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RESEARCH ARTICLE

Neutralization of *Pseudomonas aeruginosa* enzymatic activity by antibodies elicited with proteins of *Larrea divaricata* Cav.

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Abstract

Larrea divaricata Cav. (Jarilla) is a bush widely used in folk therapy for the treatment of several pathologies. Partially purified proteins of crude extract (JPCE) cross-react with proteins of Gram-negative bacteria, including *Pseudomonas aeruginosa*, which is an opportunistic pathogen that causes several intrahospitalary infections. This bacterium produces many proteins with enzymatic activity, including hemolysins and proteases that play a major role in acute infection caused by this bacterium. The aim of our work was to investigate if antibodies against with *L. divaricata* neutralize the hemolytic and proteolytic activity of *P. aeruginosa*. The hemolytic activity of soluble cellular proteins was inhibited 100% and extracellular proteins (EP) showed an inhibition between 44 and 95% when both bacterial fractions were treated with anti-JPCE serum. Also, in EP the neutralization was directed towards the active site of the hemolysin. When protease activity of extracellular products was tested, bands of 217, 155, 121, 47 and 27 kDa were observed in native zymograms. Neutralization between 55 and 70% of the bands of 217, 155 and 121 kDa was observed when EP were treated with anti-JPCE serum. In conclusion, our data clearly demonstrate that antibodies elicited with *L. divaricata*' proteins are able to neutralize the hemolytic and proteolytic activity of *P. aeruginosa* cellular and extracellular proteins. Our study constitutes the first report that associates the immunogenicity of plant proteins and bacterial proteins with enzymatic activity. These findings could be relevant in the development of alternatives therapies for patients suffering intrahospitalary opportunistic infections with *P. aeruginosa*.

Keywords: *Larrea divaricata* Cav., cross-reaction, aqueous extract immunogenic proteins, *Pseudomonas aeruginosa*, immune stimulants

Introduction

Larrea divaricata Cav. (Jarilla) is a plant that belongs to the Zygophyllaceae family. It is widely spread from the Middle West of North America to all the extension of South America.[1] This plant is widely used in folk medicine for the treatment of microbial infections, wounds, rheumatism, gastric disturbances, and tumors among others. Experimental studies with *L. divaricata* extracts have demonstrated fungotoxic and antimicrobial activity,[2–4] antitumoral effects,[5–7] immunostimulatory effects[8,9] and cross-reaction with proteins of Gram-negative bacteria.[10]

Pseudomonas aeruginosa is a Gram-negative bacterium considered as an opportunistic pathogen which can cause severe and lethal infections in vulnerable hosts due to its ability to secrete many extracellular factors that are associated with the virulence.[11] It is a major health problem in hospitals, especially with immunocompromised patients (i.e. transplanted, patients with cancer, severe burns or AIDS).[12] This organism has high intrinsic resistance to antibiotics due to the low permeability of the outer membrane and the presence of numerous multiple drug efflux pumps.[13–15] *P. aeruginosa* produces several extracellular products, including hemolysins (phospholipase

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C and rhamnolipid) and proteases (elastase, 33 kDa and alkaline protease, 53 kDa) which play a major role in acute infection caused by this bacterium.[16,17] Various cell-associated and secreted antigens have been the subject of vaccine development, but to date there is not a single candidate vaccine antigen that can be identified as having an overwhelming advantage over other potential protective antigens.[15,18]

The aim of our work was to investigate if antibodies elicited with *L. divaricata* that cross-react with proteins of *P. aeruginosa* neutralize the enzymatic activity of these bacterial proteins.

Materials and methods

Plant material

Leaves and tender branches of *L. divaricata Cav*. were collected in Nogolí, San Luis, Argentina. The plant was identified in the herbarium of the National University of San Luis, where the voucher specimen number UNSL # 467 is deposited.

Crude extract

The crude extract from fresh material in PBS pH 7.4 at 15% w/v was obtained. The material was left at 4°C during 24 h, then ground in a mortar and the aqueous fraction was filtered with a filter paper (Whatman N° 40).

