mM dithiothreitol, or 0.25 M urea. Furthermore, when the > 200-kDa purified protein was passed through a sucrose gradient, several bands with proteolytic activity were found: 62, 90, 190, and 540 kDa. The proteolytic activity was increased in the presence of calcium or zinc and was not affected after being heated at 90°C for 5 min. Proteolytic activities were also observed in CSN from all *A. pleuropneumoniae* serotypes and biotypes. The purified protease hydrolyzed porcine IgA and IgG in vitro. In addition, by immunoblot the protease was recognized by serum of naturally infected pigs with serotypes 1 and 5, and by serum of pigs experimentally infected with serotypes 1, 2, 8, or 9. Serum of a pig vaccinated with CSN of a serotype 3 strain also recognized the protease, but not sera of pigs vaccinated with a bacterin (serotype 1). Proteins from CSN of all the serotypes, which were precipitated with 70% $(NH_4)_2SO_4$, were recognized by a polyclonal antibody raised against the purified protease.

Taken together these results indicate that an antigenic protease is produced in vivo by all the serotypes of *A. pleuropneumoniae*. The results indicate that proteases could have a role in the disease and in the immune response of pigs infected with *A. pleuropneumoniae*.

RÉSUMÉ

Une enzyme protéolytique d'*Actinobacillus pleuropneumoniae* sérotype 1 de haut poids moléculaire a été purifiée à partir d'un surnageant de culture (SCN) par chromatographie au DEAE-cellulose et sépharose 4B-gélatine. Dans un gel de SDS-polyacrylamide 10 % copolymérisé avec de la gélatine porcine, une seule bande (> 200 kDa) ayant une activité protéolytique est retrouvée. Lorsque l'électrophorèse est effectuée en présence de 5 % de ß-mercaptoéthanol, de 50 mM de dithiothréitol, ou de 0.25 M d'urée, on observe plusieurs bandes de faible poids moléculaire ayant des propriétés protéolytiques. En outre, quant la protéine purifiée de 200 kDa fut soumise à un gradient de sucrose, l'activité protéolytique était retrouvée au niveau des bandes de 62, 90, 190 et 540 kDa.

L'activité protéolytique est accrue en présence de calcium ou de zinc mais n'est pas affectée par un chauffage à 90 °C pendant 5 minutes. Une activité protéolytique est aussi observée dans le SNC de tous les sérotypes et biotypes d'*A. pleuropneumoniae*. La protéase purifiée hydrolyse les IgA et IgG porcins in vitro. De plus, elle est reconnue par les sérums de porcs infectés naturellement avec les sérotypes 1 et 5, et par le sérum de porcs infectés expérimentalement avec les sérotypes 1, 2, 8 et 9. Le sérum d'un porc vacciné avec le SNC du sérotype 3 reconnaît aussi la protéase, mais pas le sérum de porcs vaccinés avec la bactérine du sérotype 1. Les protéines du SNC précipitées avec du $(NH_4)_2SO_4$ 70 % ont été reconnues par l'anticorps polyclonal dirigé contre la protéase purifiée.

Tous ces résultats confirment la présence d'une protéase antigéniquement commune à tous les sérotypes, ainsi que sa synthèse in vivo. Les protéases pourraient jouer un rôle dans la maladie et dans la réponse immunitaire des porcs infectés par *A. pleuropneumoniae*.

INTRODUCTION

Actinobacillus pleuropneumoniae is a gram-negative bacterium that causes an acute hemorrhagic pneumonia in growing and finishing pigs (34). Pathogenesis of this disease is not totally understood. As in all pathogenic bacteria, *A. pleuropneumoniae* virulence is multifactorial,

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TABLE I. *Actinobacillus pleuropneumoniae* strains

Serotypes	Reference strain	Field strain
1	Shope 4047	158, SCI p8
2	1536	
3	1421	11507, SCI p4
4	M62	
5a	K17	192ff, SCI p6
5b	L20	
6	Femϕ	
7	WF53	53, SCI p10
8	405	85-10053, SCI p2
9	CVI 13261	38, SCI-2 p11
10	D13039	
11	56153	
12	6329	
1, biotype 2	N273	
2, biotype 2	N282	

and it is associated with several factors: capsular polysaccharide, lipopolysaccharide, outer membrane proteins, exotoxins, and fimbriae (8,10, 16,17,18,33,40,42). All the serotypes cause porcine contagious pleuropneumonia (PCP) in susceptible pigs, with the same characteristic lesions, but some serotypes appear to be more virulent than others (32). This difference in virulence has been attributed to the presence or absence of ApX toxins (10).

Some pathogenic bacterial species which colonize mucosal surfaces, are known to produce extracellular enzymes that cleave or totally degrade immunoglobulin molecules or other substrates (19,25,28,29,35). It has been suggested that these extracellular bacterial proteases may play an important role in virulence (9,11,25, 39). The presence of secreted proteases in *A. pleuropnemoniae* was first detected by Kilian et al (20), when they demonstrated porcine IgA degradation. Recently, we have described secreted proteases into the culture medium of *A. pleuropnemoniae* serotype 1, that were able to cleave porcine IgA, as well as to degrade other substrates (24).

