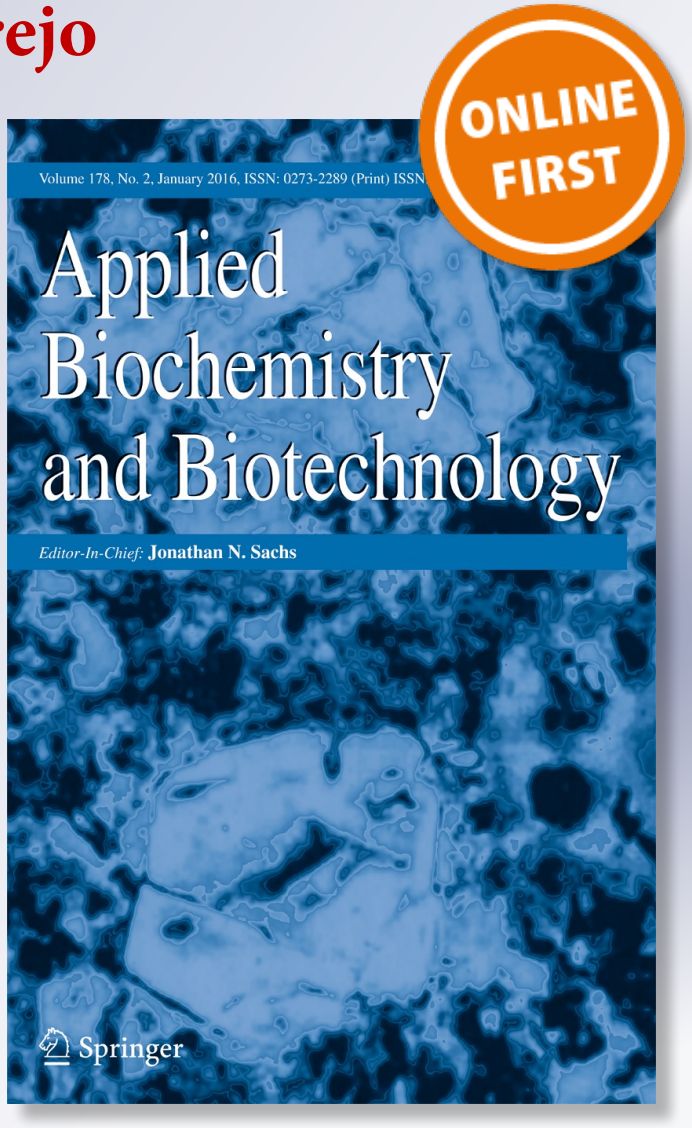
The Proteolytic Activity of Philibertia gilliesii *Latex. Purification of Philibertain g II*

Cynthia Sequeiros, María J. Torres, Marina L. Nievas, Néstor O. Caffini, Claudia L. Natalucci, Laura M. I. López & Sebastián A. Trejo

Applied Biochemistry and Biotechnology
Part A: Enzyme Engineering and Biotechnology

ISSN 0273-2289

Appl Biochem Biotechnol
DOI 10.1007/s12010-016-1997-8



Springer

Appl Biochem Biotechnol
DOI 10.1007/s12010-016-1997-8

The Proteolytic Activity of *Philibertia gilliesii* Latex. Purification of Philibertain g II

Cynthia Sequeiros[1] · María J. Torres[2] · Marina L. Nievas[1] · Néstor O. Caffini[3] · Claudia L. Natalucci[3] · Laura M. I. López[4] · Sebastián A. Trejo[5]

Received: 13 October 2015 / Accepted: 22 January 2016

Abstract The latex from the patagonic plant *Philibertia gilliesii* Hook. et Arn. (Apocynaceae) is a milky-white suspension containing a proteolytic system constituted by several cysteine endopeptidases. A proteolytic preparation (philibertain g) from the latex of *P. gilliesii* fruits was obtained and characterized to evaluate its potential use in bioprocesses. Philibertain g contained 1.2 g/L protein and a specific (caseinolytic) activity of 7.0 Ucas/mg protein. It reached 80 % of its maximum caseinolytic activity in the pH 7–10 range, retained 80 % of the original activity after 2 h of incubation at temperatures ranging from 25 to 45 °C and could be fully inactivated after 5 min at 75 °C. Philibertain g retained 60 % of the initial activity even at 1 M NaCl and was able to hydrolyze proteins from stickwater one, of the main waste effluents generated during fishmeal production. Furthermore, as a contribution to the knowledge of the proteolytic system of *P. gilliesii,* we are reporting the purification of a new peptidase, named philibertain g II (pI 9.4, molecular mass 23,977 Da, N-terminus LPESVDWREKGVVFPXRNQ) isolated from philibertain g through a purification scheme including acetone fractionation, cation exchange, molecular exclusion chromatography, and ultrafiltration.

✉ Laura M. I. López
lmiilopez@yahoo.com.ar

[1] CENPAT-CONICET, Centro Nacional Patagónico, Blvd. Brown N° 2915, 9120 Puerto Madryn, Chubut, Argentina

[2] Centro de Investigaciones y Transferencia del Noroeste de la provincia de Buenos Aires, UNNOBA-CONICET, Junín, Argentina

[3] CIPROVE, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CC 711, 1900 La Plata, Argentina

[4] CITEC, Instituto Nacional de Tecnología Industrial, Ciencias de la Salud - Universidad Nacional Arturo Jauretche, Buenos Aires, Argentina

[5] Servei de Proteòmicai Biologia Estructural (SePBioEs), Universitat Autònoma de Barcelona, Campus Universitari, 08193 Bellaterra, Cerdanyola del Vallès, Barcelona, Spain

Published online: 06 February 2016

Keywords Apocynaceae · Chromatography · Cysteine peptidase · Fish protein hydrolysates · Stickwater

Introduction

Peptidases are, from the point of view of industry, the most important type of enzymes because they represent ca. 60 % of all commercialized enzymes in the world [1]. Peptidases play an important role in biotechnology, given that proteolysis changes the chemical, physical, biological, and immunological properties of proteins. Hydrolysis of food proteins is carried out for various reasons: improvement of the nutritional characteristics, retarding deterioration, modification of different functional properties, prevention of undesired interactions, change of flavors and odors, and removal of toxic or inhibitory factors, among others. Although most peptidases used in industrial processes come from microbial sources, some plant cysteine proteinases, namely, papain, bromelain, and ficin, are still preferred in a number of cases and are currently used in the food industry for cheese and beer manufacture, tenderization of meat, production of emulsifiers, and other uses [2].

Enzyme preparations from plant extracts have been used in industrial processes for a long time, even before much was known about the nature and properties of the enzymes [3]. Proteolytic enzymes from plant sources have received special attention because they are active over a wide range of temperatures and pH, as well as in the presence of surfactants, organic solvents and denaturating agents. This stability enables their use in processes that restrict the use of conventional enzymes for industrial applications [4].