Obtention of Jarilla partially purified proteins of crude extract (JPCE)

The crude extract was filtered through a 0.45-μm membrane and sterilized by a 0.22-μm membrane. The proteins were concentrated and partially purified by using membrane concentrators (Centriplus Amicon) with a 10 kDa cut off.[19] JPCE were employed subsequently for (i) immunizations, (ii) SDS-PAGE, (iii) Western Blot, (iv) ELISA and (v) enzymatic assays.

Bacterial strain and inocula

The reference strain of *P. aeruginosa* ATCC 27853 (type strain) was used, obtained from the American Type Culture Collection (Maryland USA), and kindly provided by Bioq.Olga Aliendres of Centorbi (Laboratory of Microbiology UNSL, San Luis, Argentina). *P. aeruginosa* was isolated on MacConkey agar, at 37°C for 24 h. Colonies were collected to obtain a suspension comparable with N°5 pattern of the McFarland scale ($1–2 \times 10^8$ CFU/mL). suspension (1 mL) was added to 150 mL of Eagle medium free of proteins and incubated at 37°C with constant stirring for 24 h.

Obtaining of cellular proteins of P. aeruginosa

The cellular proteins were obtained as described by Michiels et al.[20] The cells were separated from the medium by centrifugation at 8000 ×g for 10 min and the cells were washed 2 times with sterile PBS. The cell suspension was adjusted to $OD_{600} = 1$ and then sonicated in a Vibra Cell sonicator (Sonics & Materials Inc., Dambury, Connecticut, USA) for 4 min at 20 kHz in a discontinuous way (50% cycles) in sonication buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, pH 8). To obtain the soluble cellular proteins (SCP), the sonicate was centrifuged 1 or 2 times at low speed (20 min at 1000 ×g) to remove cellular debris and whole cells. The supernatant was used as **antigenic** extract and stored at –20°C until use.

Obtaining of extracellular proteins (EP) of P. aeruginosa

The extracellular proteins were obtained as described by Daza et al.[12] After the centrifugation of the cells, the supernatant of the culture was filtered through 0.22 μm membranes and dialyzed against distilled water for a period of 24 h at 4°C. Then, it was lyophilized and resuspended in 2 mL of sterile saline. The EP were kept at –20°C until use. The **protein** content EP was determined by Lowry's method[21] employing a standard of 0.2 mg/mL albumin. EP were employed subsequently for enzymatic assays.

Animals

Groups of Rockland mice of both sexes (18–21 g) were employed for the trials of active immunization. Animals were housed and cared for at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, in accordance with institutional guidelines.

Active immunization

Animals were immunized twice subcutaneously with a 3-week interval, with 0.2 mL of JPCE (0.3 mg/mL) in $AlPO_4$ (1:1) as adjuvant. Fifteen days after the second dose, serum from **submandibular** blood was obtained.

Neutralization assays of the enzymatic activity of *P. aeruginosa* with anti-JPCE serum

Hemolytic activity

The hemolytic activity was determined for EP, SCP and JPCE as described by **Mattar et al**.[19] The human red blood cells at 1% plus PBS were used as negative control. The samples with **hemolytic activity** were subjected to incubation 1 h at 37°C with anti-JPCE serum (titer 1:1600, **immune serum anti-JPCE more representative**) at several dilutions (1/50, 1/100, 1/200 and 1/400), centrifuged at 16.000 rpm and the supernatant tested for hemolytic activity. The neutralization test was evidenced by the decrease or disappearance of the hemolytic activity.

Protease activity: Zymography

The protease activity was determined for EP and JPCE. Polyacrylamide gels at 12% containing 1% of gelatine were used. The samples were subjected to native (sample buffer composition: 6.25 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, and 0.025% bromophenol blue; incubated 30 min at 37°C) or denaturing conditions (sample buffer with 5% β-mercaptoethanol; boiled 4 min). Gels were run at 4°C on a Mini-protean