In this work, we describe the purification of a high molecular-mass (> 200 kDa) protease from culture supernatants (CSN) of *A. pleuropnemoniae* serotype 1. This protease was recognized by immune sera of pigs infected with PCP, but not by sera of pigs vaccinated with a formalinized bacterin. Such antigenic protease could be useful to distinguish between infected and vaccinated pigs, when whole cells are used as vaccines.

MATERIALS AND METHODS

BACTERIAL STRAINS

A. pleuropneumoniae serotype 1 strain 35 was reported previously (24). It was preserved by lyophilization and subcultured on brain-heart infusion agar (BHI, Difco Labs., Detroit, Michigan, USA), supplemented with 10 μg/mL NAD (Sigma Chemical, St. Louis, Missouri, USA). The field strains isolated from pigs with PCP were donated by Dr. R.F. Ross (Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, USA). Reference strains were donated by Dr. E.M. Kamp (Department of Bacteriology, Central Veterinary Institute, Lelystad, The Netherlands). All the reference and field strains used in this work are listed in Table I.

CONCENTRATION OF EXTRACELLULAR PROTEASES

Bacteria were grown in BHI-NAD broth 48 h at 37°C with agitation (60 rpm, in a Lab-line shaking bath), and centrifuged 30 min (12 000 × *g*) at 4°C (Sorvall rotor SS34). CSN was filtered through Millipore membranes (type HA 0.45 μm), concentrated by precipitation with 70% $(NH_4)_2SO_4$, or by ultrafiltration (XN50 Amicon, Lexington, Massachusetts, USA) and dialyzed against 50 mM Tris-HCl (pH 8), plus 10 mM $CaCl_2$ and 0.001% NaN_3 (buffer 1). All the chemicals and immunoreagents (except where indicated) were from Sigma. Protein concentration was determined by the method of Bradford (3).

PURIFICATION OF THE > 200-kDa PROTEASE SECRETED FROM *A. pleuropneumoniae* SEROTYPE 1

Proteins from CSN, obtained by precipitation with 70% $(NH_4)_2SO_4$, were dialyzed against 50 mM glycine-NaOH buffer (pH 9.2), and passed through a DEAE-cellulose column, which was washed with the same buffer. The retained proteins were eluted with 2 M NaCl in buffer 1. The fractions containing proteolytic activity were pooled, dialyzed against buffer 1, and concentrated by lyophilization or ultrafiltration. This material was then further purified by chromatography in a Sepharose 4B porcine-gelatin column, previously equilibrated with buffer 1. The column was prepared using CNBr-activated Sepharose 4B (Pharmacia) coupled with porcine gelatin (Sigma). The protease was eluted using 50 mM acetate buffer (pH 4.5), plus 10 mM EDTA. The proteolytic activity was determined using a synthetic substrate: 0.8 mM N-benzoyl-DL-arginine-4-nitroanilide-hydrochloride (DL-BAPA, Merck), dissolved in dimethylsulfoxide (DMSO) in the presence of 10 mM $CaCl_2$. Ten μL of substrate was added to 0.5 mL of each fraction eluted, incubated at 37°C for 1 h, and read in a spectrophotometer (Beckman DU 7500) at 405 nm. The activity was also determined by using azocasein (Sigma) or azoalbumin (Sigma) as substrates, according to Uitto et al (41).

SUBSTRATE GELS

The electrophoresis in substrate gels was performed in 10% SDS-polyacrylamide gels copolymerized with 0.1% gelatin, as described previously (15,24). Samples were not treated with ß-mercaptoethanol (ß-ME) or boiled. Five μg of the purified protein from CSN (serotype 1) or 15 μg of concentrated CSN of the different serotypes were loaded by well. A concentrated medium control was included. The effect of 0.25 M urea, 5 mM dithiothreitol (DTT), 5% ß-ME (mixed with sample buffer), or heating in the presence of 1% SDS and 5 mM EDTA, was also assayed (4,35).

DETERMINATION OF OPTIMUM pH

The purified enzyme (2 μg) was incubated in duplicate at 37°C with DL-BAPA or azoalbumin at different pHs. The following buffers were used: 50 mM acetate (pH 4–6), 50 mM Tris-hydrochloride (pH 7–9), and 50 mM carbonate (pH 10–11). A blank with substrate, but without enzyme, was included for each pH, and its optical density value was subtracted in those samples containing enzyme. Assays were performed at least 3 times.

EFFECT OF METAL IONS, INHIBITORS AND TEMPERATURE

To observe the effect of metal ions, the purified protease plus 50 mM acetate buffer (pH 6) was incubated for 30 min at 37°C in the presence of different salts ($CoCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, $CuSO_4$, $FeCl_2$ or $CaCl_2$) or inhibitors: ethylenediaminetetraacetate

(EDTA), ethyleneglycol-bis (ß-aminoethyl ether) tetraacetate (EGTA), DTT, p-hydroxymercuribenzoate (pHMB), N-ethylmaleimide (NEM), or phenyl-methyl sulfonyl-fluoride (PMSF), before the proteolytic activity on DL-BAPA or azoalbumin was assayed. The protease was also heated for 1 h at 40, 50, 60, 70 and 80°C, or in boiling water for 5 or 10 min in the presence or the absence of 10 mM $CaCl_2$, before the proteolytic activity was assayed.

