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Penduliflorain I: A Cysteine Protease Isolated from *Hohenbergia penduliflora* (A.Rich.) Mez (*Bromeliaceae*)

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Abstract Penduliflorain I, a new plant endopeptidase, was isolated and characterized from *Hohenbergia penduliflora*. Crude extract was obtained from stems. A partially purified enzyme preparation was obtained by ethanol precipitation. This preparation showed maximum activity between pH 7.5 and 8.5, was stable at ionic strength (20% decrease in proteolytic activity could be detected after 2 h in 0.4 M sodium chloride solution), and exhibited high thermal stability (inactivation required heating for 20 min at 75 °C). Inhibition and activation assays indicated the cysteine nature of the enzymatic preparation. Penduliflorain I was purified by anion exchange chromatography (Q-Sepharose HP) by FPLC system. Homogeneity was confirmed by mass spectroscopy. Molecular mass of the enzyme was 23 412.847 Da (MALDI-TOF–MS). Kinetic parameters were determined for PFLNA ($K_m = 0.3227$ mM and $k_{cat} = 4.27\ s^{-1}$). The N-terminal sequence (AVPQSIDWRDYGAVTTDKNQ) of isolated protease showed considerable similarity to other cysteine proteases obtained from stems or fruits of different *Bromeliaceae* species.

Keywords *Bromeliaceae* · *Hohenbergia penduliflora* · Chromatography purification · Stems · Thiol proteases

Abbreviations

AMPSO	3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid
BLAST	Basic local alignment search tool
CAPS	3-(ciclohexylamino)-L-propanesulfonic acid
E-64	*Trans*-(epoxysuccinyl)-L-leucylamido-(4-guadino) butane
EDTA	Ethylendiaminetetraacetic acid
FPLC	Fast protein liquid chromatography
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MES	2-(N-morpholino) ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
PFLNA	L-pyroglutamyl-L-phenylalanyl-L-leucine-p-nitroanilide
Pepstatin A	Isovaleryl-L-valil-L-valil-4-amino-3-hidroxy-6-methylheptanoil-L-alanil-4-amino-3-hidroxy-6-methylheptanoic acid
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecyl sulfate
TAPS	N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TFA	Triflouroacetic acid

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1 Introduction

Cysteine proteases are widely distributed in animals, microorganisms and plants [50]. These enzymes are involved in several physiological and developmental processes in plants [17]. *Bromeliaceae* is a plant family whose members usually produce large amounts of cysteine proteases [43, 44]. To date, a number of cysteine proteases from species belonging to *Bromeliaceae* family have been isolated and characterized: stem and fruit bromelain, ananain and comosain, obtained from *Ananas comosus* (L.) Merr. [32, 33, 37, 43, 44], as well as proteases from fruits of *Bromelia pinguin* L. [1, 49]; *Pseudananas macrodontes* (Morr.) Harms [27]; *Bromelia hieronymi* Mez [7–9], *Bromelia balansae* Mez [38], *Bromelia hemispherica* Lam., *B. palmeris* Mez, *B. sylvestris Willd. ex Sims.* [12, 21] and *B. plumieri* (E.Morren) L.B.Sm. [29].

Crude extracts of *Bromeliaceae* plants with high content of proteolytic enzymes have been used for a long time in folk medicine. More recently, cysteine endopeptidases are frequently used in food, biotechnology and pharmaceutical industries. They have been used for treatment of cancer, digestion disorders, viral diseases, inflammatory processes and immunemodulation problems [13, 23, 26, 36, 48]. Moreover, proteases are regarded as a food complement to soften meat, produce beer and dehydrate eggs [10, 15]. They are also frequently used for culture medium formulation [20]. For these reasons, special attention has been focused on the possibility to find new sources to obtain proteases.

Hohenbergia penduliflora Mez. (*Bromeliaceae*) is a terrestrial or epiphytic plant. It is acaulescent and it has wide leaves in rosette. Each plant has a unique inflorescence. The species is broadly distributed in Central America and Caribbean Islands [46].

The present paper deals with the isolation, purification and characterization of penduliflorain I, a new protease from stems of *Hohenbergia penduliflora* Mez.

2 Materials and Methods

2.1 Plant Material

Stems of *Hohenbergia penduliflora* (A.Rich.) Mez were obtained from plants grown in Modesto Reyes (Ciego de Avila, Cuba). A voucher specimen of the plant (N° 10 460) was deposited by Ph.D. Reinaldo Trujillo Sánchez at the "Julián Acuña" herbarium, CEMAEA of Camagüey, Cuba.

2.2 Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc., Cleveland, OH. AMPSO, CAPS, cysteine, DTT, E-64, EDTA, iodoacetic acid, MES, MOPS, pepstatin A, PMSF, TAPS and Tris were purchased from Sigma Chemical Company, St. Louis, MO, Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide, low molecular weight markers, and Bio-Lyte carrier ampholytes were obtained from Bio-Rad, Hercules, CA. Isoelectric point markers (low pI kit), and Q-Sepharose HP were purchased from Amersham Biosciences, Pittsburgh, PA.

2.3 Crude Preparation

Crude extracts were obtained according to Hernández et al. [22]. Stems of *Hohenbergia penduliflora* were homogenized (1:1.5 w/v) with the extractive solution (pH 3) in a domestic blender (2 L), the homogenate was filtered through a two-folded piece of gauze to remove plant debris and the suspension centrifuged at 15 000 *g* (Beckman J-21, Palo Alto, CA) for 30 min at 4 °C. All operations were carried out at 0–4 °C.