vertical slab electrophoresis cell (Bio-Rad Laboratories, Richmond, CA, USA). Later, the gels were washed with 2,5 % Triton X-100 in buffer TNC (50 mM Tris–HCl pH 7,5; 0,15 M NaCl; 10 mM $CaCl_{2;}$ 0,02% NaN_3) and incubated 12–24 h at 37°C in incubation buffer (50 mM Tris–HCl pH 7,5; 0,15 M NaCl; 10 mM $CaCl_{2;}$ 0,02% $NaN_{3;}$ 1% Tritón X-100). The degradation was revealed by staining the gel with Coomassie Blue, where the white bands corresponded to the degradation zones caused by the enzymatic activity. The bands' molecular masses (Mm) were identified by comparison with patterns whose Mm are known (kDa): 116 (β-galactosidase), 66.2 (bovine serum albumin), 45 (egg albumin), 35 (lactic dehydrogenase), 25 (Bsp981 REasa), 18.4 (β-lactoglobulin), 14.4 (lysozyme) (Fermentas). To determine the neutralizing activity of the anti-JPCE antibodies, dilutions of 1/50, 1/100 and 1/200 were used. The neutralization was evidenced by an increase in staining compared with the positive control (proteins without incubation with anti-JPCE serum).[17]

Analysis of bands on Zymography

The bands were digitalized with a scanner (UMAX S-GE) with Corel Photo-Paint X5 version 15.0.0.489 (C) 2010 Corel Corporation and the relative intensity (RI) of individual bands was analyzed. The RIs were expressed as percentage of absorbance of the band compared with the background.

Data analysis

All the experiments were performed independently at least two times. Statistical significance was determined by one-way analysis of variance and for further comparisons, we used the Tukey's test. For non-homogeneous variance, we used the Student's *t*-test. A $p \leq 0.05$ was considered statistically significant.

Results

Determination of enzymatic activity of JPCE, SCP and EP of P. aeruginosa. Neutralization tests using sera from animals immunized with JPCE

Specific hemolytic activity (SHA) of JPCE, SCP and EP

The SHA of JPCE, SCP and EP was assessed on human red blood cells (RBC) at 1%. The results obtained are shown in Table 1. High values of SHA were observed for JPCE, while the lowest SHA was obtained with SCP.

Table 1. Hemolytic activity of JPCE and cellular and extracellular fractions of P. aeruginosa against human red blood cells at 1%.

Antigen	HU_{50}	Protein (μg/100 μL)	SHA
JPCE	256	17.1	15
SCP	1	89	0.01
EP	13	23	0.56

The results are expressed as hemolytic units 50 (HU50) and specific hemolytic activity (SHA = HU50/μg protein) of JPCE, SCP and EP.

Neutralizing assays of hemolytic activity of SCP, EP and JPCE with anti-JPCE sera

We investigated the presence of neutralizing anti-hemolysin antibodies in the serum of mice immunized with JPCE. The immune serum anti-JPCE more representative (titer 1/1600) was serially diluted from 1/50 to 1/400. The immune sera obtained with different concentrations of protein preparations of SCP, EP and JPCE were incubated. After centrifugation at high speed revolutions, the percentage of hemolytic activity in the supernatants was determined. The results are expressed in Figure 1 and Table 2. The results showed a total inhibition (100%) of the hemolytic activity present in SCP when it was treated with the four dilutions of anti-JPCE serum. When EP were treated with anti-JPCE serum, the percentage of hemolytic activity inhibition was between 44 and 95%. When JPCE were treated with anti-JPCE serum, a marked decrease in hemolytic activity between 39 and 96% was observed, depending on the dilution of antibody. On the other hand, there was a dose–response effect regarding the hemolytic activity and protein concentration for the same proteic fraction.

Given that the EP and JPCE presented hemolytic activity and it was neutralized in the presence of anti-JPCE, the percentage of hemolysis in the pellet obtained after centrifugation of the immunoreaction was determined, to test if the immunocomplexes possessed hemolytic activity. The dilution of anti-JPCE serum tested was 1/200 and protein concentrations for JPCE and EP were 684,40 and 706,25 μg/mL, respectively. Both concentrations showed 100% and 87% of hemolysis in the presence of negative control serum (pre-immune serum). In Figure 2A, the results show that the hemolytic activity of JPCE was neutralized with anti-JPCE (well 4, supernatant of the immunoreaction) resulting in a lower hemolytic activity regarding the maximum activity observed in well 3 (JPCE not treated with anti-JPCE). However, in well 5 (pellet of the immunoreaction), we observed hemolysis similar to the well 3.