PROTEASE MOLECULAR MASS DETERMINATION

A fifty μg (1 mL) sample of protease purified as described above, was layered on a 10 mL sucrose gradient (0–23%), and centrifuged at 143 000 × *g* at 4°C for 17 h in a Beckman ultracentrifuge, according to the method of Martin and Ames (23). Molecular mass of the protein was determined with the methods of Martin and Ames (23) and Griffith (12). Bovine serum albumin (BSA, 66 kDa), phosphorylase B (97.6 kDa), myosin subfragment 1 (102 kDa), heavy meromyosin (205 kDa) and horse apo-ferritin (480 kDa, Mann Research Lab.) were used as standards. Molecular mass was also determined by electrophoresis in SDS-polyacrylamide gels copolymerized with porcine gelatin, using the same protein standards.

PORCINE IgA AND IgG DEGRADATION

Porcine IgA (5–10 μg) or IgG (10 μg), which were prepared as was described previously (24), were incubated in the presence of the purified protease (5 μg) at 37°C in 50 mM acetate buffer (pH 6) with 10 mM $CaCl_2$. After incubation, samples were run in 7% SDS-PAGE, and stained with Coomassie brilliant blue. All experiments were done at least in triplicate.

PRODUCTION OF POLYCLONAL ANTIBODIES

Two New Zealand rabbits were injected subcutaneously with 50 μg of the protease, purified by affinity chromatography, at 15-day intervals, until a good immune response was obtained (1:1024) in ELISA assays. Rabbits were bled before being injected and their sera were used as negative controls. Titermax was used as adjuvant for the 1st immunization, and incomplete