2.4 Partially Purified Preparation

Owing to the high carbohydrate concentration, 100 mL of crude extract was treated with an equal volume of cold (−20 °C) ethanol. The pellet was discarded, and then 4 volumes (400 mL) of ethanol were added to the supernatant. In both steps, the homogenate was treated with gentle agitation and left to settle for 20 min prior to centrifugation at 15,000*g* for 20 min at 4 °C. The final ethanol precipitated was redissolved with 50 mM Tris–HCl buffer, pH 8, and immediately frozen at −20 °C until analysis [47].

2.5 Protein Determination

Protein concentration was determined by the Coomassie Blue dye-binding method [6], using bovine serum albumin as standard. The protein content of chromatography eluates were estimated by absorbance measurement at 280 nm during separation.

2.6 Proteolytic Activity Assays

Reaction mixture contained 0.1 mL of enzyme solution and 1.1 mL of 1% (w/v) casein solution in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM cysteine. The mixture was incubated at 37 °C for 2–30 min, according to enzyme concentration. The reaction was stopped by the addition of 1.8 mL of 5% (w/v) trichloroacetic acid (TCA). Blanks were prepared by adding TCA to the enzyme solution, and then adding the substrate. The test tubes were centrifuged at 7,000*g* for 20 min, and the absorbance of the supernatants was measured at 280 nm. An arbitrary enzyme unit (Ucas) was used to express proteolytic activity [34].

2.7 Determination of Optimum pH

Solutions of 1% casein containing 10 mM cysteine were prepared within the pH range 6–10 using 25 mM sodium salts of the following "Good" buffers: MES, MOPS, TAPS, AMPSO and CAPS [16].

2.8 pH Stability

Samples were incubated for 0, 5, 10, 20, 40, 60, 90, and 120 min at pH 6, 7, 8, 9, and 10, using 25 mM sodium salts of the following "Good" buffers: MES, MOPS, TAPS, AMPSO and CAPS [16]. The residual caseinolytic activity was measured as described above.

2.9 Thermal Stability

To determine the effect of temperature, samples were incubated for 0, 5, 10, 20, 40, 60, 90 and 120 min at 20, 25, 37, 45, 55, 65, and 75 °C. The residual caseinolytic activity was measured as indicated before after stopping the reaction by immersing the tubes in an ice-water bath.

2.10 Ionic Strength Stability

Samples were incubated for 0, 5, 10, 20, 40, 60, 90, and 120 min with different sodium chloride concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1 M) and the residual caseinolytic activity was measured as mentioned.

2.11 Effect of Inhibitors

The effect of specific inhibitors [45] on proteolytic activity was determined by measuring the residual activity on casein or azocasein at pH 8.0 after preincubation at 37 °C for 30 min in the presence of *trans*-(epoxysuccinyl)-L-leucylamido-(4-guadino) butane (E-64; 1 mM), iodoacetic acid (10 and 100 mM), phenylmethanesulfonyl fluoride (PMSF, 100 mM), isovaleryl-L-valil-L-valil-4-amino-3-hidroxy-6-methylheptanoil-L-alanil-4-amino-3-hidroxy-6-methylheptanoic acid (pepstatin A; 0.1 μM) or 1,10-phenantroline (100 mM). Inhibition reversion was tested by addition of cysteine (20 mM) after the incubation period. Controls were prepared by preincubating the enzyme solution with the appropriate solvent used to dissolve the inhibitors. Caseinolytic activity was measured as mentioned.

3 SDS–PAGE

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed at 12.5% of polyacrylamide in a Miniprotean III Cell (Bio-Rad) according to Laemmli [24]. Electric current was kept constant at 30 mA during stacking and then increased to 150 mA and kept constant for 60 min. Coomassie brilliant blue R-250 were used for staining gels.

3.1 Isoelectric Focusing and Zymogram

Isoelectric focusing (IEF) was developed on 5% polyacrylamide gels containing broad pH range ampholytes (Bio-Lyte 3–10, Bio-Rad) in a Mini IEF Cell (Model 111, Bio-Rad). Samples were precipitated with 5 volumes of cold (−20 °C) acetone, centrifuged, and the protein sediments redissolved in deionized water. Focusing was carried out under constant voltage conditions in a stepped procedure: 100 V for 15 min, 200 V for 15 min and 450 V for 60 min. Gels were fixed and then stained with Coomassie Brilliant Blue R-250. In order to visualize proteolytic activity, zymograms were performed. An agarose gel was imbibed during 20 min with substrate solution (1% casein in Tris–HCl buffer, pH 8.0, 20 mM cysteine) and then washed twice with distilled water. Gels were analyzed with Electroban software [3]. Unstained IEF gels were contacted for 15 min at 55 °C with the agarose gel. Proteolytic compounds became visible as clear bands on the stained agarose gels [51].

3.2 Anion Exchange Chromatography

The chromatography was performed in a FPLC System (Pharmacia Biotech) using a column (Amersham Biosciences XK 16/40, with AK16 adaptors) packed with Q-Sepharose High Performance (Amersham Biosciences) and equilibrated with 50 mM Tris–HCl (pH 7.6) containing 5 mM $Na_2S_4O_6$. After washing the column with the same buffer, the retained proteins were eluted with 120 mL of two different linear sodium chloride gradients (0–0.15, 0.15–0.6 M) in the starting buffer. All steps were performed at 20 °C.