Laticifers usually contain a higher concentration of peptidases (up to 50 % of total proteins). Apocynaceae is a family of flowering plants widely distributed in almost the whole world that includes trees, shrubs, herbs, and lianas. Many of these plants have milky latex, and most of them contain proteolytic enzymes. Up to date, a number of latex peptidases from species belonging to Apocynaceae have been isolated and characterized [5–13]. Additionally, we have purified and characterized several peptidases from latex of other Apocynaceae species [14–23].

Philibertia gilliesii Hook. et Arn. (Apocynaceae) is a native species which grows from Bolivia to North of Patagonian region of Argentina [24]. In the present article, the proteolytic system present in latex from *P. gilliesii* fruits has been characterized and a novel cysteine endopeptidase has been isolated and purified, as part of a program dealing with the isolation of new peptidases from regional plants that are currently not used in agriculture, a situation that could have positive effects for local economies.

Materials and Methods

Plant Material

Fruits of *P. gilliesii* Hook. *et* Arn. were collected in the early summer in the nearness of Puerto Madryn, province of Chubut, Patagonia, Argentina. The corresponding voucher specimen was deposited in the CENPAT Botanical Garden herbarium (*P. gilliesii* JBPE 1146).

Chemicals

N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO), bovine serum albumin, N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), carboxypeptidase B, bovine milk casein, cysteine, EDTA, 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino) propane-1-sulfonic acid (MOPS), L-Pyroglutamyl-L-phenylalanyl-L-leucine-p-nitroanilide (PFLNA), sinapic acid, N-Tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), Tris, and glycine were purchased from Sigma Chemical Company, St. Louis. Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide and broad molecular weight markers were obtained from Bio-Rad, Hercules, CA. LMW-SDS markers kit, SP-Sepharose HR, Ampholytes Pharmalyte 8–10.5, and pI markers were purchased from GE Healthcare, Life Sciences, Uppsala, Sweden. All other chemicals were obtained from commercial sources and were of the highest purity available.

Clarified Preparation (Philibertain g)

Latex from *P. gilliesii* was obtained by superficial incisions made on fruits and received on 0.1 M Tris–HCl buffer, pH 7.5, containing 5 mM EDTA and 5 mM cysteine as protector agents. The latex suspension was frozen and stored at −20 °C. After defrosting, suspension was centrifuged at 32,000*g* for 90 min at 4 °C, the supernatant obtained was named philibertain g.

Protein Concentration

Protein content was determined by the Bradford's method [25] using bovine serum albumin as standard. The protein profile in the chromatogram was obtained by absorbance measurement at 280 nm.

Proteolytic Activity Assays on Casein

The reaction mixture contained 1.1 mL of 1 % casein solution and 0.1 mL of sample, both in 0.1 M Tris–HCl buffer (pH 8.0), containing 10 mM cysteine. The test was developed according to López et al. [26].

Optimum pH of Philibertain g

Caseinolytic activity of philibertain g was measured at 37 °C using sodium salts of the "Good" buffers [27]. Solutions of 1 % casein in 25 mM Good buffers (MES, MOPS, TAPS, AMPSO, and CAPS) with 10 mM cysteine were prepared at a pH range from 6.0 to 11.0. Trials were performed as indicated earlier.

Stability of Philibertain g in the pH Range with Higher Proteolytic Activity

The effect of pH on philibertain g stability was determined by incubating the enzyme preparation for 0, 15, 30, 60, and 120 min at 4 °C with 25 mM Good buffers at four different pH values [7–10] and then the residual caseinolytic activity was measured as indicated before.

Thermal Stability

To determine the effect of heating on the stability of philibertain g at the optimum pH, the enzyme preparation was kept for 0, 5, 10, 30, 60, 90, and 120 min at 25, 37, 45, 55, 65, and 75 °C. The reaction was stopped by dipping in an ice bath and the residual caseinolytic activity was measured as described before.

Stability of Philibertain g Against Increasing Ionic Strength

The effect of ionic strength on the proteolytic activity of philibertain g was determined on 1 % casein (pH 8.0) in the presence of increasing quantities of sodium chloride (0.00, 0.25, 0.50, 1.00, 1.50, and 2.00 M).

Effect of Some Chemicals on Proteolytic Activity of Philibertain g

The effect of different chemicals (10 mM EDTA, 1 % SDS, 1 % Triton X-100, 4 M urea, 10 mM calcium chloride, and 0.3 M sodium chloride) on philibertain g was determined by incubating the enzyme preparation for 30 min at 4 °C and then the residual caseinolytic activity was measured as indicated before.

Effect of the Storage Conditions on Proteolytic Activity

In order to investigate the effect of storage time on enzymatic stability, part of the aqueous extracts of latex suspension and philibertain g were stored at 4 and −20 °C. Additionally, philibertain g was lyophilized and stored for different periods of time. After treatments, residual caseinolytic activity was determined.

Action of Philibertain g on Fish Proteins

Obtaining and Characterization of Stickwater

Stickwater was collected from a fishmeal plant (Harengus S.A.) located in Puerto Madryn, Chubut, Argentina. The stickwater came from processing of four seasonal captured species: hake (*Merluccius hubbsi* and *Macruronus magellanicus*), choicy ruff (*Seriolella porosa*), and shrimp (*Pleoticus muelleri*). Sample was taken and immediately transported to the laboratory where it was allowed to cool and 0.002 % (*w/v*) tetracycline was added [28]; then aliquoted, frozen, and stored at −20 °C for further analyses.

Controlled Hydrolysis of Fish Protein

The hydrolysis reaction was performed using stickwater which contains 6.1 % (*w/v*) proteins, and an enzyme/substrate ratio of 8.9 caseinolytic units (Ucas) per gram of protein substrate. The mixture containing 25 mM Cys was brought to pH 9 with 1 N NaOH and incubated at 45 °C; finally, the enzymatic preparation (philibertain g) was added. The suspension pH was maintained by adding 1 N NaOH. Inactivation of the peptidase was achieved by acidification (pH 4.2) of the reaction mixture with 2 N HCl and then heating the samples for 10 min at 90 °C.

Electrophoretic Analysis

Aliquots (200 μL) of the hydrolysis mixture were withdrawn at 0, 2, 5, 10, 30, 60, 90, and 180 min and mixed with 1 mL of cold acetone. The test tubes were centrifuged and the precipitates redissolved by adding sample buffer, boiled for 5 min, and centrifuged. Samples were subjected to denaturing electrophoresis in tricine gels composed of a stacking gel (4 % T, 3 % C), a spacer gel (10 % T, 3 % C), and a separating gel (16.5 % T, 3 % C), which is especially suitable to resolve the mixture of peptides produced [29]. Gel images were processed by means of a specific software (Scion Image Beta 4.03 for Windows; Scion Corporation, Frederick, MD, USA) to obtain the corresponding densitograms.