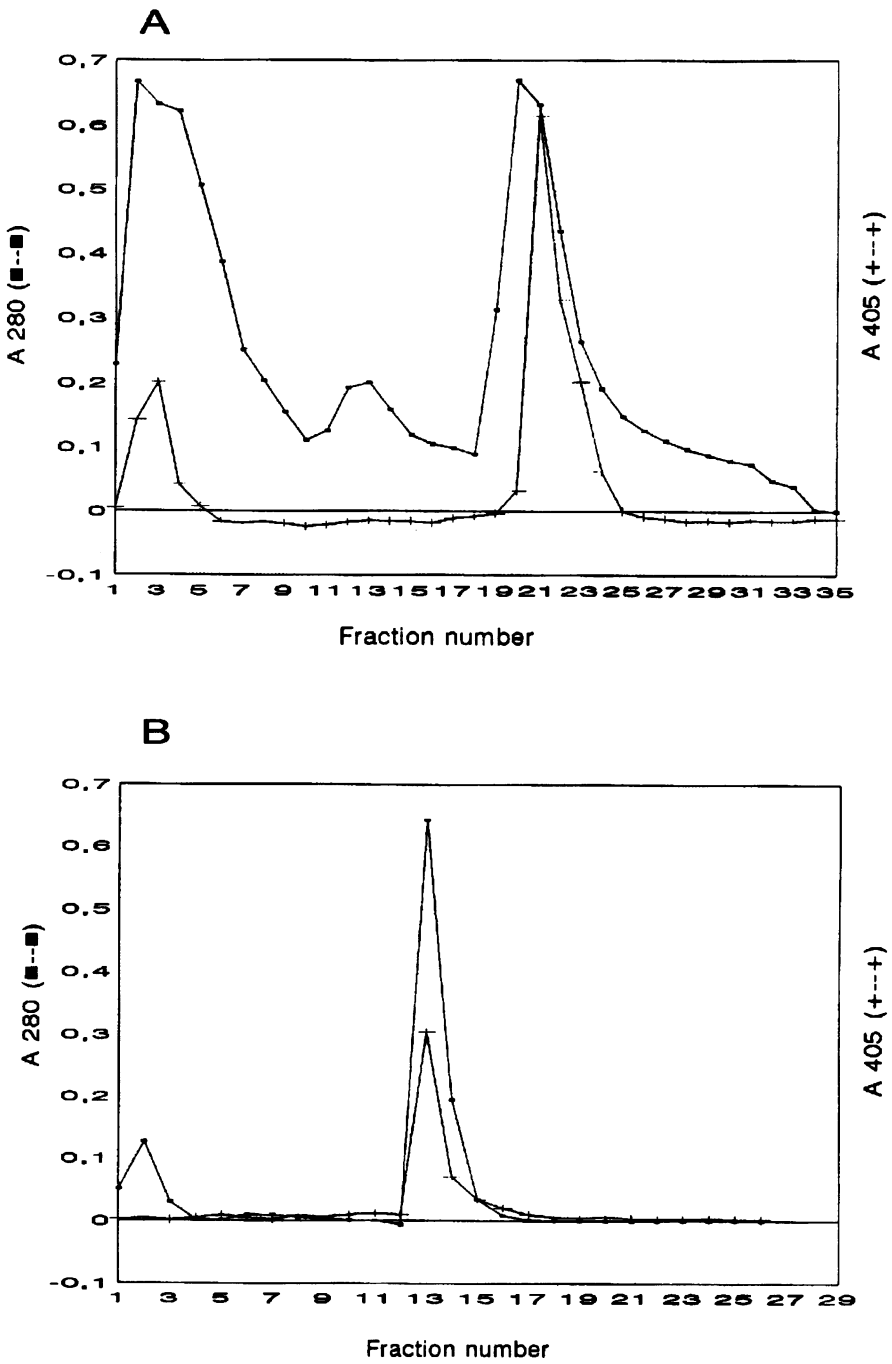

Figure 1. Elution profile of the *A. pleuropneumoniae* protease. (A) DEAE-Cellulose ion-exchange chromatography. (B) Sepharose 4B-porcine gelatin affinity chromatography. Symbols: ■ A_{280}, + protease activity.

TABLE II. Purification summary

Purification stage	Protein (mg)	Total Activity (U)[b]	Specific activity (U/mg)	Purification recovery (fold)	Purification recovery (%)
Culture supernatant	20.18[a]	37400	1853.3		100.0
70% $(NH_4)_2SO_4$ precipitation	14.78	33592	2272.8	1.2	89.8
DEAE-cellulose chromatography	8.99	27540	3063.4	1.6	73.6
Sepharose 4B-gelatin chromatography	0.216	17368	80407.4	43.4	46.4

[a] Per liter of culture medium

[b] One unit is the proteolytic activity that degrades 1 nmol of N-benzoyl-DL-arginine-4-nitroanilide hydrochloride in an hour

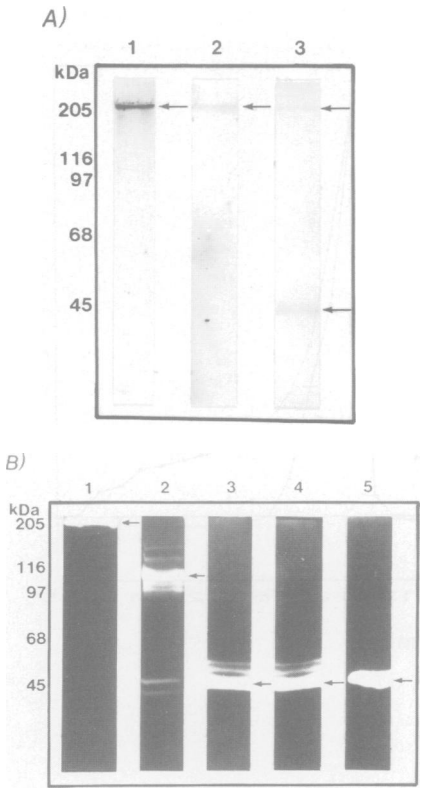

Figure 2A. Purified protease stained with silver (lane 1); its immune recognition by sera of infected pigs with PCP, by blotting to nitrocellulose membrane (lane 2); purified protein after being heated 10 min in the presence of 0.25 M urea, 5% ß-ME and 1% SDS (lane 3).

Figure 2B. Proteolytic activity of the > 200-kDa purified protease in 10% SDS-PAG copolymerized with 0.1% porcine gelatin. Lane 1, without addition; lane 2, in the presence of 0.25 M urea; lane 3, in the presence of 0.25 M urea plus 5 mM DTT or 5% ß-ME (lane 4); lane 5, after been heated 10 min in the presence of 0.25 M urea, 5% ß-ME and 1% SDS.

TABLE III. Effect of pH, metal ions, inhibitors, and temperature on the proteolytic activity

A. pH effect[a]		B. Effect of ions[c]	
pH	Activity (%)	Metal ion without addition	Activity (%) 100.0 = 936 U
4.0	34.0 ± 2.2	Ca^{2+} 10.0 mM	159.0 ± 2.7
5.0	58.8 ± 2.0	Zn^{2+} 2.5 mM	145.0 ± 3.2
6.0	100.0 = 1489 U[d]	Mn^{2+} 2.5 mM	100.0 ± 1.0
7.0	93.0 ± 2.6	Fe^{2+} 2.5 mM	100.0 ± 0.5
8.0	60.0 ± 1.2	Mg^{2+} 2.5 mM	36.0 ± 1.8
9.0	52.0 ± 3.0	Cu^{2+} 2.5 mM	12.0 ± 1.0
10.0	14.0 ± 1.8	Co^{2+} 2.5 mM	0
11.0	2.0 ± 1.5		

C. Effect of inhibitors[c]		D. Effect of temperature and calcium[c]		
Inhibitor without inhibitor	Activity (%) 100.0 = 1489 U	Temperature[c]	Activity (%) with Ca^{2+}	Activity (%) without Ca^{2+}
EDTA 10 mM[b]	0	37°C	100.0 = 1489 U	100.0 = 936 U
EGTA 10 mM[b]	0	40°C	87.9 ± 1.8	83.8 ± 2.0
DTT 5 mM	42 ± 4.0	50°C	65.9 ± 1.3	63.1 ± 2.3
pHMB 10 mM	47 ± 2.0	60°C	59.0 ± 3.3	36.1 ± 2.0
NEM 5 mM	99 ± 1.0	70°C	60.0 ± 3.0	23.5 ± 1.0
PMSF 2 mM	104 ± 5.3	80°C	65.0 ± 2.2	33.5 ± 2.8