When EP were tested (Figure 2B), in well 5 (immunoreaction pellet) an activity of 49% of hemolysis was observed. This percentage was lower than the obtained when EP were not treated with anti-JPCE (percentage of maximum hemolysis: 87%).

Neutralization assays of protease activity of JPCE and EP with anti-JPCE serum.

P. aeruginosa produces enzymes with protease activity, such as elastase and alkaline protease, which in turn are virulence factors secreted outside the bacteria. So we investigated the protease activity in EP and the presence of neutralizing antibodies against proteolytic enzymes in serum of mice immunized with JPCE. The immune serum anti-JPCE more representative (dilution 1/1600) was serially diluted from 1/50 to 1/200. The immune serum obtained was incubated with EP (0.22 mg/mL), whose concentration presented protease activity in the absence of the serum. After centrifugation at high speed, the percentage of proteolytic activity in the supernatant

was determined. The same was evidenced by the presence of five bands corresponding to the MM of 217, 155, 121, 47 and 27 kDa (Figure 3).

When EP were incubated with anti-JPCE serum, there was a neutralization of the proteolytic activity of the bands of 217, 155 and 121 between 70 and 55% with serum anti-JPCE diluted 1/50, while for the dilution 1/100, the neutralization was 18 to 25% depending on the band. No neutralization of proteolytic activity was observed when EP was incubated with serum anti-JPCE 1/200. In bands of 47 and 27 kDa, no neutralization was observed with any dilution of anti-JPCE tested (Table 3).

To assess the enzymatic activity of proteins in denatured state, EP was treated with sample buffer containing β-mercaptoethanol and subjected to 94°C for 4 min. EP was incubated with dilutions of anti-JPCE serum (1/50, 1/100 and 1/200). After incubation, the supernatant under denaturing conditions was analyzed. The zymography obtained is shown in Figure 4 where three proteolytic bands of 47, 37 and 32 kDa were observed, which were not neutralized by anti-JPCE antibodies. There were no bands of molecular mass of 217, 155, 121 and 27 kDa in EP with and without anti-JPCE that were found in the native zymography.

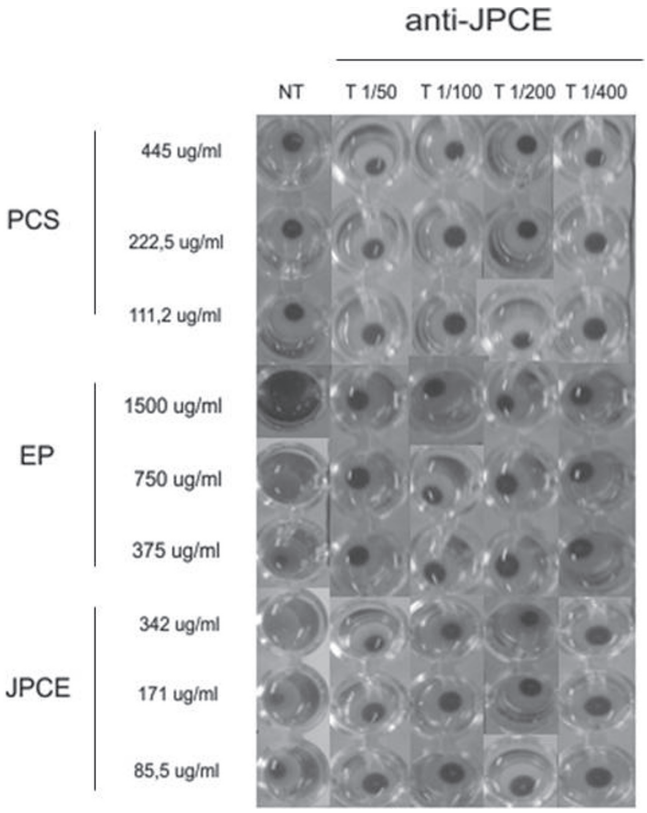

Figure 1. Neutralization assay of hemolytic activity of different protein concentrations of SCP, EP and JPCE treated with different dilutions of anti-JPCE (**T**). **NT**: hemolytic activity of proteic fractions in different concentrations not treated with serum anti-JPCE.

Table 2. Neutralization assay of hemolytic activity of different proteic concentrations of SCP, EP and JPCE with sera from animals immunized with JPCE.