3.3 Mass Spectrometry

Homogenity and molecular mass of the purified endopeptidase were determined by mass spectrometry (MALDI-TOF–MS). Mass spectra were acquired on a Bruker Ultraflex spectrometer equipped with a pulsed nitrogen laser (337 nm), in linear positive ion mode, using a 19 kV acceleration voltage. Samples were prepared by mixing equal volumes of a saturated solution of the matrix (3,5-dimethoxy-4-hydroxycinnamic acid) in 0.1% TFA in water/acetonitrile 2:1, and a 1–10 μM protein solution. From this mixture, 1 μL was spotted on MTP 384 target plate polished steel (Bruker Daltonik GmbH) and allowed to evaporate to dryness. A protein of known molecular mass (trypsinogen) was used as standard for mass calibration.

3.4 N-Terminal Sequence

The N-terminal sequence of the purified fraction was determined by Edman's automated degradation using an Applied Biosystems (Procise 492) peptide sequencer. Protein homology searches were performed using the basic local alignment search tool (BLAST) network service [2].

3.5 Titration of the Active Site with E-64

Titration of the active site was performed as described by Barrett and Kirschke [4] with minor modifications. The enzyme (5 μM) was preincubated with activation buffer (50 mM Tris–HCl, pH 8.0, containing 20 mM cysteine). Fractions (150 μL) were incubated with 50 μL of different concentrations (0–10 μM) of E-64 for 30 min at 37 °C and the residual activity then was measured on PFLNA according to Filippova et al. [14]. The enzyme concentration was established by determining both protein content [6] and molecular mass value (mass spectrometry).

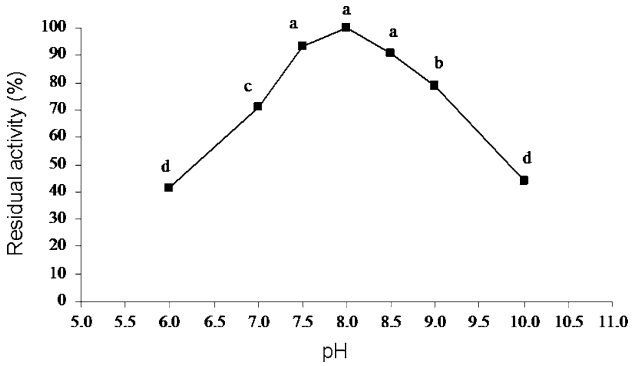

Fig. 1 Effect of pH on caseinolytic activity of partially purified preparation. 25 mM sodium salts of the following Goods buffers were used: MES (pH 5.5–6.7), MOPS (pH 6.5–7.9), TAPS (7.7–9.1), AMPSO (pH 8.3–9.7) and CAPS (pH 9.7–11.1). Data points represent the mean value of five determinations. The experiment was repeated three times. Results with the same *letter* are not statistically different (Kruskal–Wallis, Dunnett's C, $p > 0.05$)

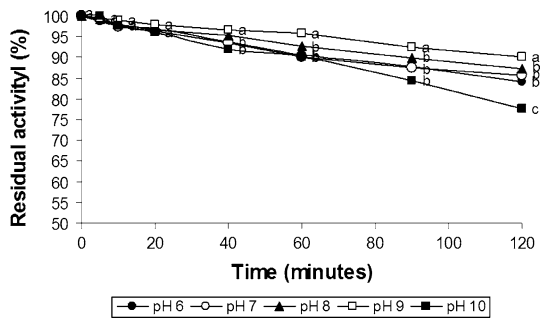

Fig. 2 pH stability of partially purified preparation. Caseinolytic activity was measured after incubating solutions of partially purified preparations at pH 6, 7, 8, 9 and 10. Samples were taken after 5, 10, 20, 40, 60, 90 and 120 min of incubation. Data points represent the mean value of five determinations. The experiment was repeated three times. At each evaluation time, results with the same *letter* are not statistically different (Kruskal–Wallis, Dunnett's C, $p > 0.05$)

3.6 Kinetic Studies

Initial rates of hydrolysis were measured spectrophotometrically at 410 nm according to Filippova et al. [14] at pH 6.5 and 45 °C for concentrations of PFLNA ranging from 0.25 to 0.6 mM in the reaction mixture. Amidolytic

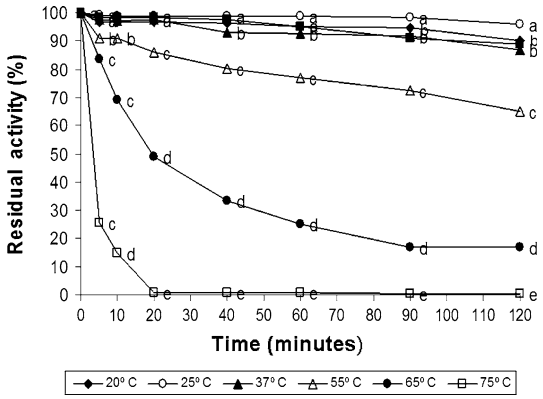

Fig. 3 Thermal stability of partially purified preparation. Caseinolytic activity was mesuared after incubating solutions of partially purified preparation at 20, 25, 37, 45, 55, 65 and 75 °C Sample were taken after 5, 10, 20, 40, 60, 90 and 120 min of incubation. Data points represent the mean value of five determinations. The experiment was repeated three times. At each evaluation time, results with the same *letter* are not statistically different (Kruskal–Wallis, Dunnett's C, $p > 0.05$)