[a] pH effect was measured at 37°C and 10 mM $CaCl_2$; buffers used were 50 mM acetate (pH 4–6); 50 mM Tris-HCl (pH 7–9) and carbonate (pH 10–11)

[b] Proteolytic activity was measured at 37°C in the absence of $CaCl_2$

[c] At temperatures lower than 37°C the activity was not measured, but samples conserved proteolytic activity in SDS-PAGE, copolymerized with gelatin

[d] One unit is the proteolytic activity that degrades 1 nmol of N-benzoyl-DL-arginine-4-nitroanilide hydrochloride in an hour

[e] Assays were realized in 50 mM acetate buffer, pH 6.0

Freund's adjuvant was for subsequent injections of the antigen.

WESTERN BLOTTING

The protease purified from CSN of *A. pleuropneumoniae* serotype 1 (strain 35), and the proteins from the CSN precipitated with 70% $(NH_4)_2SO_4$ from the different serotypes, were separated by electrophoresis in a 10% SDS-PAG (without ß-ME or heat), and then transferred to nitrocellulose membranes (Sigma) according to Towbin (38) for 1 h at 260 mA. Membranes were blocked with 5% BSA in 0.1 M phosphate saline buffer (pH 7.4) for 2 h. Then, they were placed in contact with the rabbit polyclonal antibodies (1:1000) against the purified protease, or with immune sera of pigs naturally infected with *A. pleuropneumoniae* serotypes 1 or 5, or with sera from pigs experimentally infected with reference serotypes 1, 2, 8, 9, or experimentally vaccinated with cells from reference serotype 1, or experimentally vaccinated with CSN of serotype 3. All the pig sera were diluted 1:100. The sera of animals experimentally infected with serotype 1 were obtained by exposure to bacterial aerosols (36); the sera from serotypes 2, 3, 8, and 9 were kindly donated by Dr. E. Kamp. The sera from vaccinated pigs were obtained by intraperitoneal injection with formalinized bacteria, as was described (36). A rabbit anti-pig IgG peroxidase (1:1000) (Sigma) or peroxidase protein A (1:1000) (Sigma), with diaminobenzidine hydrochloride and H_2O_2 were used to observe the reaction.

RESULTS

PURIFICATION OF THE > 200-kDa PROTEASE ACTIVITY FROM *A. pleuropneumoniae* SEROTYPE 1

Previously, we have observed several proteolytic activities in CSN of *A. pleuropneumoniae* and showed that the highest molecular mass proteolytic activity (i.e. > 200 kDa) was always present, although minor activities at low molecular mass were also present (24). The > 200-kDa protease activity was obtained by concentrating 10 L of CSN by precipitation with 70% $(NH_4)_2SO_4$, or by ultrafiltration (20-fold). This > 200-kDa protein was retained in the DEAE-cellulose chromatography column at pH 9.2 (Fig. 1A) and eluted with 2 M NaCl. Following elution, 9 mg of total protein was obtained. The pooled fraction containing the proteolytic activity was further purified by Sepharose 4B-gelatin chromatography (Fig. 1B). Forty-six percent of the total initial activity was retained (Table II). The purity of this protein was checked by staining with silver nitrate (Fig. 2A, lane 1); additionally, the activity was determined by using synthetic substrates (data not shown).

PROTEOLYTIC ACTIVITY

Degradation of porcine gelatin by the purified protease was observed as a white zone in a blue background in substrate gels stained with Coomassie brilliant blue (Fig. 2B, lane 1). In addition to the main zone of clearing (> 200 kDa), several lower molecular-mass bands were observed in the substrate gels; apparently they could be

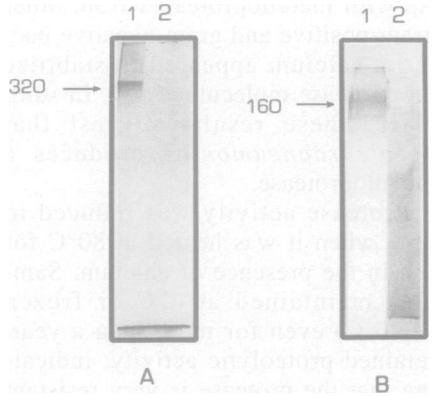

Figure 3. Immunoglobulin degradation. SDS-PAGE of the immunoglobulin cleavage products due to *A. pleuropneumoniae* purified protease. (A) 7% SDS-PAGE of porcine IgA (5 μg) incubated with 5 μg protease; (B) 10% SDS-PAGE of porcine IgG (10 μg) incubated with 5 μg protease. 1A and 1B are the IgA and IgG without protease, respectively; 2A and 2B are the immunoglobulin degradation after incubation in the presence of the purified protease.

originated from the highest protease activity. Consistent with this notion, lower molecular-mass regions of proteolytic activity were observed when this major band was taken out from the gel and electrophoresed in the presence of 0.25 M urea, or 0.25 M urea plus 5% ß-ME, or 0.25 M urea plus 50 mM DTT (Fig. 2B, lanes 2, 3, and 4). Under these conditions, polypeptides with a molecular mass of approximately 100–110 kDa and 45–50 kDa respectively, were observed, the 47 kDa being the most active. All the activities were inhibited by 10 mM EDTA or EGTA and reactivated in the presence of 10 mM $CaCl_2$ (data not shown). When the sample was boiled in the presence of EDTA, urea, and SDS, and was reactivated with calcium after the electrophoresis, a single proteolytic band of 47 kDa was observed (Fig. 2B, lane 5).