	Protein	NT	T 1/50		T 1/100		T 1/200		T 1/400	
Antigen	(μg/ml)	%H	%H	% I	%H	% I	%H	%I	%H	%I
SCP	445	20	0	100	0	100	0	100	0	100
SCP	222.5	3	0	100	0	100	0	100	0	100
SCP	111.2	0	0	0	0	0	0	0	0	0
EP	1.500	>100	28	72	54	46	51	49	56	44
EP	750	>100	13	87	33	67	33	67	32	68
EP	375	87	5	95	22	78	23	77	21	79
JPCE	342	75	26	74	52	48	61	39	—	
JPCE	71	1	4	6	6	4	19	81	—	—
JPCE	85.5	25	4	96	19	81	18	82	—	—

The results are expressed as percentage of hemolytic activity (%H) and percentage of inhibition (% I).
NT, not treated with anti-JPCE serum; T, treated with dilutions 1/50, 1/100, 1/200 and 1/400 of anti-JPCE serum.

In the case of JPCE, the protease activity was evidenced by a native zymography. We used a concentration of 2190 µg/mL of JPCE and it was incubated with different dilutions of anti-JPCE serum. The results are expressed in Figure 5, where four bands with protease activity were observed with JPCE untreated corresponding to MM 75, 57, 23 and 18 kDa. When protease activity was assessed in the supernatant obtained after reaction of JPCE with anti-JPCE serum, a neutralization of proteolytic activity in the bands of 75 kDa and 57 kDa was observed, and the activity remaining in the bands of 23 and 18 kDa.

When JPCE was tested under denaturing conditions, no proteolytic activity was observed (figure not shown).

Discussion

Determination of enzymatic activity of JPCE and cellular and extracellular fractions of *P. aeruginosa*. Neutralization tests using anti-JPCE serum

Hemolytic activity of JPCE, SCP and EP

The hemolysins of *P. aeruginosa* are exotoxins, like most of the hemolysins produced by other bacteria.

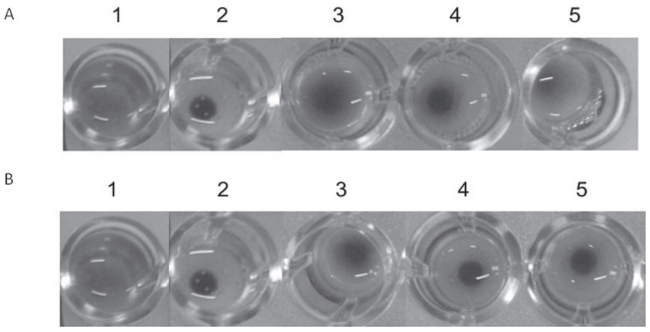

Figure 2. Neutralization assay of hemolytic activity of **(A)** JPCE and **(B)** EP with serum from animals immunized with JPCE (anti-JPCE 1/200). Well 1, C (+) of hemolysis = RBC + water + SDS 1%, well 2, C (–) of hemolysis = RBC + PBS; well 3, maximum hemolytic activity in the presence of negative control serum (pre-immune serum, serum C (–)); well 4, hemolytic activity in supernatant after treatment with anti-JPCE serum, and well 5, hemolytic activity in pellet after treatment with anti-JPCE serum.

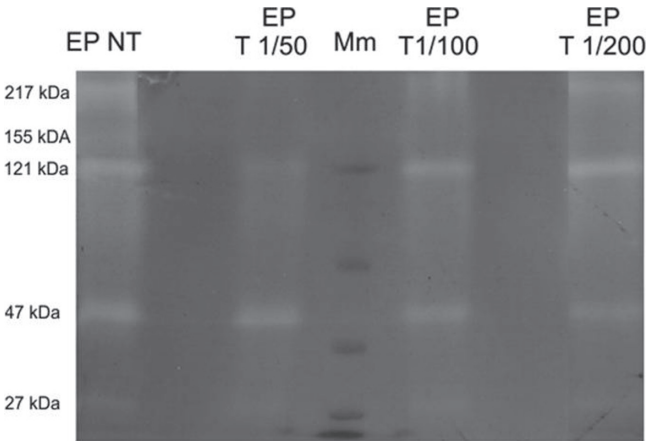

Figure 3. Zymography neutralization assay of proteolytic activity of EP with serum from animals immunized with JPCE (anti-JPCE) at dilutions 1/50, 1/100 and 1/200. **T;** EP treated with serum anti-JPCE; **NT**, EP not treated with serum anti-JPCE (100% of proteolytic activity). The zymography was carried out under native conditions. Mm, molecular mass markers expressed in kDa.