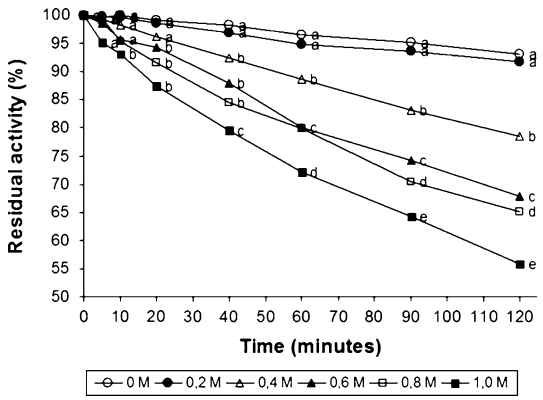

Fig. 4 Ionic strength stability of partial purified preparation. Caseinolytic activity was mesuared after incubating solutions of partially purified preparation at 0.2, 0.4, 0.6, 0.8 and 1 M. Sample were taken after 5, 10, 20, 40, 60, 90 and 120 min of incubation. Data points represent the mean value of five determinations. The experiment was repeated three times. At each evaluation time, results with the same *letter* are not statistically different (Kruskal–Wallis, Dunnett's C, $p > 0.05$)

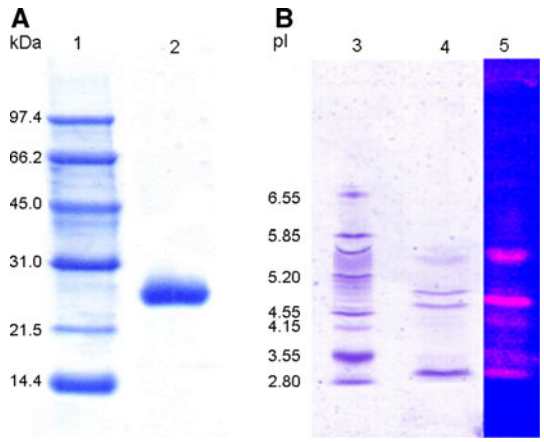

Fig. 5 SDS–PAGE, IEF and zymogram of partial purified preparation. **a** SDS–PAGE on 12.5% polyacrylamide gel. **b** IEF and zymogram. *Lane 1* molecular weight standards: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase bovine (31.0 kDa), ovalbumin (45.0 kDa), serum albumin bovine (66.2 kDa), and phosphorylase b (97.4 kDa); *lane 2*, partial purified preparation; *lane 3*, isoelectric point markers (low pI): pepsinogen (pI 2.80), amyloglucosidase (pI 3.50), glucose oxidase (pI 4.15), trypsin inhibitor (pI 4.55), *β*- lactoglobulin A (pI 5.20), carbonic anhydrase B, bovine (pI 5.85), and carbonic anhydrase B, human (pI 6.55); *lane 4*, partial purified preparation and *lane 5*, partial purified preparation zymogram

activity was expressed in an arbitrary enzyme unit (U_{PFLNA}), defined as the amount of peptidase that released 1 μmol of *p*-nitroanilide. Initial rates of velocity were calculated at different substrate concentrations from the product concentration vs. time curves. K_m and V_{max} were calculated by regression analysis using the nonlinearized form of the Michaelis–Menten equation (Sigma Plot 8.02). k_{cat} (V_{max}/enzyme concentration) was estimated using the functional enzyme concentration obtained on the basis of site active titration.

4 Results and Discussion

Crude extract from stems of *Hohenbergia penduliflora* contained 0.89 mg/mL of proteins and showed a caseinolytic activity of 0.98 Ucas/mL. Proteolytic activity of *Bromeliaceae* plants have been generally evaluated with casein and haemoglobin [43].

The redissolved ethanol precipitate retained 90.8% of the initial activity and 83% of the original protein content. Partially purified preparation exhibited maximum caseinolytic activity in the neutral–weak alkaline pH zone: higher

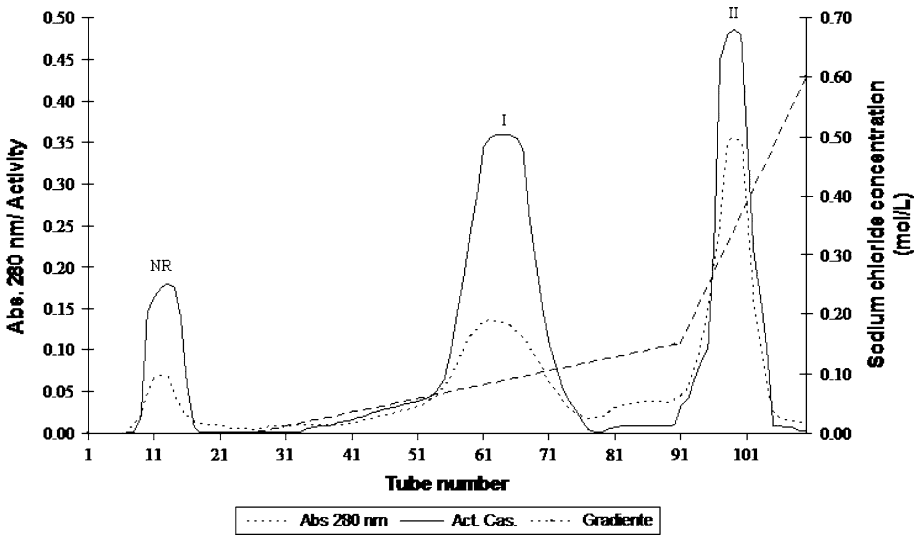

Fig. 6 Anion exchange chromatography (Q-Sepharose high performance) of partially purified preparation. Column was equilibrated with 50 mM Tris–HCl (pH 7.6), 5 mM Na2S4O6. The unbound material was eluted by washing with one column volume of the equilibrating buffer (flow rate: 0.75 mL/min), and the bound proteins were eluted with two different NaCl linear gradients (0–0.15, 0.15–0.60 M) in the same buffer (flow rate: 0.5 mL/min). Fractions of 2.0 mL were collected. All steps were performed at 20 °C