Purification and Characterization of Philibertain gII

Cation Exchange Chromatography

The partial purified preparation obtained by acetone fractionation [19] was applied (3 mL) onto a SP-Sepharose Fast Flow column (K 16/40, Pharmacia Biotech) equilibrated with 50 mM Gly–NaOH buffer 50 mM (pH 8.6). After an isocratic washing with NaCl 35 mM, a first gradient (35–100 mM) was applied; then a short gradient was used (100–170 mM NaCl) followed by an isocratic phase 170 mM NaCl; finally, after a small gradient (170 to 200 M NaCl), the slope was changed (from 200 to 350 M NaCl). In all process, NaCl was disolved in the start buffer and the flow rate was 1.5 mL/min. The absorbance at 280 nm as well as the caseinolytic activities in all fractions were tested. The fractions showing proteolytic activity were pooled and stored at −20 °C for further studies.

Molecular Exclusion Chromatography

A pool of I–IV fractions obtained by cationic exchange chromatography was treated with 4 volumes of acetone and the precipitate redissolved in 50 mM Gly-NaOH buffer (pH 8.6) containing 5 mM sodium tetrathionate and 0.3 M NaCl and subjected to molecular exclusion chromatography on an FPLC system (Pharmacia) using a XK 16/40 column (Pharmacia) packed with Sephacryl S-100 equilibrated and eluted (0.5 mL/min) using the same buffer.

Isoelectric Focusing and Zymogram

One milliliter of philibertain g II was treated with 3.5 vol acetone and the precipitate was redissolved with 100 μL of deionized water. Five percent polyacrylamide gels were used, containing alternatively Pharmalyte broad range pH 3–10 (GE Healthcare Life Sciences). A Mini isoelectric focusing (IEF) Cell (Model 111, Bio-Rad) was employed to carry out isoelectric focusing.

Unstained IEF gels were contacted for 10 min in an oven at 60 °C with an agarose gel imbibed with a 1 % casein solution. After incubation, the agarose gel was dehydrated and stained by Coomassie Brilliant Blue R-250. Unstained bands evidence proteolytic activity [30].

SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed in a Miniprotean II Cell (Bio-Rad) according to Laemmli [31]. Current was kept constant at 40 mA during stacking and then increased to 60 mA and kept constant for 40 min. Gels (12.5 % polyacrylamide) were stained by Coomassie Brilliant Blue R-250.

Mass Spectrometry

The molecular mass and homogeneity of philibertain g II was determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The sample was spotted on an MTP 384 GroundSteel and mixed with freshly prepared matrix solution (sinapic acid) and processed in a UltrafleXtrem MALDI-TOF mass spectrometer (Bruker Daltonics, Germany), using bovine carbonic anhydrase as standard.

N-terminal Sequence

The N-terminal sequence of philibertain g II was determined by Edman's automated degradation using an Applied Biosystems (model 476) peptide sequencer. The Basic Local Alignment Search Tool (BLAST) network service [32] was used to perform peptidase homology searches.

Results and Discussion

Characterization of Philibertain g

Plant extracts with a high content of proteolytic enzymes can be applied in a variety of processes such as the manufacture of food, and biomedical and environmental applications [3]. Clarified latex extracts of *Calotropis gigantea* and *Calotropis procera* have been recently used for biomedical applications [33–35], and those of *Carica papaya* to biodegrade protein effluents [36] and to hydrolyze raw hide [37]. Philibertain g obtained from latex of *P. gilliesii* fruits containing several endopeptidases [21] was extensively characterized, taking into account its potential use in industrial processes.

Philibertain g contained 1.2 mg/mL protein, its caseinolytic activity was 8.5 Ucas/mL, thus its specific activity was 7.0 Ucas/mg protein. This specific activity was 2 and 1.4 times the specific activity presented by *C. papaya* (3.5 Ucas/mg protein) and *Vasconcellea quercifolia* (5 Ucas/mg protein) preparations, respectively [38]. Philibertain g reached 80 % of its maximum caseinolytic activity at pH 7–10. While maximum activity was reached at pH 9, a shoulder could be detected at pH 7, typical for a non-purified enzyme preparation, where more than one enzyme may be present (data not shown). The highest activity at neutral and alkaline pH is a common feature of most peptidases belonging to the family Apocynaceae. Philibertain g remained stable (100 % residual activity) when it was incubated for 2 h at pH values ranging from 7 to 10. The stability of the enzyme preparation in the pH range in which has the highest proteolytic activity is an important feature for its use in biotechnological processes.

The assessment of the temperature effects on the stability of philibertain g revealed that it retained 80 % of the original activity after 2 h of incubation between 25 and 45 °C. The

enzyme preparation was fully inactivated after 5 min at 75 °C, an advantageous behavior for its use in industrial processes. Comparison of thermal stability of philibertain g with similar preparations from other Apocynaceae species (Table 1), shows that *Asclepias fruticosa* [17] exhibits a similar behavior (both are virtually inactive after 2 h at 60 °C). In contrast, peptidases from *Funastrum clausum* [19], *Morrenia brachystephana* [18], and *Morrenia odorata* [16] show a high thermal stability even at 70 °C.

Although the activity of most enzymes is not affected at low ionic strength values, it frequently decreases when the concentration of salt (NaCl) exceeds 0.2 M [39]. In our work, the salt concentration increase produced a gradual reduction on the activity of philibertain g, while retaining 60 % of the initial activity even at 1 M NaCl. This feature shows that philibertain g has a great potential for use in enzymatic processing of marine raw material.

For enzymes to be effectively used in industrial processes, they must be active in a wide range of conditions, such as addition of surfactants, reducing agents, high salt concentrations, changes in pH and temperature, and addition of preservatives [40]. The behavior of philibertain g against different salts and denaturing surfactants has been tested. Detergents as Triton X-100 and SDS drastically reduced the enzyme activity to nearly undetectable values. In contrast, EDTA, NaCl, and $CaCl_2$ were almost innocuous, retaining high values of the enzymatic activity (98, 85, and 76 %, respectively). However, 4 M urea increased enzyme activity (160 %), behavior that was previously reported for other cysteine peptidases like papain, ficin, and stem bromelain [41], ervatamin C [42], ervatamin B [43], and procerain [44]. In the case of stem bromelain, it was reported that 5 M urea produces an intermediate state with enhancement of activity due to local conformational changes accompanied by increase dynamics in the active site [45].

