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Enzymatic activities and protein profile of latex from *Calotropis procera*

Cleverson Diniz T. Freitas [a], Jefferson Soares Oliveira [a],
Maria Raquel A. Miranda [a], Nívea Maria R. Macedo [a], Maurício Pereira Sales [b],
Laurival A. Villas-Boas [c], Márcio Viana Ramos [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, Campus do Pici,
Cx. Postal 6033, Fortaleza, Ceará CEP 60451-970, Brazil*
[b] *Departamento de Bioquímica e Centro de Biociências da Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil*
[c] *Instituto de Biologia, Departamento de Biologia Geral, Universidade Federal da Bahia, Salvador, Bahia, Brazil*

Abstract

The laticifer fluid of *Calotropis procera* is rich in proteins and there is evidence that they are involved in the pharmacological properties of the latex. However, not much is known about how the latex-containing proteins are produced or their functions. In this study, laticifer proteins of *C. procera* were pooled and examined by 1D and 2D electrophoresis, masses spectrometry (MALDI-TOF) and characterized in respect of proteolytic activity and oxidative enzymes. Soluble laticifer proteins were predominantly composed of basic proteins (PI > 6.0) with molecular masses varying between 5 and 95 kDa. Proteins with a molecular mass of approximately 26,000 Da were more evident. Strong anti-oxidative activity of superoxide dismutase (EC 1.15.1.1) (1007.74 ± 91.89 U g^{-1} DM) and, to a lesser extent ascorbate peroxidase (EC 1.11.1.1) ($0.117^d \pm 0.013$ μMol H$_2$O$_2$ g^{-1} min^{-1}), were detected. However, catalase (EC 1.11.1.6) was absent. The strong proteolytic activities of laticifer proteins from *C. procera* were shown to be shared by at least four distinct cysteine proteinases (EC 3.4.22.16) that were isolated by gel filtration chromatography. Serine and metaloproteinases were not detected and aspartic proteinase activities were barely visible. Chitinases (EC 3.2.1.14) were also isolated in a chitin column and their activities quantified. The presence of these enzymatic activities in latex from *C. procera* may confirm their involvement in resistance to phytopathogens and insects, mainly in its leaves where the latex circulates abundantly.
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1. Introduction

Latex is widely distributed in plants and 12,000–35,000 species have been reported to contain it [14,20]. It is a milky fluid composed of a liquid serum holding, either in suspension or solution, constituting a complex mixture of molecules [25]. A wide range of proteins is found in latex fluids, such as carbohydrate-binding proteins (lectins) and *N*-acetyl-β-ᴅ-glucosaminidases.

These were purified from *Hevea brasiliensis* latex [17,18] and chitinases were detected in *Carica papaya* and *Ficus microcarpa* latexes [4,36]. Proteolytic enzymes, mainly serine and cysteine types, are more abundant proteinases found in latex fluids [27]. Cysteine proteinases have been recently purified to homogeneity from distinct latex fluids [11,24,31] and papain, found in the latex of *C. papaya*, has been continuously studied since it was discovered [5].

Evidences have supported the possible involvement of laticifer proteins (LP) in the plant defense mechanisms [28]. However, additional information about its occurrence, biological activities and structure of latex proteins is still required to confirm this hypothesis.

The shrub *Calotropis procera* is found mainly in tropical regions and to a lesser extent in sub-tropical areas. The plant is well-known for its great capacity of producing latex which exudates from damaged leaves. There is a growing demand in popular medicine for the different parts of the plant, including the latex, and as a consequence a vast literature describes its related potential. However, limited information about the biochemical properties of the latex of *C. procera* is available. A bacteriolytic enzyme and recently the purification of a cysteine proteinase from *C. procera* latex have been described [12,35], however, these are the only laticifer proteins from *C. procera* of which there is information. The motivations factor for this study is the lack of information available about latex proteins from *C. procera*. Therefore, preliminary bioassays testing the potential of these proteins against insects were recently concluded in our laboratory. The results suggested that the laticifer proteins could be part of the defensive strategies against insects. In an attempt to investigate this hypothesis, a partial biochemical and enzymatic characterization was performed to obtain new information on the occurrence and biological activities of proteins from the latex of *C. procera*.

2. Results and discussion

2.1. Protein content

Six independent samples of the whole latex from *C. procera* were independently prepared as described in Section 4.2.1. The dry mass of rubber in 20 ml was estimated and as shown in Table 1, rubber corresponded to more than 80% of the dry mass while the remaining soluble fraction comprised less than 20%. The content of soluble proteins was shown to be approximately $8\ mg\ ml^{-1}$. This suggests that the latex from *C. procera* is a rich source of proteins. Soluble proteins were thus, used to characterize the biochemical and enzymatic profile of the latex. Two-dimensional electrophoresis (2D) analyses revealed that LP exhibits a large range of proteins with relative molecular masses varying from 5000 to 95,000 Da. This information was further checked by masses spectrometry (Fig. 1). In both figures (A and B), an important protein component with an approximate molecular mass of

26,000 Da was seen. Most proteins, by 2D, were distributed in the pH range above 5.5 with prevalence of basic characters. LP was fractionated by gel filtration in a Sephacryl S-100 column (Fig. 2). The fractions recovered were divided in sub-fractions according to the absorbance profile and examined by electrophoresis. As expected, the main protein peak was represented by proteins with molecular mass corresponding to 26,000 Da. These fractions were further tested for proteolytic activities. It was interesting to note that a high molecular weight protein barely detected in LP fraction was seen clearly in electrophoresis (Fig. 2). This protein appeared in PI after the fractionation of LP on Sephacryl. A discrete peak with 95,745 Da was also observed in the masses spectra (Fig. 1).