Table 1 Purification scheme of penduliflorain I

Step	Vol. (mL)	Protein (mg/mL)	Total prot. (mg)	Cas. Act. (Ucas/mL)	Total. cas. Act. (Ucas)	Specific. act. (U/mg)	Yield (%)	Purification (*n*-fold)
PPP*	4.0	0.739	2.950	0.889	3.556	1.205	100.0	1.00
No retained	8.0	0.042	0.336	0.041	0.328	0.979	9.25	0.81
Peak I (penduliflorain I)	18.0	0.016	0.288	0.112	2.016	6.998	56.7	5.80
Peak II	10.0	0.066	0.660	0.101	1.010	1.530	28.4	1.27

* Partial purified preparation

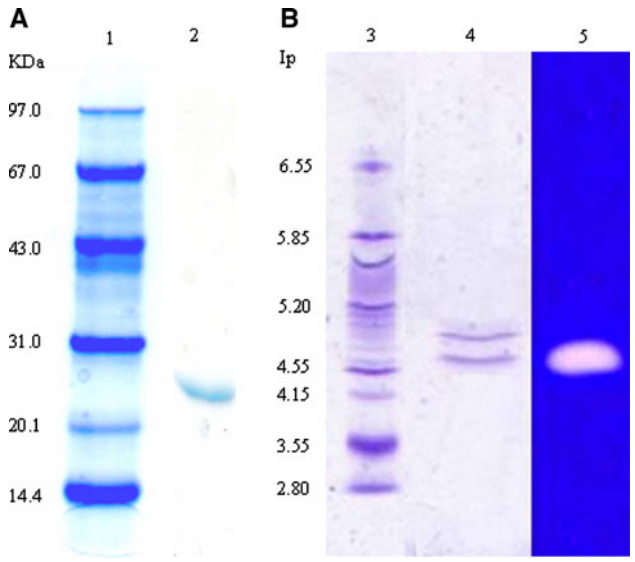

Fig. 7 SDS–PAGE, IEF and zymogram of penduliflorain I. **a** SDS–PAGE on 12.5% polyacrylamide gel. **b** IEF and zymogram. *Lane 1* molecular weight standards: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase bovine (31.0 kDa), ovalbumin (45.0 kDa), serum albumin bovine (66.2 kDa), and phosphorylase b (97.4 kDa); *lane 2*, penduliflorain I; *lane 3*, isoelectric point markers (low pI): pepsinogen (pI 2.80), amyloglucosidase (pI 3.50), glucose oxidase (pI 4.15), trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), carbonic anhydrase B, bovine (pI 5.85), and carbonic anhydrase B, human (pI 6.55); *lane 4*, penduliflorain I and *lane 5*, penduliflorain I zymogram

than 90% of maximum activity between pH 7.5 and 8.5 (Fig. 1) and was very stable at pH 6–9 (conserved up to 85% of residual activity after 120 min, Fig. 2).

The preparation was very stable (more than 80% of caseinolytic activity) between 20 and 45 °C after 120 min of incubation. Thermal inactivation was achieved after 20 min of incubation at 75 °C (Fig. 3). The partially purified preparation was very stable at high salt concentrations: conserved up to 55% of residual activity after 120 min of incubation in 1 M sodium chloride solution (Fig. 4).

The preparation was irreversibly inhibited by E-64 (1 mM), a selective inhibitor for the "papain-like" cysteine endopeptidases [44] and iodoacetic acid (100 mM), and activated by 20 mM cysteine. The obtained results suggest that *Hohenbergia penduliflora* proteases belong to the cysteine-type proteases, as all other endopeptidases isolated from *Bromeliaceae* species [39]. SDS–PAGE of the partially purified preparation revealed the presence of only one band with molecular mass around 25,000 Da (Fig. 5a). IEF followed by zymogram showed several protein bands (three of them with proteolytic activity) between pI 2.8 and 5.85 (Fig. 5b).

Characterization of the partially purified preparation demonstrates its potential application in biotechnological processes such as production of protein hydrolysates for

