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Biochemical analysis of a papain-like protease isolated from the latex of *Asclepias curassavica* L.

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Most of the species belonging to *Asclepiadaceae* family usually secrete an endogenous milk-like fluid in a network of laticifer cells in which sub-cellular organelles intensively synthesize proteins and secondary metabolites. A new papain-like endopeptidase (asclepain cII) has been isolated and characterized from the latex extracted from petioles of *Asclepias curassavica* L. (*Asclepiadaceae*). Asclepain cII was the minor proteolytic component in the latex, but showed higher specific activity than asclepain cI, the main active fraction previously studied. Both enzymes displayed quite distinct biochemical characteristics, confirming that they are different enzymes. Crude extract was purified by cation exchange chromatography (FPLC). Two active fractions, homogeneous by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and mass spectrometry, were isolated. Asclepain cII displayed a molecular mass of 23,590 Da, a pI higher than 9.3, maximum proteolytic activity at pH 9.4–10.2, and showed poor thermostability. The activity of asclepain cII is inhibited by cysteine proteases inhibitors like E-64, but not by any other protease inhibitors such as 1,10-phenantroline, phenylmethanesulfonyl fluoride, and pepstatine. The N-terminal sequence (LPSFVDWRQKGVVFPIRNQGQ CGSCWTFSA) showed a high similarity with those of other plant cysteine proteinases. When assayed on N-α-CBZ-amino acid-p-nitrophenyl esters, the enzyme exhibited higher preference for the glutamine derivative. Determinations of kinetic parameters were performed with N-α-CBZ-L-Gln-p-nitrophenyl ester as substrate: $K_m = 0.1634$ mM, $k_{cat} = 121.48$ s^{-1}, and $k_{cat}/K_m = 7.4 \times 10^5$ s^{-1}/mM.

Keywords *Asclepias curassavica*; *Asclepiadaceae*; cysteine proteinase; latex; laticifers**

Introduction

Proteases are enzymes that catalyze the degradation of peptides and proteins, and occupy a significant role in numerous physiologic processes in the living beings, as well as by their use in different industrial processes. It has been verified that proteases direct specific and selective modifications of proteins, such as the activation of proenzymes, sanguineous coagulation, digestion of fibrin clots, secretory protein processing and transport through membranes, germination, senescense, defense against plant pathogens (especially fungi and insects), and acquisition of nutrients and apoptosis [1–7].

In the world, the industries that apply enzymes for their products invest annually near a trillion of dollars in their commercialization. Of them, 75% belong to hydrolytic enzymes and of this percentage, the proteases represent 60% of total of the world-wide sales [2]. The vast diversity of proteases, with its specification, has attracted the attention of the scientists worldwide that has taken them to explore its multiple physiologic and industrial applications. In relation to this last aspect, the proteases develop to a protagonist role in the biotechnological processes because they maintain their enzymatic activity within a wide range of pH and temperature [8].

At the moment several examples of the use of enzymes and of specifically proteases in different areas from the industry can be mentioned: modified proteins for the food industry, baking, beer elaboration, cheese production, detergent dust preparation, treatment of industrial effluents,

textile industry, manufacture of leather, pharmaceutical industry, cleaning of surgical supplies, and biomedicals [9–12].

Species belonging to the *Asclepiadaceae* family usually contain proteolytic enzymes in latex. Latex is a milky fluid with a complex mixture of constituents, like proteins, vitamins, carbohydrates, lipids, terpenes, alkaloids, and free amino acids. The presence of certain enzymes like chitinases and proteases in latex vacuoles suggest that they may help plants for defense against pathogens, parasites, and herbivores by attacking the invader once the plant cell is lysed [13].

Our group has been largely devoted to screening new plant sources from the local flora, which could provide new proteases, as plant proteases used in Argentina are usually imported. Many members of the *Asclepiadaceae* family have shown to contain very active proteases in the latex. The group has previously reported in the purification and characterization of proteases present in lattices of several members of the *Asclepiadaceae* family [14–22].

Asclepias curassavica L. is native of South America and grows from Mexico to Argentine but has become a naturalized weed in tropical and subtropical areas throughout the world. The stems of this species exude large quantities of latex when leaves are cut off, which has been used in folk medicine as emetic, vermifuge, and antigonorrheic [23,24]. The main proteolytic component, named asclepain cI, was isolated from the latex in a previous study [25]. In this article the biochemical characteristics of a second protease is presented, which despite of being the minor proteolytic component in the latex, it showed higher specific activity than asclepain cI. Additionally, this new enzyme exhibited different properties that could be profitable when used in industrial processes.