2.2. Enzymes

2.2.1. Anti-oxidative enzymes

Both abiotic and biotic stresses may enhance the production of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [3,16]. Most ROS are generated in plants due to dismutation of superoxide, which is formed as a result of a single electron transfer to molecular oxygen in an electron transfer chain, mainly in the chloroplasts. Hydrogen peroxide plays an important role in plant defense mechanisms as its generation is one of the earliest responses to phytopathogenic microorganism invasion. H_2O_2 generated during the oxidative burst under biotic stress is involved in restricting pathogen growth and inducting pathogenesis related (PR) proteins, such as phytoalexins and anti-oxidant enzymes [7,21].

The activity of the anti-oxidant enzymes in LP fraction is presented in Table 1. Superoxide dismutase (SOD) activities are high in latex, thus SOD might play an important role in dismutation of superoxide radicals. Catalasic (CAT) activities were not detectable, although there were ascorbate peroxidase (APX) activities indicating that APX contributes, to some extent, in H_2O_2 detoxification in the latex of *C. procera*. Enzymes involved in anti-oxidative metabolism have been described as occurring in other laticifer materials [8,38]. Although the absence of catalase activity agrees with the results of other studies that failed to detect such activity in latex

Table 1
Recoveries of rubber and proteins from the whole latex of *C. procera* and anti-oxidative enzyme activities investigated in LP fraction from *C. procera*

Samples[a]	Dry mass of rubber		Dry mass of lyophilized proteins		Total mass of protein in the whole latex ($mg\ ml^{-1}$)	Total soluble proteins in LP ($mg\ ml^{-1}$)[b]
	mg	%	mg	%		
X	1.314	82.52	277.83	17.48	13.88	8.85
SD	±0.14	±3.63	±62.31	±3.63	±3.12	±1.87

Enzymes	Activity
Ascorbate peroxidase	$0.117^d \pm 0.013\ \mu Mol\ H_2O_2\ g^{-1}\ min^{-1}$
Catalase	Not detected
Superoxide dismutase	$1007.749 \pm 91.892\ U\ g^{-1}\ DM$

[a] Six independent samples of 20 ml of latex from *C. procera* were prepared as described in Section 4.2.1. Dry mass of lyophilized proteins (LP) was considered as the fraction free of rubber.
[b] Soluble proteins were estimated by Bradford using a $10\ mg\ ml^{-1}$ solution of LP.
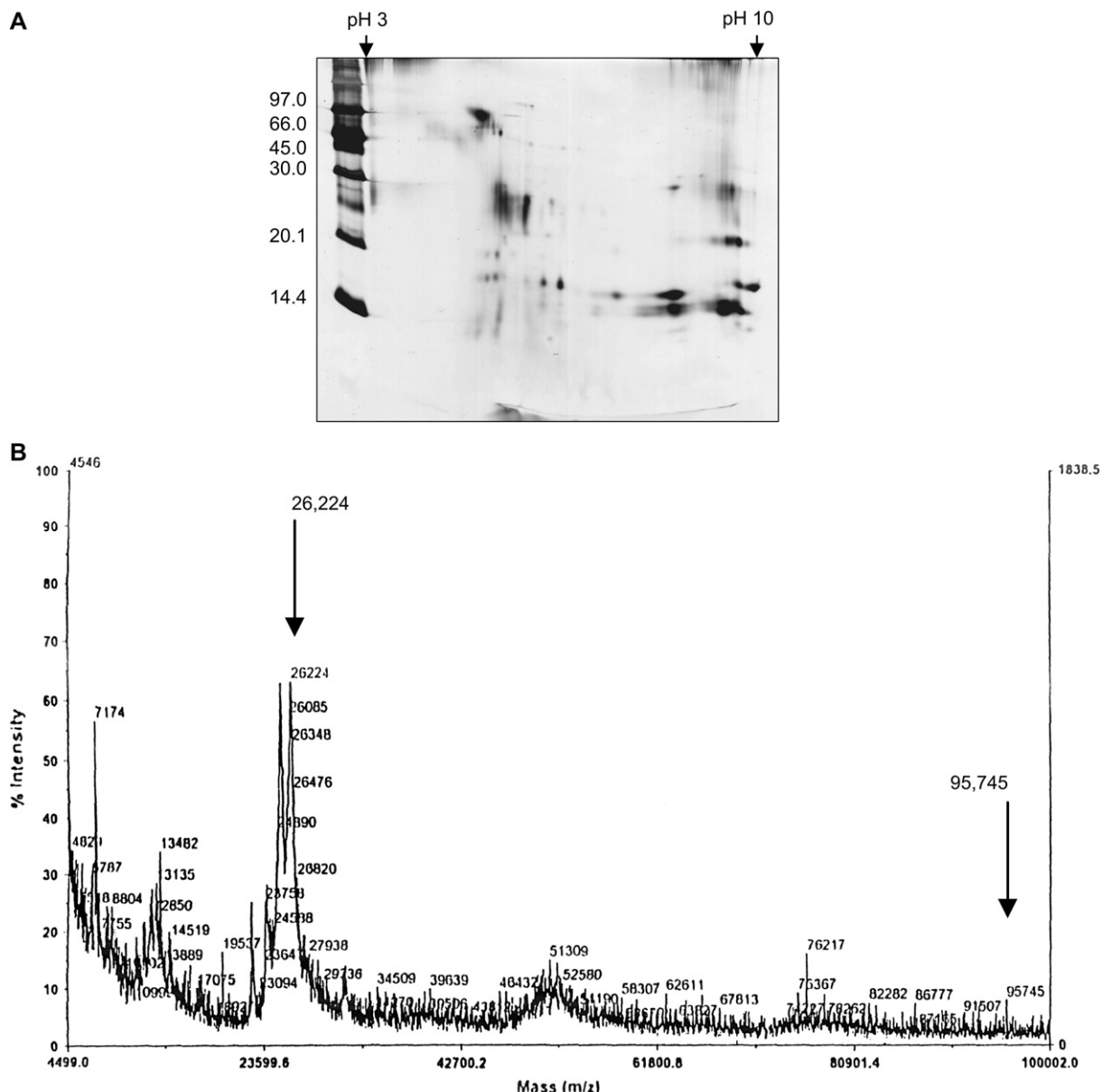
A