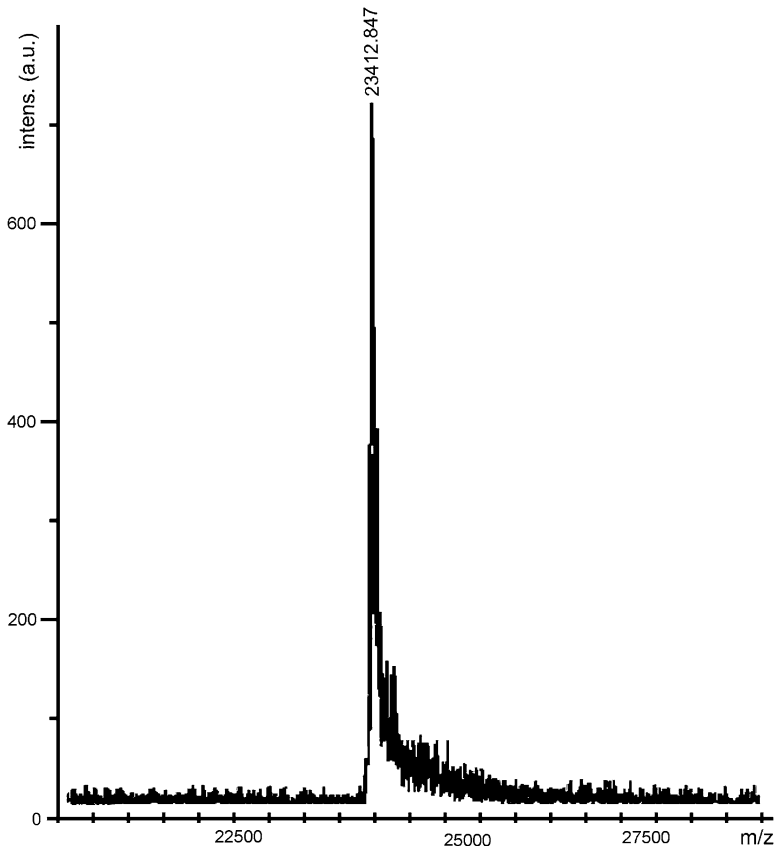

Fig. 8 Mass spectrometry of penduliflorain I

Table 2 N-terminal amino acid sequences of some cysteine plant endopeptidases

Plant protease	Source	N-terminal sequence #																	Identity (%) (*; **)	Reference
Penduliflorain I	*Hohenbergia penduliflora*	A	V	P	Q	S	I	D	W	R	D	Y	G	A	V	T	T	D	K	N	Q			
			V	P	Q	S	I	D	W	R	D	Y	G	A	V	T	T	D	K	N	Q			
Stem bromelain	*Ananas comosus*	A	V	P	Q	S	I	D	W	R	D	Y	G	A	V	T	**S**	V	K	N	Q		90; 85	[41]
Fruit bromelain	*Ananas comosus*	A	V	P	Q	S	I	D	W	R	D	Y	G	A	V	**N**	**E**	V	K	N	Q		85; 80	[52]
Macrodontain I	*Pseudananas macrodontes*	A	V	P	Q	S	I	D	W	R	D	Y	G	A	V	**N**	**E**	V	K	N	Q	G	85; 80	[27]
Macrodontain II	*Pseudananas macrodontes*	A	V	P	Q	S	I	D	W	R	D	Y	G	A	V	**N**	**E**	V	K	N	Q		85; 80	[28]
Balansain I	*Bromelia balansae*	A	V	P	**E**	S	I	D	W	R	D	Y	G	A	V	T	**S**	V	K	N	Q		85; 80	[38]
Ananain	*Ananas comosus*		V	P	Q	S	I	D	W	R	D	**S**	G	A	V	T	**S**	V	K	N	Q		80; 85	[25]
Comosain	*Ananas comosus*		V	P	Q	S	I	D	W	R	**N**	Y	G	A	V	T	**S**	V	K	N	Q		80; 85	[33]
Hieronymain I	*Bromelia hieronymi*	A	**L**	P	**E**	S	I	D	W	R	A	**K**	G	A	V	T	**E**	V	K	**R**	Q	D	65; 60	[7]
Papain	*Carica papaya*		**I**	P	**E**	**Y**	**V**	D	W	R	**Q**	**K**	G	A	V	T	**P**	V	K	N	Q	G	55; 60	[11]

Letters in bold: different aa residue in relation to the sequence of penduliflorain

* Homology for first sequence of penduliflorain I

** Homology for second sequence of penduliflorain I

food industry, culture media and nutritional supplements [20, 30, 31].

On the basis of these results anion exchange chromatography was selected for the next purification step, which afforded three proteolytic fractions (Fig. 6). The non-retained fraction was the minor one. When the sodium chloride gradient reached 0.15 M, the main proteolytic component eluted (Fig. 6, peak I). Finally a single peak of activity against casein was obtained (Fig. 6, peak II). The first peak, named penduliflorain I, was selected for the study shown here, due to its high specific activity. The purification scheme is presented in Table 1.

Yielding of penduliflorain I was 56.7% and the purification factor was 5.80-fold. The purification factor of penduliflorain I is low, but this is common in plant organs with high proteolytic activity, where proteases represent the bulk of protein content of crude extracts [27].

Homogeneity of the purified enzyme was checked by SDS–PAGE and MS (Figs. 7a, 8). The molecular mass of penduliflorain I determined by SDS–PAGE was 23.8 kDa, whereas by MS was 23412.847 Da, similar to those indicated for cysteine proteases isolated from other *Bromeliaceae* species [5] and lying in the range of 21–30 kDa reported for most of the plant papain-like cysteine proteinases [18]. As can be seen in Fig. 7b, IEF showed two bands at pI 4.7 and 5.0 probably owned to the presence of isoforms, a frequent fact in endopeptidases isolated from species belonging to the *Bromeliaceae* family [42], which can be interpreted as chemical modifications (deamidation or hydroxilation) with no significant molecular weight difference but with slightly charge variations, affording different pI bands [8, 9, 19].