As conservation is a very important aspect for an industrial product, different forms of storage were also evaluated. The behavior was similar for latex suspension and philibertain g after 6 months at −20 °C: in both cases, 75 % activity was retained. Notably, philibertain g retained about 70 % of the initial activity after 34 months of storage at −20 °C. On the contrary, storage at 4 °C during 6 months affected the samples in a different way: latex suspension retained 60 % but philibertain g only 35 % of the initial activity. The lyophilization process decreased 25 % philibertain g activity, while after 6 months, the lyophilized sample retained 60 % of its initial activity.

Action of Philibertain g on Fish Proteins

Stickwater is the liquid effluent from fish meal factories, which may be used to obtain protein hydrolysates for the food industry. Composition of stickwater is highly variable and depends

Table 1 Thermal stability of different enzyme preparations obtained from latex from Apocynaceae species after two hours of incubation at the indicated temperatures

Plant species	(%) Residual activity			Reference
	45 °C	60 °C	70 °C	
Philibertia gilliesii	85	5	0	
Asclepias fruticosa	77	0	0	[17]
Funastrum clausum	90	75	65	[19]
Morrenia brachystephana	95	80	45	[18]
Morrenia odorata	95	70	22	[16]

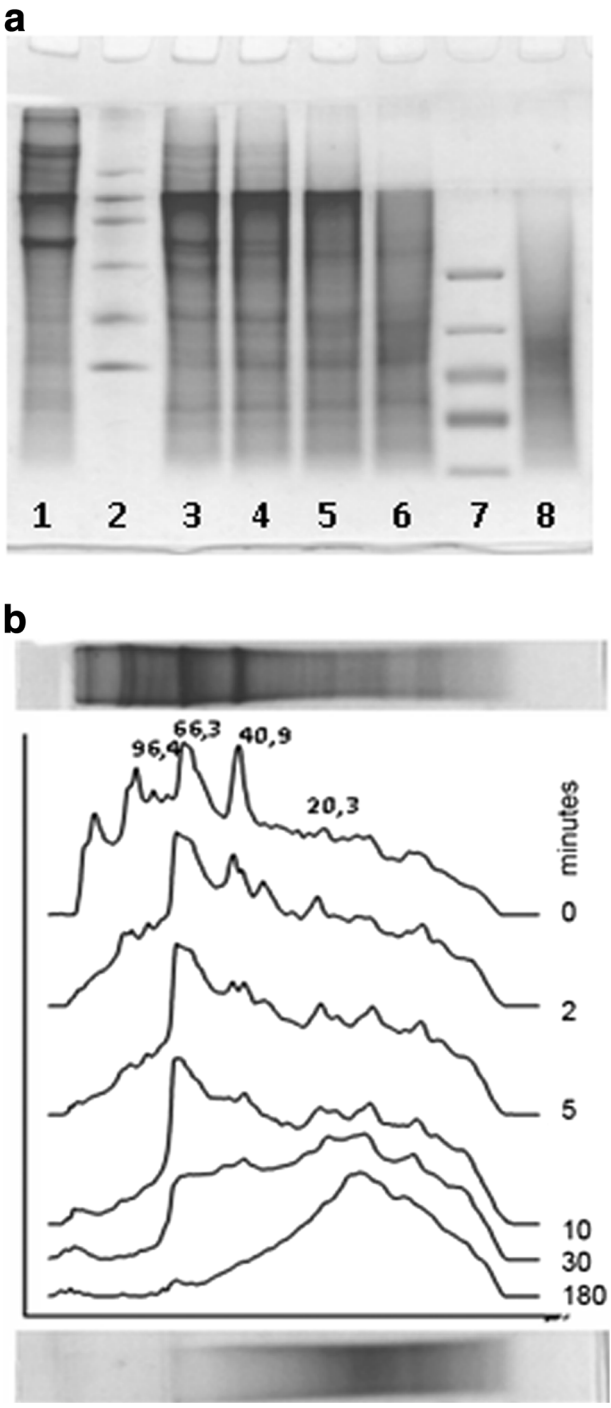


Fig. 1 **a** Electrophoresis of fish protein hydrolysates (pH 9, 45 °C, enzyme/substrate ratio of 8.9 caseinolytic units (Ucas) per g of protein substrate) with philibertain g. *Lane 1* unhydrolyzed stickwater; *Lane 2* molecular weight markers (97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kDa). *Lane 7* peptide markers (26.0, 17.0, 14.4, 6.5, and 3.5 kDa). *Lanes 3–6 and 8* sample hydrolysates at 2, 5, 10, 30, and 180 min of reaction, respectively. **b** Densitogram corresponding to electrophoresis shown in part **a**. The upper and lower strips correspond to 0 and 180 min hydrolysis, respectively. The *numbers above the peaks* correspond to calculated kiloDalton for each polypeptide

on the raw material and the conditions of storage and operation of the fish meal plant [28]. The stickwater used in this study contained 6.9 % of total solids, 6.1 % proteins, and 1.0 % ash,

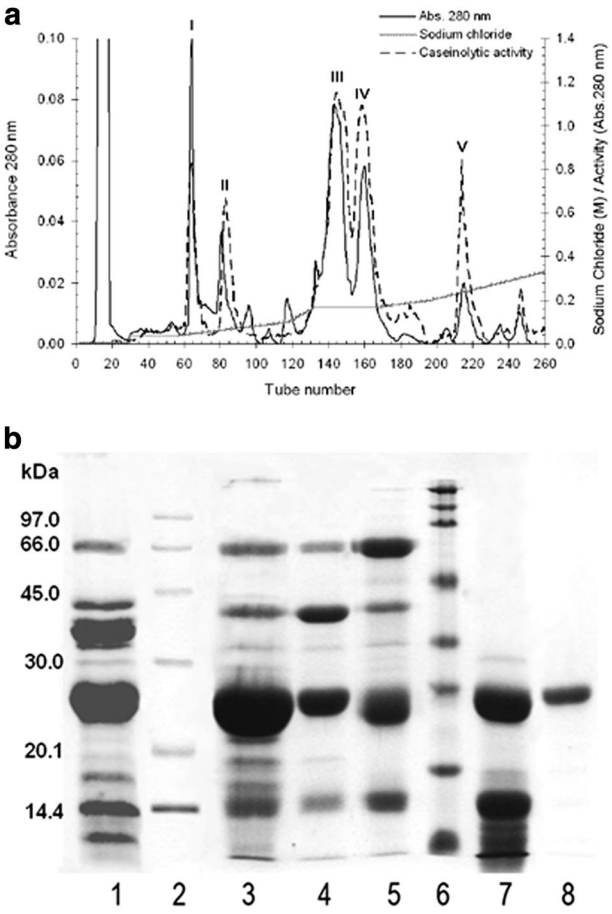


Fig. 2 **a** Cation exchange chromatography of the acetone partially purified preparation of philibertain g. Column: SP-Sepharose; buffer: 50 mM Gly-NaOH pH 8.6; variable gradient from 35 to 350 mM NaCl; flow rate: 1.5 mL/min. **b** SDS-PAGE of the fractions obtained from cation exchange chromatography of the acetone partially purified preparation of philibertain g. *Lane 1* acetone partially purified preparation of philibertain g; *lane 2* molecular mass markers; *lane 3* fraction I; *lane 4* fraction II; *lane 5* fraction III; *lane 6* broad molecular mass markers (220, 120, 100, 55, 38, 29, 20, and 7 kDa); *lane 7* fraction IV; *lane 8* fraction V