B

Fig. 1. (A) Two-dimensional polyacrylamide gel electrophoresis (12.5%) of laticifer proteins separated in the pH ranges of 3–10. Molecular weight markers were from Amersham Biosciences (product code 17-0446-01). Samples of 60 μg of proteins were applied in the dry strips as detailed in the materials and methods. (B) Masses spectra of laticifer proteins recorded in a Matrix Assisted Laser Desorption Ionization (MALDI) and Time of Fly (TOF) analysis. Arrows indicate the most abundant protein species found in polyacrylamide electrophoresis (26,224 Da) and the high molecular weight protein appearing in PI of Sephacryl S-100 gel filtration (95,745 Da) observed in (Fig. 2).

fluids, a very recent study of the latex of *Euphorbia characias* mentions a catalase-like activity [29]. Both ascorbate peroxidase and glutathione peroxidase were found in the latex of *H. brasiliensis* [22]. The authors suggested that both APx and GPx, involved in the detoxification of reactive oxygen species in vivo, might play a role in stress resistance and/or regulate the duration of latex flow since regular exploitation of rubber trees by tapping, a wounding process leads to an increase in the production of reactive oxygen species. Other studies involving anti-oxidant metabolism of *C. procera* are not available. However, the presence of both anti-oxidant enzymes, described here, may confirm latex as having

a protective role against possible oxidative damage which begins after the leaves are wounded.

2.2.2. Chitinolytic activity

Chitinolytic activity was measured in LP and fractions obtained after fractionizing LP in a chitin column. Two peaks were obtained; one corresponding to unbound proteins washed in the starting buffer (PI) and another, corresponding to bound proteins that were recovered after washing the column with 1 M acetic acid (PII). As shown in Fig. 3, LP exhibited a two-fold increase in the chitinolytic activity found in the whole latex. The total activity was separated in both PI and PII, but PII
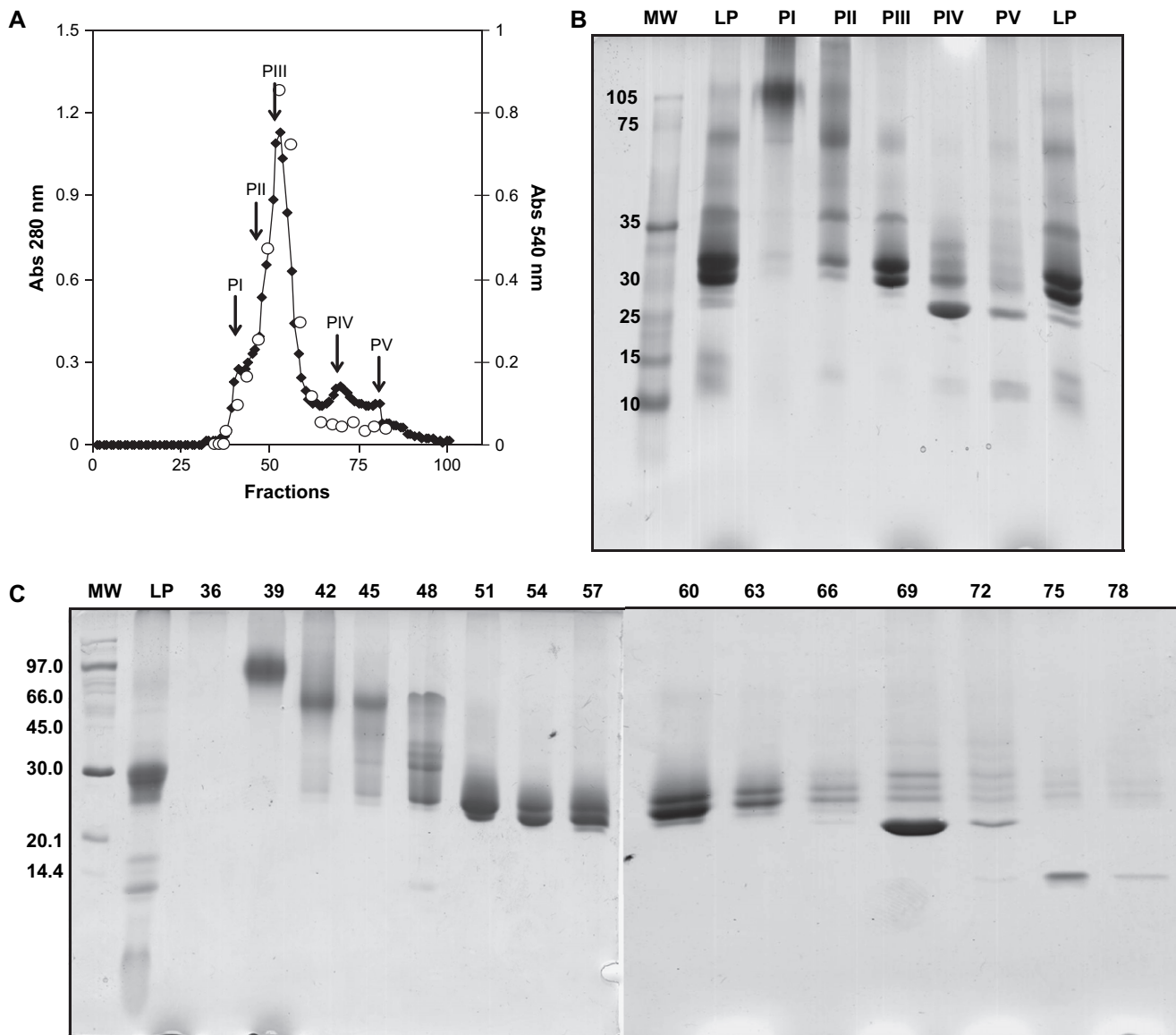
Fig. 2. (A) Gel filtration chromatography on Sephacryl-100 (104 × 2 cm) column and proteolytic activity (○) in the fractions using BANA as substrate. Laticifer proteins (30 mg ml⁻¹) were eluted with 50 mM ammonium bicarbonate buffer pH 7.9. Flow rate: 0.5 ml/min. Fractions: 2.5 mL. Arrows indicate intervals of fractions used to separate and poll the peaks. (B) Polyacrylamide gel (12.5%) electrophoresis of the peaks recovered after gel filtration. Molecular weight markers were from Amersham Biosciences Product N. RPN 800. (C) Polyacrylamide gel (12.5%) electrophoresis of sequential fractions recovered during gel filtration on Sephacryl S-100. Numbers correspond to fractions collected. Molecular weight markers were from Amersham Biosciences Product N. 17-0446-01. Proteolytic activity against BANA was detected in fractions: 42–69 with a maximum of 51.