Two different N-terminal sequences of penduliflorain I were obtained, differing only in the first amino acid (AVPQSIDWRDYGAVTTDKNQ and VPQSIDWRDYGAVTTDKNQ), one of them starting with Ala and the other one with Val. Similar behavior was observed for the N-terminal sequence of bromelain, where 25% started with Ala and 75% with Val [41], results which could be attributed to different cleavages of the proenzyme during activation. The N-terminal sequence of penduliflorain I was compared to those of other *Bromeliaceae* cysteine proteases (Table 2). A remarkable similarity (90 and 85% identities) between penduliflorain I and stem bromelain N-terminus can be observed, as well as with those of fruit bromelain, and macrodontain I and II (85 and 80% identities). The high correspondence showed by penduliflorain I N-terminus with those belonging to other cysteine proteases included in the clan CA, family C1, according to MEROPS [40], confirms that the *Bromeliaceae* endopeptidases are more closely related to each other than to other members of the papain family, suggesting relatively recent evolutive divergence [43]. The presence of a Gln residue in

Table 3 Kinetic parameters obtained for penduliflorain I toward synthetic substrates

Substrate	K_m (mmol L^{-1})	V_{max} (mmol L^{-1} s^{-1}) (10^{-3})	k_{cat} (s^{-1})	k_{cat}/K_m ($mmol^{-1}$ L s^{-1})
N-α-CBZ-ala-*p*-nitrophenyl ester*	0.07	0.45	11.06	153.45
PFLNA**	0.33	0.17	4.27	13.22
Z-Phe-Arg-pNA**	0.53	0.16	3.28	6.16
Bz-Phe-Val-Arg-pNA**	0.78	0.10	3.29	4.20

* The protease was assayed by its esterolytic activity against the synthetic protease substrate N-α-CBZ-ala-*p*-nitrophenyl ester. One unit of protease activity was defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenol per minute from the substrate in the assay system described in kinetic studies under Sect. 2

** The protease was assayed by its amidolytic activity against the synthetic protease substrates**.** One unit of protease activity was defined as the amount of enzyme which releases of 1 μmol of *p*-nitroanilide per minute from the substrate in the assay system described in kinetic studies under Sect. 2

the 19 position is important for catalytic activity by helping the catalytic triad in the formation of the "oxoanion hole", an electrophilic center that stabilizes the tetrahedral intermediate. The existence of Pro2 is also a common feature in the mature proteases of family C1, and it is suggested that it prevents attack by aminopeptidases, due to the resistance offered by the Xaa-Pro bond to such enzymes [44]. SIDWR and GAV motifs are also present in all *Bromeliaceae* proteases sequences.

E-64 and cysteine endopeptidases react each other on an equimolecular basis, so this inhibitor can be used for titration of the enzyme active site [4]. In the titration assay (data not shown) the residual enzyme activity gave a straight line (residual activity vs. E-64 concentration) that intersected the abscissa at 1.97 μM E-64, corresponding to 40% of active enzyme. This performance has been previously reported for other *Bromeliaceae* cysteine peptidases: hieronymain I and II showed that almost half of both enzymes are in inactive form [7, 8].

Kinetic parameters (K_m, V_m, and k_{cat}) were determined for both esterolytic and amidolytic activities (Table 3). The ratio k_{cat}/K_m, sometimes called the specificity constant, is the best way to compare the catalytic efficiency of a enzyme [35]. The best results were obtained on the *N*-CBZ-*p*-nitrophenyl ester derivative of Ala ($k_{cat}/K_m = 153.45$ seg^{-1} mM^{-1}), one order of magnitude higher than those obtained on PFLNA ($k_{cat}/K_m = 13.22$ seg^{-1} mM^{-1}). PFLNA is a typical substrate for cysteine endopeptidases [14]. Non-detectable activity could be found on Z-Arg-Arg-p-nitroanilide, but the enzyme exhibited slightly activity on Z-Phe-Arg-pNA and Bz-Phe-Val-Arg-pNA. Rowan and Buttle [43] reported that ananain and fruit bromelain, proteases isolated from pineapple plants, preferred Bz-Phe-Val-Arg-pNA and did not degradated Z-Arg-Arg-p-nitroanilide. In our experiments, penduliflorain I showed similar substrate preferences.

Results of SDS–PAGE, isoelectro-focusing, mass spectrometry, kinetic parameters and N- terminal amino acid sequence showed that the purified penduliflorain I could belong to the¨papain-likë family of proteases.

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References

1. Abreu Payrol J, Obregón WD, Trejo SA, Caffini NO (2008) Protein J 27:88–96
2. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Nucleic Acids Res 25:3389–3402
3. Báez E, Guerra M, Rodríguez P, Hernández Z, Nieves N (1996) Cult Trop 17:45–47
4. Barrett AJ, Kirschke H (1981) Meth Enzymol 80:535–561
5. Barrett AJ, Rawlings ND (2004) In: Barrett AJ, Rawlings ND, Woessner JF (eds) Handbook of proteolytic enzymes, vol II, 2nd edn. Academic Press, London, p 2368
6. Bradford MM (1976) Anal Biochem 72:248–254
7. Bruno MA, Pardo MF, Caffini NO, López LM (2003) J Prot Chem 22:127–138
8. Bruno MA, Trejo SA, Avilés XF, Caffini NO, López LM (2006) Protein J 25:224–231
9. Bruno MA, Trejo SA, Caffini NO, López LM (2008) Protein J 27:426–433
10. Burica O, Vitez L (1989) Biotechnol 22:26–80
11. Cohen LW, Coghlan VM, Dihel LC (1986) Gene 48:219–227
12. Cruz MT, Oliver MC, Del Castillo LM, Castañeda-Agulló M (1974) Rev Latinoamer Quím 5:18–25
13. Engwerda CR, Andrew D, Ladhams A, Mynott TL (2001) Cell Imm 210:66–75
14. Filippova IY, Lysogorskaya EN, Oksenoit ES, Rudenskaya GN, Stepanov VM (1984) Anal Biochem 143:293–297
15. Foda Y, Bassiouny S, Abdalla M, Shehata M (1980) Mesopotamian J Agricul 15:93–111
16. Good NE, Izawa S (1972) Meth Enzymol 24:53–68
17. Granell A, Cercos M, Carbonell J (1998) In: Barrett AJ, Rawlings ND, Woessner JF (eds) Handbook of proteolytic enzymes. Academic Press, San Diego, pp 578–583
18. Grzonka Z, Jankowska E, Kasprzykowski F, Kasprzykowska R, Lankiewicz L, Wiczk W, Wieczerzak E, Ciarkowski J, Drabik P, Janowski R, Kozak M, Jaskólski M, Grubb A (2001) Acta Biochim Polonica 48:1–20
19. Harrach T, Eckert K, Maurer H, Macheleidt I, Nuck R (1998) J Protein Chem 17:51–61