with a pH value of 6.7. Although the feedstock was different, our values are consistent with those reported by Bechtel [46] for stickwater. The protein hydrolysis of the stickwater was carried out by using philibertain g at pH 9. In order to examine the peptide profile of the hydrolysates, samples were taken at different times and were submitted to Tricine-SDS-PAGE. As shown in Fig. 1a, after 2 h of hydrolysis, low MW peptides only remain. In addition, Fig. 1b shows the densitogram obtained from gel electrophoretogram of stickwater hydrolysates at different times. After 2 min of philibertain g treatment, the proteins of the highest molecular weight were significantly degraded, while the 66.3 kDa band remained virtually unchanged. After 180 min, mostly low MW peptides were present confirming the usefulness of the philibertain g to hydrolyze the proteins of the stickwater.

Purification and Characterization of Philibertain g II

As a first step of purification, philibertain g was submitted to acetone fractionation. This preparation retained 95.4 % of initial proteolytic activity and 80.4 % of protein content [21]. Cation exchange chromatography allowed the separation of five active fractions via a complex elution program involving different ionic strength conditions (Fig. 2a). Electrophoretic

comparison between acetone partially purified preparation of philibertain g and the main fractions (I–V) obtained by cation exchange chromatography is shown in Fig. 2b. It can be noted that fractions I–III (lanes 3–5) have similar electrophoretic profiles, in which polypeptides of higher (65.5 and 44.0 kDa) and lower molecular mass coelute with the band expected for cysteine peptidases (around 25 kDa). On the other hand, fraction IV (lane 7) contains mainly this band and polypeptides with lower molecular mass. Fraction V (lane 8) is the only pure one and may correspond to philibertain g I [21]. These findings could indicate the existence of strong interactions between the proteins present in the sample, which are presumably diminished or eliminated by increasing the ionic strength along the chromatography. This kind of interaction has not been described in the literature related to peptidases, nor has been observed previously in our laboratory.

On the basis of the aforementioned, peaks I to IV were pooled since all of them contained the band of about 25 kDa and showed proteolytic activity. The pool was treated with 4 volumes of acetone and the precipitate redissolved in 50 mM Gly-NaOH buffer (pH 8.6) containing 5 mM sodium tetrathionate and 0.3 M NaCl to minimize protein-protein interaction, after which a molecular exclusion chromatography was performed (Fig. 3a). The elution was performed at a constant ionic strength (0.3 M NaCl). Figure 3b shows the electrophoretic profile of the fractions from molecular exclusion chromatography; the first two fractions, lacking proteolytic activity,

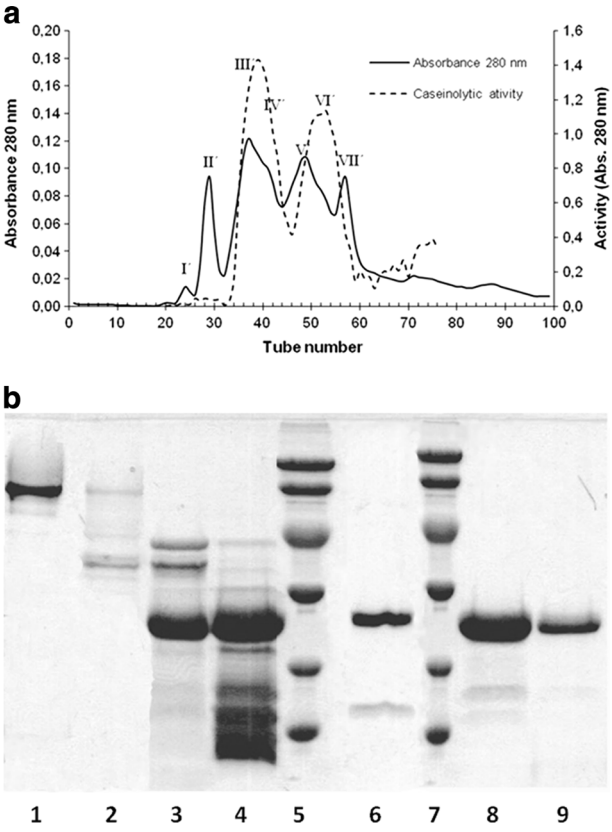


Fig. 3 **a** Molecular exclusion chromatography. Sample: pool of I–IV fractions obtained by cationic exchange chromatography in NaCl 0.3 M; column: sephacryl S-100 equilibrated with 50 mM Gly-NaOH buffer (pH 8.6) containing 5 mM sodium tetrathionate and 0.3 M NaCl. The column was eluted (0.5 mL/min) using the same buffer. **b** SDS-PAGE of the fractions obtained from molecular exclusion chromatography. *Lane 1* fraction I ; *lane 2* fraction II ; *lane 3* fraction III ; *lane 4* fraction IV ; *lanes 5 and 7* molecular mass markers (97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kDa); *lane 6* fraction V ; *lane 8* fraction VI ; *lane 9* fraction VII