was more active. However, saturation of the column was considered because activity in PI was considerably diminished while PII was similar, when the chromatography was developed with 25 mg of LP instead of 50 mg. Class I chitinase has been found in the latex of *F. microcarpa* and other latex fluids and this activity has been shown to exhibit antifungal activity [36]. Class II chitinases have been described in *C. papaya* latex [4]. The physiological role of plant chitinases is thought to protect plants against phytopathogenic fungi by degrading chitin, the major component of the cell walls of many fungi. A chitin-binding protein named hevein was first detected in the latex of *H. brasiliensis*. This short peptide is a potent anti-fungal agent but devoid of chitinolytic

activity. Following this view, chitinases present in the latex of *C. procera* are thought to form part of the defensive strategy of the plant.

2.2.3. Proteolytic activity

The ability of LP to digest substrates for endoproteinases, such as azocasein, BANA and BApNA was determined in different pH conditions (Fig. 4). The proteolysis in the presence of azocasein was more intense in pH 5.0. However, when LP was activated with DTT prior to the proteolysis, the total activity was greatly increased and the optimum pH was observed at pH 6.0. This result confirms the presence of a strong
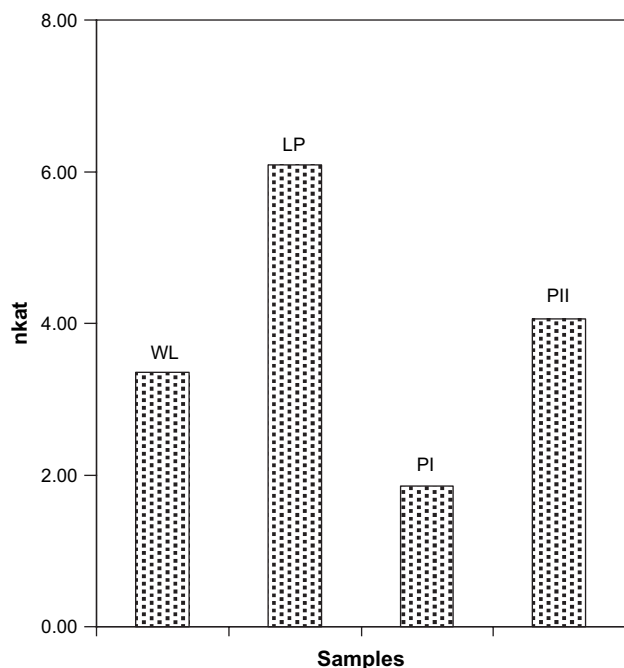
Fig. 3. Chitinolytic activity content in the whole latex of *C. procera* (WL), LP and chromatographic (PI) and (PII) peaks obtained after passing through a chitin column.

proteolysis in this latex which is mediated by cysteine proteinases activated by DTT [12].

Proteolysis of LP on BANA, a cysteine proteinase specific substrate, was higher at pH 6.0, however, lower to that of azocasein. This information and the fact that LP has ability to degrade azocasein without prior activation with DTT suggest the presence of proteolytic activity other than that of cysteine proteinases. Such an activity could not be attributed to serine proteases since no proteolysis was detected when LP was assayed against BApNA.