BIOCHEMICAL CHARACTERIZATION

The optimum pH, heat tolerance, and the effect of activators and inhibitors were determined using the purified protease. The protease was more active in the pH range of 6.0–7.0, and less active (20–30%) at pH 4 or at a pH higher than 9 (Table IIIA). Knowing that calcium increases the proteolytic activity of *A. pleuropneumoniae* (24), we also tested the effect of different divalent metal ions; Ca^{2+} or Zn^{2+} increased the proteolytic activity (50%) and Co^{2+} completely inhibited

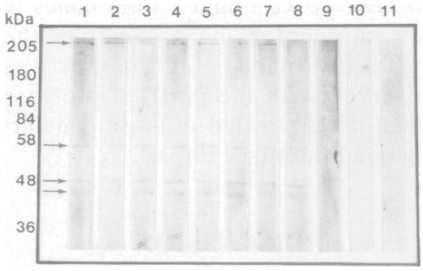

Figure 4. Immune recognition of the *A. pleuropneumoniae* purified protease by sera of infected or vaccinated pigs. (A): (lane 1) serotype 1; (2) 1; (3) 2; (4) 3; (5) 5; (6) 8; (7) 9; (8) polyclonal serum against the purified protease of serotype 1; (9) negative control (preimmune porcine serum); (10–11) sera of pigs vaccinated with a bacterin (serotype 1). Samples were heated 5 min in the presence of 0.25 M urea and 5% ß-ME before electrophoresed.

it (Table IIIB). Consistent with this, in the presence of 10 mM EDTA or EGTA, the activity was totally inhibited. The activity was less inhibited by DTT or pHMB. Neither NEM nor PMSF had an effect on the activity (Table IIIC). In the presence of calcium, the protease was stable at temperatures up to 80°C (Table IIID). On the other hand, when the molecular mass of the purified protease activity was determined by sucrose gradient, several bands with proteolytic activity were found: 62, 90, 190 and approximately 540 kDa (data not shown).

IMMUNOGLOBULIN DEGRADATION

Since immunoglobulins are considered important defense molecules, we tested the degradation of porcine IgA and IgG by the > 200-kDa protease activity. These immunoglobulins were totally degraded when incubated in the presence of the protease for more than 24 h (Fig. 3).

RECOGNITION OF THE PURIFIED PROTEASE BY SERA OF PIGS WITH PCP

We tested various swine sera for the presence of anti-protease antibody by Western blot. Antibodies recognizing the protease purified from *A. pleuropnuemoniae* serotype 1 were detected in sera from: a) pigs naturally infected with serotypes 1 and 5 (Fig. 4, lanes 1 and 5); b) pigs experimentally infected with the serotypes 1, 2, 8, and 9 (lanes 2, 3, 6 and 7); and c) a pig vaccinated with CSN of serotype 3 (lane 4). Our antibody raised against the purified protease showed similar recognition to that of

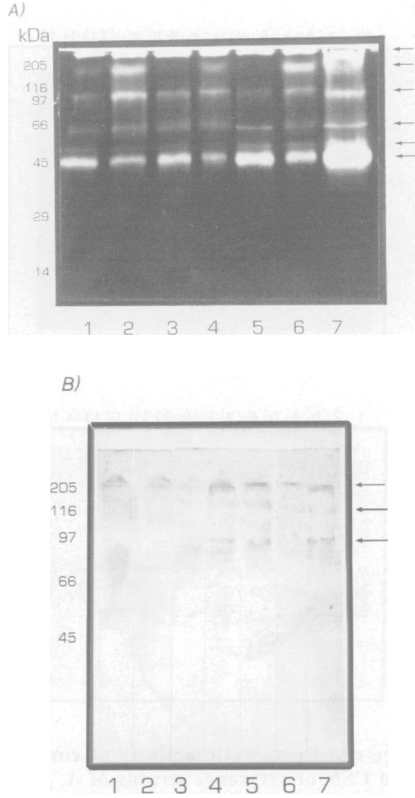

Figure 5A. Proteolytic activity of concentrated culture supernatans from different *A. pleuropneumoniae* serotypes (field strains) in porcine gelatin-SDS-PAG. (Lane 1) serotype 1; (2) serotype 3; (3) serotype 5; (4) serotype 7; (5) serotype 8; (6) serotype 9; (7) strain 35 serotype 1. Arrows indicate the positions of proteolytic activity.