Table 3. Neutralization assay of proteolytic activity of EP with serum from animals immunized with JPCE (anti-JPCE) at dilutions 1/50, 1/100 and 1/200.

	NT	T 1/50		T 1/100		T 1/200	
Band (kDa)	% PA	% PA	% I	% PA	% I	%PA	% I
217	100	45	55	100	-	100	-
155	100	30	70	75	25	100	-
121	100	35	65	82	18	100	-
47	100	100	-	100	-	100	-
27	100	100	-	100	-	100	-

The results are expressed as the percentage of proteolytic activity (% PA) and percentage of inhibition (% I) in regard to the relative intensity (RI) of the band corresponding to 100% of proteolytic activity.
NT, EP not treated with anti-JPCE serum; **T**, EP treated with dilutions 1/50, 1/100 and 1/200 of anti-JPCE serum.

Quoted in literature *P. aeruginosa* produces at least two toxins with hemolytic activity, phospholipase C and rhamnolipid.[22] We observed that anti-JPCE antibodies recognized proteins with hemolytic activity present in cellular and extracellular fractions of *P. aeruginosa*. These results demonstrate that JPCE has immunogenic determinants that are present in enzymes of *P. aeruginosa* with hemolytic activity. In the case of JPCE, the hemolytic activity was inhibited with anti-JPCE serum. Moreover, we observed that the anti-JPCE antibodies are not directed toward the active site of the JPCE enzyme. On the other hand, in the case of EP, anti-JPCE neutralized the hemolytic activity present in the pellet, thus indicating the presence of antibodies that recognize epitopes in the active site of the enzyme. However, antibodies did not fully inhibit the enzymatic activity. This result suggests that the antigenic determinant of the hemolysin may be distinct from the active portion of the protein molecule. The degree of neutralization of the active site may depend on the distance from the antigenic site on the molecule.[23,24]

Protease activity: zimography

Native and denaturing zymographies were performed with EP and JPCE to detect the presence of proteolytic activity in these antigens. It was noted that EP, in native conditions, produced five bands of 217, 155, 121, 47 and 27 kDa. The bands of 155 and 47 kDa could be the 160 kDa elastase and the 53 kDa alkaline protease respectively, which have been previously described.[16,25] When EP was incubated with anti-JPCE serum, there was a neutralization of the proteolytic activity of the bands of 217, 155 and 121 kDa. This could indicate the recognition by anti-JPCE antibodies of some proteins with protease activity, including elastase, which is one of the most important virulence factors involved in the pathogenicity of *P. aeruginosa*.[17,22]

Then we investigated whether there were proteins with protease activity under denaturing conditions. We observed that EP presented three proteolytic bands of 47, 37 and 32 kDa. The band of 32 kDa could be elastase (33 kDa) that in denatured state retains its proteolytic activity and in native state forms a multimer of 160 kDa.[16,25] The band of 47 kDa could be the same observed in the

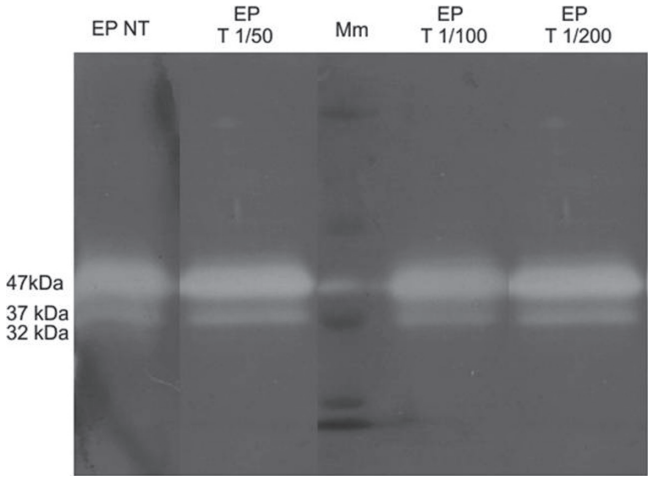

Figure 4. Zymography EP proteolytic activity in the absence of anti-JPCE (**NT**) and incubated with anti-JPCE serum dilutions 1/50, 1/100 and 1/200 (**T**). The zymography was carried out under denaturing conditions. Mm, molecular mass markers expressed in kDa.