In addition, the proteolytic activity of LP was investigated in gelatin-containing gel at different pH (data not shown). Stronger proteolysis was detected at pH 5.0. Four distinct bands were also easily identified, suggesting the presence of at least four endogenous proteinases. Multiple cysteine proteinases have been described in the latex of *Calotropis gigantea* [2,32]. Recent works by Dubey and Jagannadham [12,13] suggested the presence of at least four distinct cysteine proteinases in *C. procera* latex and thus confirm the findings of this study. Other authors suggest that the presence of cysteine proteinase in different latex fluids may somehow be related to the defensive role of latex [37].

2.3. Characterization of proteolytic activity

Further characterization of the proteolytic activities was performed in inhibitory assays using specific inhibitors of proteinases. The inhibitors PMSF, EDTA and EGTA did not alter the total proteolytic activity of LP, however, E-64, a specific inhibitor of cysteine proteinase consistently diminished the proteolysis. Lack of inhibition by PMSF, EDTA and EGTA

confirms the absence of serine and metaloproteinases in LP. However, pepstatin, which specifically inhibits aspartic proteinases was also a strong inhibitor of azocasein proteolysis (Fig. 4). This data could explain the proteolysis of azocasein at the pH optimum of 5.0, observed when LP was not activated by DTT, and highlights the presence of an aspartic proteinase.

Digestion of BANA by LP was affected by PMSF, E-64 and iodoacetamide. The two latter inhibitors are highly specific for cysteine proteinases and thus confirm the previous results. PMSF, which is a common inhibitor of serine proteases, is reported to be able to inhibit cysteine proteinases [15].

Heat treatment of LP prior to proteolysis on azocasein affected enzymatic activities at 60 °C followed by a progressive loss of activity up to 90 °C (Fig. 5). This was not observed when BANA was used as a substrate. In this case, proteolysis was progressively reduced and annulled at 90 °C.

3. Conclusions

Little information is available about the biochemical activities present in the latex of *C. procera*. This work analyzed the protein and enzymatic profile of the latex after separating the proteins and rubber fractions. The latex was shown to be a rich source of proteins with distinguished proteolytic potency and anti-oxidant capacity. Among the evaluated anti-oxidant enzymes, superoxide dismutase was mainly responsible for ROS elimination. Cysteine proteinases were the stronger enzymatic activities detected and quantified but aspartic proteinases were also present. The presence of cysteine proteinases could be related to a defensive role of latex in plants. Likewise, the presence of chitinases reinforces the probable involvement of the latex in defensive actions. As initially stated, proteins of *C. procera* latex have been shown to have interesting effects on insects when tested in preliminary bioassays. Such results will appear elsewhere in the further. It is now expected that the occurrence of the chitinase and proteolytic enzymes in the latex could explain detrimental effects observed upon insects and this hypothesis is currently being tested. In conclusion, the soluble protein phase of *C. procera* latex emerges as a very interesting source of different classes of proteins worthy of being exploited as protecting proteins.

4. Methods

4.1. Chemicals

N-Benzoyl-DL-argininyl-*p*-nitroanilide (BapNA), *N*-benzoyl-DL-arginine β-naphthylamide hydrochloride (BANA), *trans*-epoxysuccinyl-L-leucylamido (4-guanidio)-butane (E-64), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA), phenylmethylsulphonylfluoride (PMSF), 4-(dimethyl-amino)cinnamaldehyde, pepstatin (PEP) and azocasein were purchased from Sigma Chemical Co. (St. Louis, MO). Iodoacetamide (IAA), molecular weight markers and Sephacryl S-100 were from Amersham Biosciences. Others chemicals were of analytical grade.
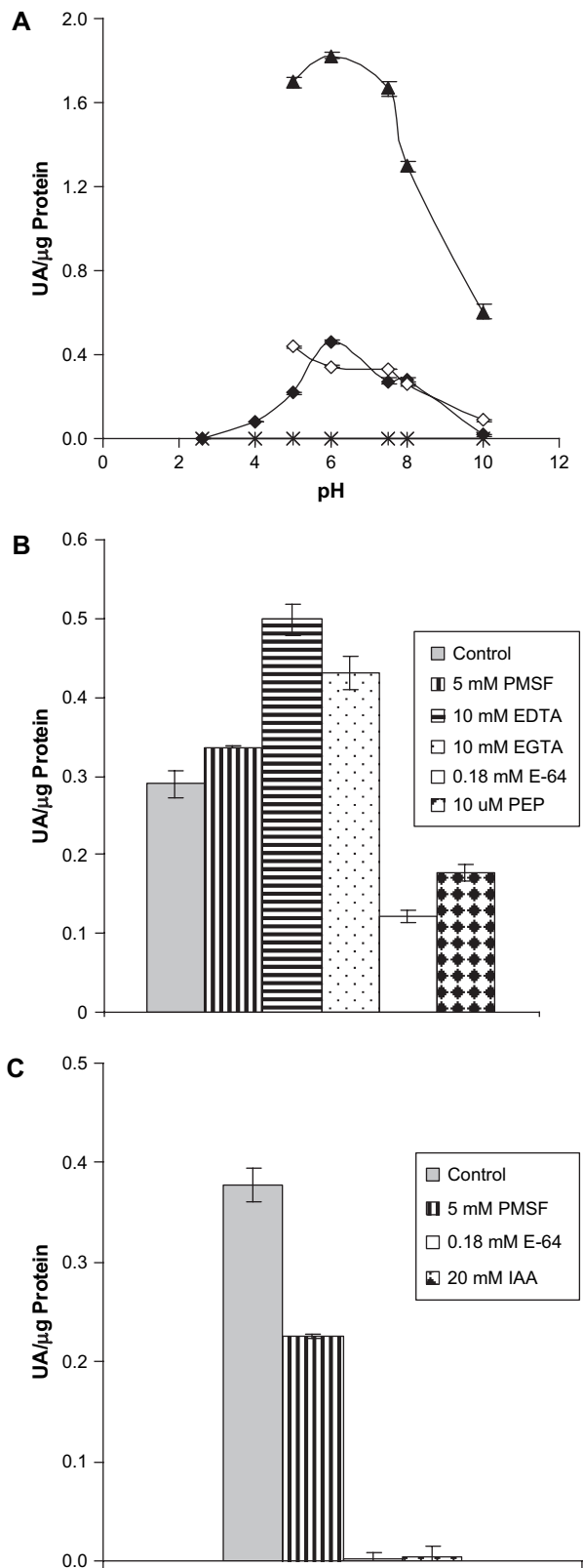
Fig. 4. Effect of pH on the proteolytic activity of LP (A) using azocasein (◇); BApNA (×); and proteolytic activity of LP pre-activated with DTT using Azocasein (▲) or BANA (●) and effects of various enzyme inhibitors on the proteolytic activity of laticifer proteins from *Calotropis procera*, using (B) azocasein and (C) BANA + DTT, as substrates. Each point has $n = 3$. Error bars indicate standard error of the mean.