Figure 5B. Immunoblot analysis of concentrated CSN from different serotypes of *A. pleuropneumoniae* reacted with the polyclonal serum against the purified protease. Samples are the same as the Fig. 5A. When samples were in the presence of 0.25 M urea and 5% ß-ME, the recognition bands are observed at similar molecular mass as are observed in Fig. 6B.

the other antisera (lane 8). There was no reaction with hyperimmune sera of vaccinated pigs with a bacterin prepared with *A. pleuropneumoniae* serotype 1 (lanes 10 and 11), or with a preimmune pig serum (lane 9). As the electrophoresis was performed in the presence of 0.25 M urea and 5% ß-ME, bands of > 200, 55, 47 and 45 kDa were recognized by the aforementioned sera and also by the polyclonal rabbit antibody against the purified protease.

PROTEASES ARE IN ALL THE SEROTYPES AND ARE RECOGNIZED BY ANTIBODIES TO THE PURIFIED PROTEASE

The $(NH_4)_2SO_4$-precipitated and dialyzed proteins were obtained from

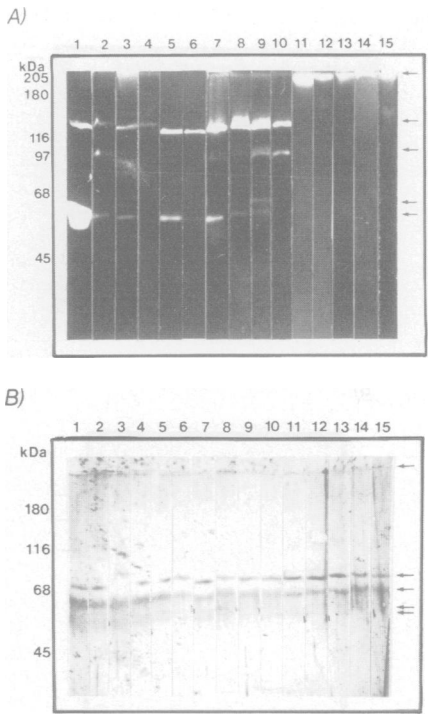

Figure 6A. Proteolytic activity of concentrated CSN of reference strains of *A. pleuropneumoniae* in porcine gelatin-SDS-PAG. (Lane 1) serotype 1; (2) 2; (3) 3; (4) 4; (5) 5a; (6) 5b); (7) 6; (8) 7; (9) 8; (10) 9; (11) 10; (12) 11; (13) 12; (14) 1B2; (15) 2B2. Arrows indicate the proteolytic activity bands.

Figure 6B. Immunoblot analysis of concentrated CSN of reference strains of *A. pleuropneumoniae* reacted with the polyclonal serum against the purified protease of serotype 1. Samples are the same as Fig. 6A. Samples were heated 5 min in the presence of 0.25 M urea and 5% ß-ME before electrophoresed. Samples without urea or ß-ME presented recognition bands to high molecular mass as was observed with the samples of Fig. 5B.

the CSN of the reference strains of all the serotypes and from selected field strains, listed in Table I, and their proteolytic activity was assayed in substrate gels. In the Fig. 5A we show the proteolytic activity of serotypes 1, 3, 5, 7, 8, and 9, which are field isolates. All the reference strains from biotype 1 and both of biotype 2 also degraded the substrate (Fig. 6A). When a rabbit polyclonal antibody raised against the purified protease was used to search antigenicity due to similar proteases in the field strains, polypeptides of > 200, 120, and 90 kDa could be detected (Fig. 5B). CSN proteins of the reference strains which were electrophoresed in the presence of 0.25 M urea and 5% ß-ME, were also recognized by the mentioned antibody (approximately at 70 kDa, 60 kDa, and 2 faint bands at low molecular mass, besides the > 200-kDa band) (Fig. 6B), as was observed with the purified protease when it was treated in those denaturing conditions (see Fig. 4).

DISCUSSION

The proteases of some pathogenic microorganisms have been shown to contribute to virulence by degrading molecules of the immune system such as immunoglobulins and the complement cascade system, interfering with the immune response (2,28,31,35,39). Bacterial proteases are also known to degrade important tissue components such as collagen and extracellular matrix elements, thereby promoting invasion and colonization (11,14,22,37).

A. pleuropneumoniae is known to secrete proteases into the culture medium when it is growing in vitro (24); but, the optimum conditions for activity were not known, nor it was known if these proteases are produced in vivo. (To address these questions a protease activity was purified and characterized from the CSN of a serotype 1 strain.) Proteolytic activity was associated with a metalloprotease of > 200 kDa. The purified protease degraded porcine IgA and IgG in vitro. Degradation of IgG was observed only with the purified protein; crude preparations did not degrade IgG, as was reported previously (24). (Since immunoglobulins have important functions such as protecting against the invasion of microorganisms (19), a protease that degrades immunoglobulins could be a virulence factor of *A. pleuropneumoniae*.)