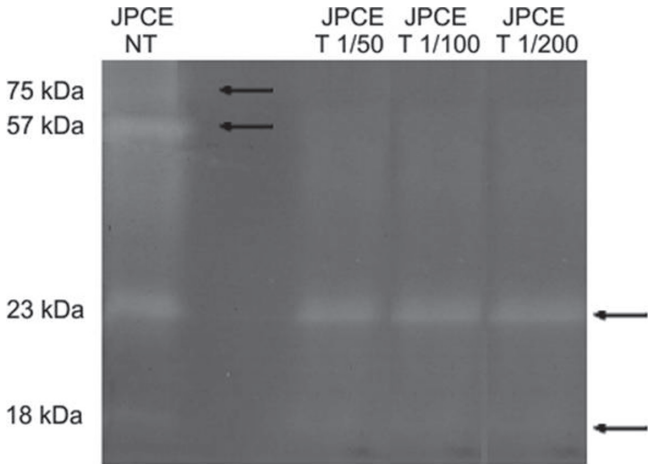

Figure 5. Zymography JPCE neutralized proteolytic activity with different dilutions of anti-JPCE serum (**JPCE T** (1/50, 1/100 and 1/200)). **JPCE NT**, JPCE in the absence of anti-JPCE serum. The zymography was carried out under native conditions. Mm, molecular mass markers expressed in kDa.

native zymography, as it may be a monomer, which is not affected by denaturing and retains its proteolytic activity, as has been observed for alkaline protease.[16]

To evaluate the participation of anti-JPCE antibodies in neutralizing protease activity, the EP antigen was incubated with anti-JPCE. The supernatant obtained after the reaction was subjected to denaturing conditions and its proteolytic activity was determined in a zymography. We observed that there was no protease activity decreased in none of the bands (47, 37 and 32 kDa) with the dilutions of the antibodies tested; therefore these proteins were not recognized by the anti-JPCE. On the other hand, we observed that the band of 32 kDa is present in the supernatant and has proteolytic activity. Given that the band of 155 kDa under native conditions was not 100% neutralized, could suggest that the minimum dilution of anti-JPCE tested (dilution 1/50) would not be binding to all the immunoreactive sites of the 155 kDa protein, so the 32 kDa band could be a denaturing product of the 155 kDa multimer, while retaining its protease activity.[16]

For JPCE, the native zymography revealed the presence of four bands with proteolytic activity of 75, 57, 23 and 18 kDa. When JPCE was incubated with anti-JPCE, there was a neutralization of proteolytic activity in the bands of 75 kDa and 57 kDa. These results show the presence of antibodies that recognize antigenic determinants in JPCE with protease activity. When we tested the proteolytic activity of JPCE under denaturing conditions, no proteolytic activity was observed, suggesting that JPCE proteases would act only in native conditions.

Conclusion

Our data clearly demonstrate that antibodies elicited with *L. divaricata* proteins are able to neutralize the hemolytic and proteolytic activity of *P. aeruginosa* cellular and extracellular proteins. Anti-JPCE antibodies recognized proteins with protease activity, including elastase (160 kDa), which is one of the most important virulence factors of *P. aeruginosa*.[22] This *in vitro* protection studies demonstrate the ability of JPCE antibodies to prevent the lytic effect of these proteins, which are relevant in *P. aeruginosa* pathogenesis[16,17] and its neutralization is very important for host survival.[26] Our study constitutes the first report that associates the immunogenicity of plant proteins and bacterial proteins with enzymatic activity. These results highlight an unknown feature of *L. divaricata* extract activity. Further studies are under way in order to determine whether the anti-JPCE antibodies are protective. These findings could be relevant in the development of alternatives therapies for patients suffering intrahospitalary opportunistic infections with *P. aeruginosa*.

Declaration of interest

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