4.2. Protein extraction and biochemical analysis

4.2.1. Latex preparation

The crude latex from *C. procera* (Ait.) R.Br. was collected from non-cultivated healthy plants and placed in distilled water (ratio of 1:1) and kept at room temperature until laboratory analysis was undertaken. The plant exsiccate (sample specimen no. 32663) as authenticated by Prof. Edson Paula Nunes, was deposited at the Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil.

Natural coagulation-like effect of the material was partially prevented by gentle agitation during collection. The samples were centrifuged at $5000 \times g$, 10 min at 4 °C and the supernatant, still containing non-precipitated rubber, was exhaustively dialyzed in distilled water and once more submitted to centrifugation as described. The clean supernatant, devoid of rubber, was lyophilized and stored until use; this fraction was denominated as laticifer proteins (LP). The dialysis step successfully precipitated 100% of the remaining rubber, and washed out small metabolites soluble in water. This was achieved using dialysis tube with a cut off of 8000 Da. After this procedure, the sample was expected to contain concentrated proteins.

4.2.2. Protein determination

Protein content was measured according to the Bradford procedure with bovine serum albumin as the protein standard [10].

4.2.3. 1D SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of SDS (PAGE–SDS) and 2-mercaptoethanol was performed as described by Laemmli [23]. Protein samples were dissolved in sample buffer [0.0625 M Tris buffer (pH 6.8) containing 2% SDS]. Runs were performed at 25 mA per gel at 25 °C for 4 h. Gels were stained with coomassie brilliant blue (R-350) solution in water:acetic acid:methanol (8/1/3.5, v/v/v) and de-colored with the same solution without the dye.

4.2.4. 2D SDS–PAGE

Eleven centimeter Immobiline DryStrips, pH 3–10 or 6–11 (Amersham Biosciences) were rehydrated overnight with buffer [7 M urea/2 M thiourea/1% CHAPS/2% appropriate IpG-buffer/bromophenol blue] containing the proteins. The electrophoretic run was performed on an Ettan IPGphor II system from Amersham Pharmacia Biotech. Electrical conditions were as suggested by the supplier. After the first-dimensional run, the IpG gel strips were incubated at room temperature to equilibrate the strips in 3 ml of equilibration buffer (50 mM Tris, 30% Glycerol, 6 M urea, 2% SDS and traces of bromophenol blue). The second dimension electrophoresis was performed on a vertical system with uniform 12.5% separating gel (14 × 14 cm), at 25 °C. Protein spots in 2-DE gels were seen by silver staining. Each experiment was conducted in duplicate. The gels were scanned using an Imager Scanner (Amersham Biosciences) with the help of LabScan software. All details of individual gels and comparative analysis
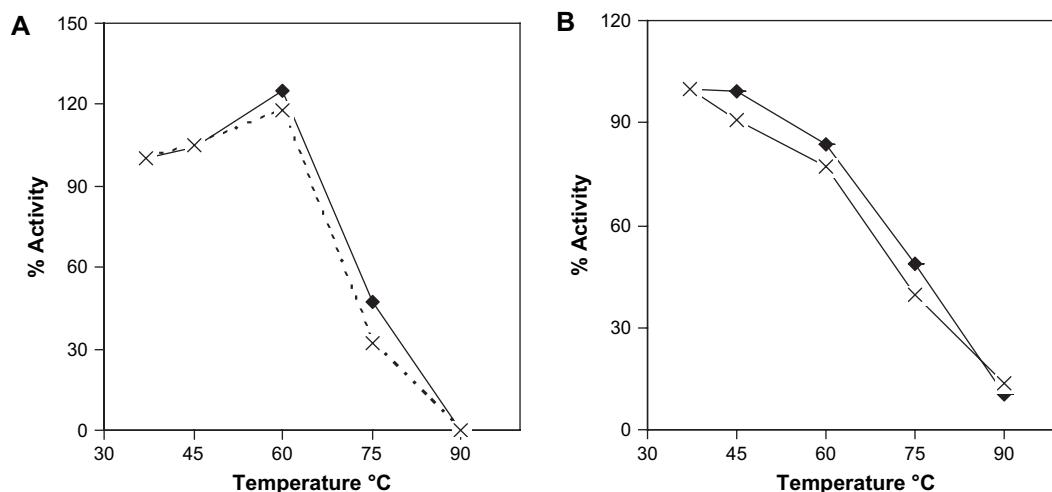
Fig. 5. Thermo-stability of the proteolytic activity of LP using (A) azocasein or (B) BANA + DTT, as substrates. (◆) 15 min and (×) 30 min. Each point has $n = 3$. Error bars indicate standard error of the mean.