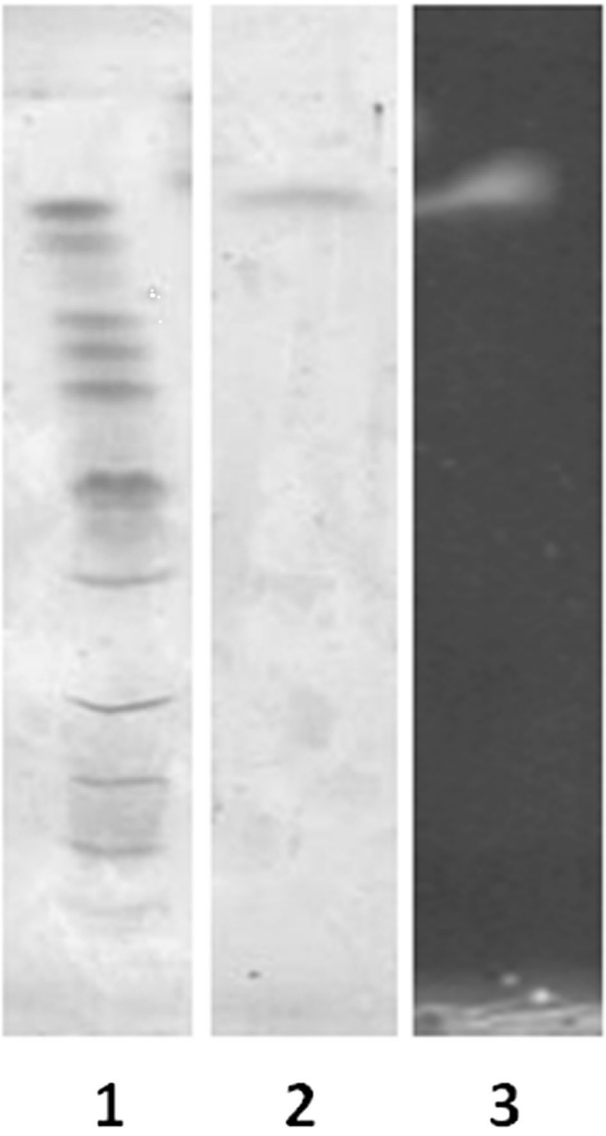


Fig. 4 Isoelectric focusing and zymogram. *Lane 1* pI markers: amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β-lactoglobulin a (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85 and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45 and 8.65), and trypsinogen (pI 9.30); *lane 2* philibertain g II; lane 3, philibertain g II zymogram

only show bands with molecular mass exceeding 25 kDa (lanes 1 and 2). While the main band in all other lanes is located in the zone expected for cysteine peptidases (around 25 kDa), fractions III and IV also contain higher and lower molecular mass polypeptides (lanes 3 and 4). Fractions V and VI (lanes 6 and 8) are devoid of higher molecular mass polypeptides and only show a few lower molecular mass polypeptides. Finally, fraction VII (lane 9) shows a main band corresponding to *ca*. 26 kDa and almost indistinguishable minor bands of low molecular mass. In brief, the use of molecular exclusion technique with 0.3 M NaCl allowed us to isolate a fraction with proteolytic activity (fraction VII) that—after ultrafiltering to remove low MW peptides and salts—was homogeneous, according to IEF-zymogram, with a pI value of 9.4 (Fig. 4). Presence of basic cysteine peptidases in the lattice of plant species is a frequent event [47], which

Table 2 Comparison of the N-terminus sequence, molecular masses and isoelectric points of philibertain g II with papain and plant cysteine peptidases belonging to the family Apocynaceae

Protease	N-terminus	Identity (%)	Molecular mass (Da)	pI	Reference
Philibertain g II	LPESVDWREKGVVFPXRNQ	100,0 (19/19)	23,977	9.4	This work
Asclepain f	LPDSVDWREKGVVFPIRNQ	89.5 (17/19)	23,652	> 9.3	(*17*)
Procerain B	LPNSVDWREKDVVFPIRNQ	84.2 (16/19)	23,802	9.25	(*50*)
Ervatamin a	LPEHVDWRAK---------	80.0 (8/10)	27,600	8,37	(*9*)
Araujiain h III	LPESVDWRKKNLVFPVRNQ	78.9 (15/19)	23,546	10.5	(*15*)
Asclepain c I	LPNSVDWRQKGVVFPIRDQ	78.9 (15/19)	23,200	> 9.3	(*20*)
Asclepain c II	LPSFVDWRQKGVVFPIRNQ	78.9 (15/19)	23,590	> 9.3	(*22*)
Funastrain c II	LPNSVDWRQKGVVSAIRNQ	73.7 (14/19)	23,636	> 9.3	(*19*)
Morrenain o II	LPDSVDWRKKNLVFPVRNQ	73.7 (14/19)	25,800	> 9.3	(*16*)
Morrenain b II	LPDSVDWRKKNLVFPVRNQ	73.7 (14/19)	26,000	> 9.3	(*16*)
Asclepain b	LPNFVDWRKNGVVFPIRNQ	73.7 (14/19)	21,000	-	(*5, 51*)
Asclepain a	LPNSIDWRQKNVVFPIKNQ	68.4 (13/19)	23,000	-	(*5, 51*)
Araujiain h II	VPDSIDWREKDAVLPIRNQ	63.2 (12/19)	23,718	8.9	(*15*)
Papain	IPEYVDWRQKGAVTPVKNQ	63.2 (12/19)	23,406	9.55	(*52, 53*)
Philibertain g I	LPASVDWRKEGAVLPIRHQ	63.2 (12/19)	23,530	> 10.25	(*21*)
Ervatamin c	LPEQIDWRKKGAVTPVWNQ	63.1 (12/19)	23,000	9.54	(*49*)
Ervatamin b	LPSFVDWRSKGAVNSIKNQ	57.9 (11/19)	23,000	9.35	(*48*)
Heynein	LPEQIDXRXXGAVNP----	53.3 (8/15)	23,000	10.8	(*10*)
Morrenain b I	VPDKIDYREKGAVLDIRNQ	52.6 (10/19)	23,205	> 9.3	(*18*)

Identical residues are highlighted

has also been found in other species belonging to the Apocynaceae family (Table 2). The new purified enzyme was named philibertain g II.

The activity of philibertain g II was increased when preactivated with 5 mM cysteine and was fully inactivated with E-64. These findings confirm that philibertain g II is a cysteine peptidase. The value of the molecular mass obtained by mass spectrometry (MALDI-TOF) was 23,977 Da, which is closely related to those of other cysteine plant peptidases from the family Apocynaceae (Table 2).

N-terminus sequence of philibertain g II (19 residues) was determined, after which it was compared (Table 2) with philibertain g I [21] and those of other peptidases isolated from latex of other Apocynaceae species, [5, 9, 10, 15–20, 22, 48–51] as well as that of papain, peptidase type of clan CA and of subfamiliy C1A [52, 53]. The N-terminus sequence of philibertain g II showed the presence of highly conserved residues and motifs of "papain-like" cysteine peptidases, as Pro2 and Ser4 residues, and the DWR motif [53]. Other highly conserved amino acid residues were Lys 10, Val13, Pro 15, and Asn 18. The higher identity degree (89.5 %) was obtained with asclepain f, isolated from *Asclepias fruticosa*, followed by 63.2 % identity with papain and philibertain g I.