We observed proteolytic activity in samples without calcium or zinc addition; this could be explained by the small amounts of these ions in the reagents, and samples for measuring activity were not treated with chelating agents. Zinc- and calcium-dependent metalloproteases have been reported in important bacterial pathogens such as *Pseudomonas, Haemophilus*, and *Streptococcus* species (11,14,29). An increase in the proteolytic activity was observed when calcium or zinc were added, and a corresponding behavior was observed by the inhibition with the chelating agents EDTA and EGTA. As with metalloproteases from other gram-positive and gram-negative bacteria, calcium appeared to stabilize the protease molecule (5,43). In summary, these results suggest that *A. pleuropneumoniae* produces a metalloprotease.

Protease activity was reduced to 65% when it was heated at 80°C for 1 h in the presence of calcium. Samples maintained at 4°C or frozen (−20°C) even for more than a year, retained proteolytic activity, indicating that the protease is very resistant to extreme temperatures.

The purified protease had an optimum pH of 6, but it was active over a broad range of pH (5–9). Extracellular proteases able to cleave different substrates at a broad range of pH have also been observed in *Porphyromonas gingivalis* (21) and other microorganisms. Activity at different pH values gives the microorganism the facility to survive in different environmental conditions.

The *A. pleuropneumoniae* protease activity was slightly diminished in the presence of DTT or pHMB, but was not affected by NEM, PMSF or TLCK, implying that -SH groups are likely present in the catalytic site of the protein. In addition, the inhibitory effect of high DTT concentrations has been demonstrated in other metalloproteases (22,37).

Under the conditions employed for growth and purification, the > 200-kDa protease seems to be present in small amounts in *A. pleuropneumoniae* CSN. In fact, we had to purify the protease from several liters of culture. However, the > 200-kDa protease is very active and easily detected in porcine-gelatin substrate gels. Two molar NaCl was used to elute the protein because it was lost easily at low NaCl concentrations.

The > 200-kDa protease appears to be a multimer of a lower molecular mass (MM) protease (≈ 47 kDa). There is evidence for this: a) all the bands electrophoresed in SDS-PAG copolymerized with gelatin were inhibited by EDTA or EGTA; b) the polyclonal antibody that was prepared against the > 200-kDa protein recognized the lower molecular mass polypeptides; c) low molecular-mass bands with proteolytic activity were observed if the > 200-kDa protein was treated with reducing agents like

ß-ME, DTT or urea; and d) when the purified protease was precipitated with acetone or methanol-chloroform-water (44), lower molecular mass forms recognized by the polyclonal antibody against the protease of > 200 kDa were also observed.

Proteases, cytotoxins and outer-membrane proteins like porins form aggregates of high molecular mass in the presence of detergents, LPS or murein (1,4,13,27). When the > 200-kDa protease was assayed by the method of Chevalier et al (4) (who obtained a monomeric form of protein H), the protease activity was present in a low MM form. This finding suggests that the protease might have a noncovalent association with an outer membrane component, as has been suggested for RTX toxins (1,13). *P. gingivalis* proteases, able to cleave immunoglobulins and other molecules, have activity at a broad range of MM and pH. Besides that, lower molecular mass activities (50–60 kDa) could be detected under reducing conditions (30,35), as was observed with our *A. pleuropneumoniae* protease. Recently, it was shown in *P. gingivalis* that different proteolytic bands originated from 2 different proteases, which are associated with other molecules (hemagglutinins or LPS) (30).

The *A. pleuropneumoniae* protease can be produced in vivo, as well as in vitro. Sera of pigs infected with this bacterium, but not those from pigs vaccinated with a bacterin, recognized our protease by immunoblotting. Bacterins are prepared with chemically inactivated whole-cell bacteria, with no components secreted to culture medium; thus, proteases should not be present. This was confirmed when we tested the serum of an animal vaccinated with the CSN of *A. pleuropneumoniae*. This serum recognized the purified protease in immunoblots. Furthermore, in preliminary studies with pulmonary tissue of pigs affected with PCP, we have observed the presence of the protease by immunofluorescence using a polyclonal antibody against the > 200-kDa purified protease.

We also found that antibodies raised against the > 200-kDa protease of *A. pleuropneumoniae* serotype 1 recognized proteins in CSN from all the 12 serotypes of the biotype 1 and the 2 serotypes of the biotype 2. Furthermore, immune sera from pigs infected naturally or experimentally with different serotypes, recognized the protease purified from the serotype 1. Thus, the protease is a common factor to all the serotypes and it is produced in vivo.

Other molecules common to the different serotypes have been recently described, and are principally from the outer membrane; these proteins are also immunogenic and can differentiate between vaccinated and infected animals (6).

We observed proteolytic activity in both reference and field strains. However, less activity was displayed by the reference strains. This could be due to the passage number in the laboratory, as was already described (24). With respect to the immune recognition, a better recognition was observed when samples were treated with chaotropic agents (compare Fig. 5B vs 6B). Probably when the proteolytic aggregate is dissociated, monomers could have a better exposure to the interaction with the antibodies.

Bacterial proteases are good antigens (19,22,29). In addition, the protective effect of secreted compounds (proteases could be included) by *A. pleuropneumoniae* has been demonstrated by using cell-free supernatants as vaccines (7,26). More studies will be neccesary to determine the importance that these kinds of proteases could have in the treatment of PCP.

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