were performed using ImageMaster 2D Platinum Software (Amersham Biosciences).

4.2.5. Masses spectrometry

The protein profile of LP was performed by Matrix Assisted Laser Desorption Ionization (MALDI) and Time of Fly (TOF) analysis to obtain a masses spectrometry map using a Vision 2000 TOF-Voyager instrument (Finningan Mat. Bremen, Germany) equipped with a 337 UV laser.

4.3. Protein isolation

4.3.1. Gel filtration chromatography (Sephacryl S-100)

Samples of LP fraction (30 mg ml^{-1}) were submitted to gel filtration in a Sephacryl S-100 column (104×2 cm) that was previously equilibrated with 50 mM ammonium bicarbonate buffer (pH 7.9). The sample, in the same buffer, was separated from insoluble material (centrifugation at $20,000 \times$ g at 4 °C for 20 min) and loaded on to the column. Protein elution was performed with the same buffer at a flow rate of 0.5 ml/min and the protein profile was measured at 280 nm. The resulting fractions were checked by electrophoresis and proteolytic activity.

4.3.2. Affinity chromatography on chitin

Samples of 50 or 25 mg of LP were dissolved in 5 ml of saline isotonic (150 mM) and subjected to centrifugation for 10 min at 10 °C and $10,000 \times$ g for removal of any insoluble material. The cleared samples were applied to a chitin column (2×15 cm) previously equilibrated with the corresponding solution and the column was washed until the absorbance at 280 nm of the eluted samples reached a value close to zero. The proteins bound to the column were eluted by washing the column with 1 M acetic acid. Pooled fractions were tested for chitinolytic activity.

4.4. Enzymatic assays

4.4.1. Anti-oxidative enzymes (SOD, CAT and APX)

The lyophilized LP fraction was investigated for antioxidant enzyme activity. Samples of 200 μg were homogenized with a mortar and pestle in 4 ml of ice-cold extraction buffer [100 mM potassium−phosphate buffer (pH 7.0) + 0.1 mM EDTA]. The homogenate was filtered through a muslin cloth and centrifuged at $12,000 \times$ g for 15 min. The supernatant fraction was used as a crude extract for enzyme activity assays and all the procedures above were performed at 4 °C. The following analyses were determined in duplicate and the results were expressed as a mean value ± SEM.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Ref. [16]. The reaction mixture (1.5 ml) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 mM NBT, 2 mM riboflavin and 50 μl enzyme extract. Riboflavin was added last and the tubes were shaken and illuminated under two 20 W fluorescent lamps. The reaction was left for 15 min, after which the lights were switched off and the tubes covered with a black cloth. The absorbance of the reaction mixture was measured at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required to cause a 50% reduction in the NBT photoreduction rate. The results were expressed as U g^{-1} of dry mass (DM).

Total catalase (CAT, EC 1.11.1.6) activity was measured according to the Beers and Sizer's method [6]. The reaction mixture (1.5 ml) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 20 mM H$_2$O$_2$ and 50 μl enzyme extract. The reaction was started by adding the enzyme extract, and the decrease in H$_2$O$_2$ was monitored at 240 nm and quantified by its molar extinction coefficient (36 M^{-1} cm^{-1}). The results were expressed as μMol H$_2$O$_2$ min^{-1} g^{-1} DM.

Total ascorbate peroxidase (APX, EC 1.11.1.1) activity was assayed according to Nakano and Asada [30]. The reaction mixture (1.5 ml) composed of 50 mM phosphate buffer (pH 6.0), 0.1 mM EDTA, 0.5 mM ascorbate, 1.0 mM H_2O_2 and 50 µl enzyme extract. The reaction was started by adding H_2O_2 and ascorbate oxidation was measured at 290 nm for 1 min. Enzyme activity was measured using the molar extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}) and the results expressed in µMol H_2O_2 min^{-1} g^{-1} DM, taking into consideration that 2 mol ascorbate are required for a reduction of 1 mol H_2O_2.

4.4.2. Chitinolytic activity

The colorimetric assay was performed using a modification of Ref. [9]. The assay mixture contained 250 µl of LP (1 mg ml^{-1}) in 50 mM sodium acetate buffer (pH 5.2) and 2 mg of colloidal chitin in a total volume of 500 µl. It was incubated at 37 °C for 60 min. The reaction was boiled for 5 min and then centrifuged ($10,000 \times g$ for 20 min). For each 300 µl of the supernatant, 100 µl of 0.6 M potassium tetraborate were added and the amount of N-acetyl-glucosamine liberated was determined according to Reissig et al. [33]. The activity was determined from a calibration curve according to Boller et al. [9]. The controls included enzyme and substrate blanks as well as internal standards.