In conclusion, we are providing valuable information about the knowledge of Patagonian plants with high proteolytic activity, a topic scarcely reported so far. The proteolytic system of *P. gilliesii*

latex showed high specific activity, good stability at a wide range of pH and temperatures, as well as high stability in the presence of some chemicals used in industrial processes. Additionally, philibertain g has revealed ability to hydrolyze stickwater proteins. Owing to all these characteristics, philibertain g has a significant potential for industrial applications as well as remediation agent. On the other hand, a novel cysteine endopeptidase, named philibertain g II (pI 9.4, molecular mass 23,977 Da, N-terminus LPESVDWREKGVVFPXRNQ), was isolated and partially characterized from *P. gilliesii* as part of a program focusing on the isolation of new peptidases from regional plants that are currently not used in agriculture, a situation that could have positive effects for local economies.

Acknowledgments The authors acknowledge Dr. Ana María Beeskow (CENPAT-CONICET) for the identification of plant material. L.M.I. López, M.J. Torres, and C. Sequeiros are members of the CONICET Researcher Career; C.L. Natalucci is member of the CICPBA Researcher Career. The present work was supported by grants from CONICET (PIP 0297), CICPBA, and University of La Plata (X-576), Argentina.

References

1. Feijoo-Siota, L., & Villa, T. G. (2011). Native and biotechnologically engineered plant proteases with industrial applications. *Food Bioprocess Technol, 4*, 1066–88.
2. Pardo, M. F., López, L. M. I., Canals, F., Avilés, F. X., Natalucci, C. L., & Caffini, N. O. (2000). Purification of balansain I, an endopeptidase from unripe fruits of *Bromelia balansae* Mez (Bromeliaceae). *J Agric Food Chem, 48*, 3795–800.
3. González-Rábade, N., Badillo-Corona, J. A., Aranda-Barradas, J. S., & Oliver-Salvador, M. D. C. (2011). Production of plant proteases in vivo and in vitro—a review. *Biotechnol Adv, 29*, 983–96.
4. Yadav, R. E., Patel, A. K., & Jagannadham, M. V. (2011). Purification and biochemical characterization of a chymotrypsin like serine protease from *Euphorbia neriifolia* Linn. *Process Biochem, 46*, 1654–62.
5. Lynn, K. R., Brockbank, W. J., & Clevette-Radford, N. A. (1980). Multiple forms of the asclepains, cisteinyl proteases from milkweed. *Biochim Biophys Acta, 612*, 119–25.
6. Ramos, M. V., Araújo, E. S., Jucá, T. L., Monteiro-Moreira, A. C., Vasconcelos, I. M., Moreira, R. A., et al. (2013). New insights into the complex mixture of latex cysteine peptidases in *Calotropis procera. Int J Biol Macromol, 58*, 211–9.
7. Ramos, M. V., Souza, D. P., Gomes, M. T., Freitas, C. D., Carvalho, C. P., Júnior, P. A., et al. (2014). A phytopathogenic cysteine peptidase from latex of wild rubber vine *Cryptostegia grandiflora. Protein J, 33*, 199–209.
8. Pal, G., & Sinha, N. K. (1980). Isolation, crystallization, and properties of calotropins DI and DII from *Calotropis gigantea. Arch Biochem Biophys, 202*, 321–9.
9. Nallamsetty, S., Kundu, S., & Jagannadham, M. V. (2003). Purification and biochemical characterization of a highly active cysteine protease ervatamin a from the latex of *Ervatamia coronaria. J Protein Chem, 22*, 1–13.
10. Patel, B. K., & Jagannadham, M. V. (2003). A high cysteine containing thiol proteinase from the latex of *Ervatamia heyneana*: purification and comparison with ervatamin B and C from *Ervatamia coronaria. J Agric Food Chem, 51*, 6326–34.
11. Tomar, R., Kumar, R., & Jagannadham, M. V. (2008). A stable serine protease, wrightin, from the latex of the plant *Wrightia tinctoria* (Roxb.) R.Br.: purification and biochemical properties. *J Agric Food Chem, 56*, 1479–87.
12. Singh, A. N., Shukla, A. K., Jagannadham, M. V., & Dubey, V. K. (2010). Purification of a novel cysteine protease, Procerain B, from *Calotropis procera* with distinct characteristics compared to Procerain. *Process Biochem, 45*, 399–406.
13. Singh, A. N., & Dubey, V. K. (2011). Exploring applications of procerain b, a novel protease from *Calotropis procera*, and characterization by N-terminal sequencing as well as peptide mass fingerprinting. *Appl Biochem Biotechnol, 164*, 573–80.
14. Priolo, N., Morcelle del Valle, S., Arribére, M. C., López, L. M. I., & Caffini, N. O. (2000). Isolation and characterization of a cysteine protease from the latex of *Araujia hortorum* fruits. *J Protein Chem, 19*, 39–48.
15. Obregón, W. D., Arribére, M. C., Morcelle del Valle, S., Liggieri, C., Caffini, N. O., & Priolo, N. S. (2001). Two new cysteine endopeptidases obtained from the latex of *Araujia hortorum* fruits. *J Protein Chem, 20*, 317–25.