20. Headon D, Walsh G (1994) Biotechnol Adv 12:635–646
21. Hernández Arana A, Rodríguez Romero A, Cruz y Victoria MT, Del Castillo LM (1983) Rev Latinoam Quim 14:95–98
22. Hernández M, Chávez M, Márquez M, Rodríguez G, Santos R, González J, Carvajal C. (1997) Cuban Patent C12 N 9/50
23. Kelly T (2005) Drug Resist Updat 8:51–58
24. Laemmli U (1970) Nature 237:680–685
25. Lee K, Albee K, Bernasconi R, Edmunds T (1997) Biochem J 327:199–202
26. Leipner J, Iten F, Saller R (2001) BioDrugs 15:779–789
27. López LM, Sequeiros C, Natalucci CL, Brullo A, Maras B, Barra D, Caffini NO (2000) Protein Express Purif 18:133–140
28. López LMI, Sequeiros C, Trejo SA, Pardo MF, Caffini NO, Natalucci CL (2001) Biol Chem 382:875–878
29. Montes C, Amador M, Cuevas D, Córdoba F (1990) Agric Biol Chem 54:17–24
30. Morris H, Almarales A, Carrillo O, Abdala RT (2001) Revista Cubana de Alimentación y Nutrición 15(2):85–89
31. Morris H, Borges L, Martínez C, Carrillo O (2002) Revista Cubana de Alimentación y Nutrición 16(1):5–12
32. Murachi T (1970) In: Arnon R (ed) Methods in enzymology, vol 14. Academic Press, New York, pp 475–485
33. Napper AD, Bennett SP, Borowski M, Holdridge MB, Leonard MC, Rogers EE, Duan Y, Laursen RA, Reinhold B, Shames SL (1994) Biochem J 301:727–735
34. Natalucci CL, Brullo A, López LM, Hilal RM, Caffini NO (1996) J Food Biochem 19:443–454
35. Nelson DL, Cox MM (2005) In: Freeeman WH & Co (ed) Lehninger principles of biochemistry, 4th edn. WH Freeeman, New York, p 207
36. Onken JE, Greer P, Calingaert B, Hale L (2008) Clinical Immunol 126:345–352
37. Ota S, Muta E, Katahira Y, Okamoto Y (1985) J Biochem 98:219–228
38. Pardo MF, López LM, Canals F, Avilés FX, Natalucci CL, Caffini NO (2000) J Agric Food Chem 48:3795–3800
39. Rawlings ND, Barrett AJ (2004) In: Barrett AJ, Rawlings ND, Woessner JF (eds) Handbook of proteolytic enzymes, vol II, 2nd edn. Academic Press, London, pp 1051–1071
40. Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ (2008) Nucleic Acids Res 36:D320–D325
41. Ritonja A, Buttle DJ, Rawlings ND, Turk V, Barrett AJ (1989) FEBS Lett 258:109–112
42. Rowan AD, Buttle DJ, Barrett AJ (1990) Biochem J 266:869
43. Rowan AD, Buttle DJ (1994) Meth Enzymol 244:555–568
44. Rowan AD (1998) In: Barrett AJ, Rawlings ND, Woessner JF (eds) Handbook of proteolytic enzymes. Academic Press, San Diego, pp 546–572
45. Salvesen GS, Nagase H (2001) In: Beynon R, Bond JS (eds) Proteolytic enzymes, 2nd edn. Oxford University Press, Oxford, pp 105–128
46. Sauget JS (1946) In: Cultural SA (ed) La Flora de Cuba. La Habana, Cuba, pp 284–297
47. Scopes RK (1984) In: Springer (ed) Protein purification. Principles and practice. Springer, New York, p 219
48. Targoni O, Tary L, Lehmann P (1999) J Autoimmunol 12:191–198
49. Toro-Goyco E, Rodriguez-Costas I, Ehrig H (1980) Biochim Biophys Acta 622:151
50. Turk V, Guncar G, Turk D (1999) In: Turk V (ed) Proteases: New perspectives. Basel, Switzerland, pp 1–16
51. Westergaar JL, Hackbarth C, Treuhaft MW, Roberts RC (1980) J Immunol Meth 34:167–175
52. Yamada F, Takahashi N, Murachi TJ (1976) J Biochem 79:1223–1234