4.4.3. Total proteolytic activity

Azocasein was used as a non-specific substrate to investigate total proteolytic activity in LP. The reaction mixture contained 50, 100, 150 or 200 µl (1 mg ml^{-1}) of the LP (pre-incubated or not with 3 mM DTT for 10 min) and 200 µl of 1% azocasein in 50 mM PBS buffer (pH 6.0). The reaction was performed at 37 °C and stopped 60 min later by adding 300 µl of 20% trichloroacetic acid (TCA). The tubes were centrifuged ($5000 \times g$ for 10 min at 25 °C) and the supernatants were alkalinized with 400 µl of 0.2 N NaOH solution. The color developed was measured by absorbance at 420 nm [39]. One unit of activity was defined as the amount of enzyme capable of increasing absorbance by 420 nm at 0.01.

4.4.4. Assays for cysteine proteinases

Assays for cysteine proteinases were conducted utilizing BANA as a substrate. Aliquots of 50, 100, 150 and 200 µl of the LP [1 mg ml^{-1} in 50 mM PBS (pH 6.0)] were pre-incubated with 40 µl of an activation solution in the presence or absence of 3 mM DTT and 2 mM EDTA for 10 min at 37 °C and 200 µl of BANA were added [1 mM in 50 mM PBS (pH 6.0)]. After 30 min, the reaction was stopped by adding 500 µl of 2% HCl in ethanol and 500 µl of 0.06% 4-(dimethyl-amino)cinnamaldehyde. After 40 min, the resulting color was measured by absorbance at 540 nm [1]. One unit of enzymatic activity was defined as the amount of enzyme that increases the absorbance at 540 nm by 0.01. All assays were performed in triplicate. The results of each series were expressed as the mean value ± SEM.

4.4.5. Assays for serine proteases

Assays for serine proteases were performed utilizing BApNA as a substrate. Aliquots of 50, 100, 150 and 200 µl of the LP [1 mg ml^{-1} in 50 mM Tris–HCl (pH 7.5)] were incubated with 50 mM Tris–HCl (pH 7.5) for 10 min at 37 °C and 500 µl of 1.25 mM BApNA solution were added. After 30 min at 37 °C, the reaction was stopped by adding 150 µl of acetic acid (30%). The resulting color was measured by absorbance at 405 nm [19]. All assays were performed in triplicate. The results of each series were expressed as the mean value ± SEM.

4.4.6. Activity versus pH

The effect of pH on enzyme stability was evaluated using the following buffers: 50 mM glycine (pH 2.6), 50 mM sodium acetate (pH 4.0 and 5.0), 50 mM sodium phosphate (pH 6.0 and 7.0) and 50 mM Tris–HCl (pH 8.0 and 10.0). The remaining activities were determined at 37 °C and both the substrates, azocasein and BANA, were tested as described above.

4.4.7. Heat stability and effect of proteinase inhibitors

For testing heat stability, latex proteins were incubated at different temperatures ranging from 37 to 90 °C in 50 mM PBS, pH 6.0 for 15 and 30 min. After these periods, the proteolytic activity was measured at 37 °C using azocasein or BANA under standard assay conditions.

To elucidate the mechanistic nature of the proteolytic activity in latex of *C. procera*, a set of inhibitors for different types of proteases was tested. Aliquots of 50 µl (1 mg ml^{-1} in 50 mM PBS, pH 6.0) of LP were pre-incubated for 30 min at room temperature in the presence of 20 µl of the following inhibitors: 0.1 M iodoacetamide, 5 mM PMSF, 5 mM TPCK, 0.18 mM of E-64 and 10 µM pepstatin, in separate aliquots. The remaining activities were determined at 37 °C pH 6.0 using BANA or azocasein as substrates.

4.4.8. Detection of proteases by Zymography

The electrophoresis was carried out according to the Schagger and Jagow methods [34]. LP was dissolved in 1 M Tris–HCl buffer (pH 6.8), containing 10% sodium dodecyl sulfate (SDS), 10% glycerol in the presence or absence of 5% 2-mercaptoethanol. The sample was separated by electrophoresis through 10% polyacrylamide gels containing 0.1% gelatin at 25 °C as in Ref. [26]. After electrophoresis, the gels were immersed in water containing 2.5% of Triton X-100 (renaturing solution) and gently shaken for 30 min at 25 °C to wash out SDS and allow protein rearrangement. The gels were then incubated in 50 mM PBS (pH 6.0) containing 3 mM DTT and 2 mM EDTA for 3 h at 37 °C. The incubated gels were stained with 0.2% Coomassie Brilliant Blue R-350 in 40% methanol and 10% acetic acid followed by treatment using the same solution without the dye. Enzymatic activity was detected as transparent bands.

4.4.9. Effect of pH on gelatinolytic activities

The following developing buffers were used to determine pH activity profiles: 50 mM glycine (pH 2.6), 50 mM sodium

acetate (pH 4.0 and 5.0), 50 mM sodium phosphate (pH 6.0 and 7.0) and 50 mM Tris−HCl (pH 8.0), containing 3 mM DTT and 2 mM EDTA. The samples were separated by electrophoresis and the gel was immersed in 2.5% Triton X-100 as before. In addition, the gels were immersed in different solutions cited above for 3 h followed by the experimental procedures already described to detect proteases.

Acknowledgements

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