16. Cavalli, S. V., Cortadi, A., Arribére, M. C., Conforti, P., Caffini, N. O., & Priolo, N. S. (2001). Comparison of two cysteine endopeptidases from latices of *Morrenia brachystephana* Griseb. and *Morrenia odorata* (Hook et Arn.) Lindley (Asclepiadaceae). *Biol Chem Hoppe Seyler, 382*, 879–83.
17. Trejo, S. A., López, L. M. I., Cimino, C. V., Caffini, N. O., & Natalucci, C. L. (2001). Purification and characterization of a new plant endopeptidase isolated from the latex of *Asclepias fruticosa* L. (Asclepiadaceae). *J Protein Chem, 20*, 469–77.
18. Vairo Cavalli, S. E., Arribére, M. C., Cortadi, A., Caffini, N. O., & Priolo, N. S. (2003). Morrenain b I, a papain-like endopeptidase from the latex of *Morrenia brachystephana* Griseb. (Asclepiadaceae). *J Protein Chem, 22*, 15–22.
19. Morcelle, S. R., Trejo, S. A., Canals, F., Aviles, F. X., & Priolo, N. S. (2004). Funastrain cII: a cysteine endopeptidase purified from the latex of *Funastrum clausum. Protein J, 23*, 205–15.
20. Liggieri, C. S., Arribére, M. C., Trejo, S. A., Canals, F., Aviles, F. X., & Priolo, N. S. (2004). Purification and biochemical characterization of asclepain cI from the latex of *Asclepias curassavica* L. *Protein J, 23*, 403–11.
21. Sequeiros, C., Torres, M. J., Trejo, S. A., Esteves, J. L., Natalucci, C. L., & López, L. M. I. (2005). Philibertain g I, the most basic cysteine endopeptidase purified from the latex of *Philibertia gilliesii* Hook. et Arn. (Apocynaceae). *Protein J, 24*, 445–53.
22. Liggieri, C., Obregón, W., Trejo, S., & Priolo, N. (2009). Biochemical analysis of a papain-like protease isolated from the latex of *Asclepias curassavica* L. *Acta Biochim Biophys Sin, 41*, 154–62.
23. Trejo, S. A., López, L. M., Caffini, N. O., Natalucci, C. L., Canals, F., & Avilés, F. X. (2009). Sequencing and characterization of asclepain f: the first cysteine peptidase cDNA cloned and expressed from *Asclepias fruticosa* latex. *Planta, 230*, 319–28.
24. Correa, M. N. (1999). Parte VI. Dicotiledóneas. In F. Patagónica (Ed.), *Gamopétalas (Ericaceae a Calyceraceae)* (p. 536). Buenos Aires: Colección Científica del INTA.
25. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*, 248–54.
26. López, L. M. I., Sequeiros, C., Natalucci, C. L., Brullo, A., Maras, B., Barra, D., & Caffini, N. O. (2000). Purification and characterization of macrodontain I, a cysteine peptidase from unripe fruits of *Pseudananas macrodontes* (Morr.) Harms (Bromeliaceae). *Protein Expr Purif, 18*, 133–40.
27. Good, N. E., & Izawa, S. (1972). Hydrogen ion buffers. *Methods Enzymol, 24*, 53–68.
28. del Valle, J. M., & Aguilera, J. M. (1991). Physicochemical characterisation of raw fish and stickwater from fish meal production. *J Sci Food Agric, 54*, 429–41.
29. Schägger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem, 166*, 368–79.
30. Westergaar, J. L., Hackbarth, C., Treuhaft, M. W., & Roberts, R. C. (1980). Detection of proteinases in electrophoretograms of complex mixtures. *J Immunol Methods, 34*, 167–75.
31. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–5.
32. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res, 25*, 3389–402.
33. Bindhu, O. S., & Singh, M. K. (2014). Hemostatic, milk clotting and blood stain removal potential of cysteine proteases from *Calotropis gigantea* (L.) R. Br. Latex. *Pharmacogn Mag, 10*, S350–6.
34. Rajesh, R., Raghavendra Gowda, C. D., Nataraju, A., Dhananjaya, B. L., Kemparaju, K., & Vishwanath, B. S. (2005). Procoagulant activity of *Calotropis gigantea* latex associated with fibrin(ogen)olytic activity. *Toxicon, 46*, 84–92.
35. Ramos, M. V., Viana, C. A., Silva, A. F. B., Freitas, C. D. T., Figueiredo, I. S. T., Oliveira, R. S. B., et al. (2012). Proteins derived from latex of *C. procera* maintain coagulation homeostasis in septic mice and exhibit thrombin- and plasmin-like activities. *Naunyn Schmiedebergs Arch Pharmacol, 385*, 455–63.
36. Biazus, J. P. M., Santana, J. C. C., & De Souza, R. R. (2006). Empirical modeling of biodegradation process of proteic effluents by enzymes of *Carica papaya* sp. *Revista Brasileira de Engenharia Agrícola e Ambiental, 10*, 436–40.
37. Pitpreecha, S., & Damrongsakkul, S. (2006). Hydrolysis of raw hide using proteolytic enzyme extracted from papaya latex. *Korean J Chem Eng, 23*, 972–6.
38. Torres, M. J., Trejo, S. A., Martin, M. I., Natalucci, C. L., Aviles, F. X., & Lopez, L. M. I. (2010). Purification and characterization of a cysteine endopeptidase from *Vasconcellea quercifolia* A. St.- Hil. latex displaying high substrate specificity. *J Agric Food Chem, 58*, 11027–35.
39. Scopes, R. K. (1984). *Protein purification. Principles and practice (pp. 219)*. New York: Springer.
40. Uhlig, H. (1998). *Industrial enzymes and their applications (pp. 351–374)*. New York: Wiley-Interscience.
41. Glazer, A. N., & Smith, E. L. (1971). Papain and other plant sulfhydryl proteolytic enzymes. In P. D. Boyer (Ed.), *The Enzymes* (pp. 501–46). New York: Academic.

42. Sundd, M., Kundu, S., Pal, G. P., & Jagannadham, M. V. (1998). Purification and characterization of a highly stable cysteine peptidase from the latex of *Ervatamia coronaria. Biosci Biotechnol Biochem, 62*, 1947–55.
43. Kundu, S., Sundd, M., & Jagannadham, M. V. (2000). Purification and characterization of a stable cysteine protease Ervatamin B, with two disulfide bridges, from latex of *Ervatamia coronaria. J Agric Food Chem, 48*, 171–9.
44. Dubey, V. K., & Jagannadham, M. V. (2003). Procerain, a stable cysteine peptidase from the latex of *Calotropis procera. Phytochemistry, 62*, 1057–71.
45. Ahmad, B., & Khan, R. H. (2006). Studies on the acid unfolded and molten globule states of catalytically active stem bromelain: a comparison with catalytically inactive form. *J Biochem, 140*, 501–8.
46. Bechtel, P. J. (2005). Properties of stickwater from fish processing byproducts. *J Aquat Food Prod Technol, 14*, 25–38.
47. Domsalla, A., & Melzig, M. F. (2008). Occurrence and properties of proteases in plant lattices. *Planta Med, 74*, 699–711.
48. Biswas, S., Chakrabarti, C., Kundu, S., Jagannadham, M. V., & Dattagupta, J. K. (2003). Proposed amino acid sequence and the 1.63 A X-ray crystal structure of a plant cysteine protease, ervatamin B: some insights into the structural basis of its stability and substrate specificity. *Proteins, 51*, 489–97.
49. Thakurta, P. G., Biswas, S., Chakrabarti, C., Sundd, M., Jagannadham, M. V., & Dattagupta, J. K. (2004). Structural basis of the unusual stability and substrate specificity of ervatamin C, a plant cysteine protease from *Ervatamia coronaria. Biochemistry, 43*, 1532–40.
50. Singh, A. N., Yadav, P., & Dubey, V. K. (2013). cDNA cloning and molecular modeling of procerain B, a novel cysteine endopeptidase isolated from *Calotropis procera. PLoS ONE, 8*, e59806.
51. Brockbank, W. J., & Kenneth, R. L. (1979). Purification and preliminary characterization of two asclepains from the latex of *Asclepias syriaca* L. (Milkweed). *Biochim Biophys Acta, 578*, 13–22.
52. Mitchel, R. E., Chaiken, R. M., & Smith, E. L. (1970). The complete amino acid sequence of papain. *J Biol Chem, 245*, 3485–92.
53. Rawlings, N. M., & Barrett, A. J. (2013). Introduction: the clans and families of cysteine peptidases. In N. D. Rawlings & G. S. Salvesen (Eds.), *Handbook of proteolytic enzymes* (pp. 1743–73). London: Academic.