Materials and Methods

Plant material
Asclepias curassavica L., '*scarlet milkweed*', (*Asclepiadaceae*) is an erect, evergreen perennial sub-shrub with woody base. Like most milkweeds, it has opposite leaves and milky sap [26]. Latex was obtained from plants grown in Rosario, Province of Santa Fe, Argentina. A voucher specimen (UNR 1130) has been deposited at the UNR herbarium (Faculty of Agricultural Sciences, University of Rosario, Argentina).

Crude enzyme extract preparation
Latex (2 ml), obtained by superficial incisions of petioles, was collected on 13 ml of 0.1 M citric phosphate buffer (pH 6.5) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and cysteine, in order to avoid phenoloxidase activity and oxidation, respectively. This suspension was first centrifuged at $16,000\,g$ for 30 min at 4°C to discard gums and other insoluble materials, and the supernatant was ultracentrifuged at $100,000\,g$ for 1 h at 4°C. This new supernatant containing soluble proteins (crude extract (CE), 12 ml), was fractionated and conserved at -20°C for further studies.

Purification of asclepain cII
The CE was applied in a column packed with SP-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with 0.05 M Tris–HCl buffer (pH 8.25). Cation exchange chromatography (FPLC, Pharmacia) was developed by adding the starting buffer (0.05 M Tris–HCl, pH 8.25), followed by a sodium chloride linear gradient (0–0.6 M) prepared in the same buffer. Cation exchange chromatography was monitored by measuring absorption at 280 nm. Caseinolytic activity was tested in on the eluted fractions, and those showing proteolytic activity were pooled and stored at -20°C for further studies (cf next section).

Proteolytic activity assays
Proteolytic assays were made using casein as substrate. The reaction mixture was prepared by mixing 0.1 ml of the purified enzyme with 1.1 ml of 1% casein containing 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 8.5). The reaction was carried out at 42°C and stopped 2 min later by the addition of 1.8 ml of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at $3000\,g$ for 20 min and the absorbance of the supernatant measured at 280 nm. An arbitrary enzyme unit (U_{cas}) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions.

Protein determination
Proteins were measured according to Bradford's method [27] using bovine albumin (Sigma Chemical Co., St. Louis, USA) as standard.

pH profile
The effect of pH on enzyme activity was determined on casein (pH range 6.0–10.5) at constant ionic strength using 0.01 M sodium salts of the following buffers [28]: 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino) propanesulfonic acid (MOPS), *N*-tris(hydroxymethyl)

methyl-3-aminopropanesulfonic acid (TAPS), 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid (AMPSO), and 3-(ciclohexylamino)-1-propanesulfonic acid (CAPS). At each pH a control assay was done without enzyme and used as a blank.

pH stability

Two milliliter of the purified enzymatic preparation was precipitated with three volumes of acetone, centrifuged at $3000\,g$ during 20 min, and then the precipitated was redissolved in the corresponding 'Good' buffer adjusted to pH 10.0. Samples (0.1 ml) were incubated at $20°C$ for $1-3$ h and the residual activity was assayed according to the method described above.

Thermal stability

After incubating the enzyme solution (0.1 ml) for 30, 60, 90, and 120 min at $40°C$, $50°C$, $60°C$, and $70°C$, the residual proteolytic activity was determined as indicated previously.

Effect of inhibitors on proteolytic activity

The effect of specific inhibitors on proteolytic activity was determined by preincubating the protease preparation on casein or azocasein [29] with inhibitors and then estimating the residual activity. The enzyme preparation (0.99 ml) was incubated with 0.01 ml of the one following inhibitors: 10 mM $(2S,3S)$-3-(N-{(S)-1-[N-(4-guanidinobutyl)carbamoyl]3-methylbutyl} carbamoyl)oxirane-2-carboxylic acid (E-64), 100 mM phenylmethanesulfonyl fluoride (PMSF), and $0.1-0.5$ mM pepstatin A, during 30 and 60 min at $30°C$. The residual proteolytic activity was determined on casein as indicated above.

Due to 1,10-phenantroline exhibits high absorbance at 280 nm, for this assay casein was changed by azocasein. The enzyme preparation (0.99 ml) was incubated with 0.01 ml of 100 mM 1,10-phenanthroline and then the residual activity was determined as follows: 0.25 ml of 2% azocasein in 0.1 M glycine—NaOH buffer (pH 9.5) containing 20 mM cysteine was added to 0.15 ml of enzyme sample and incubated at $45°C$ for 30 min. The reaction was stopped by adding 1 ml of 10% TCA. After centrifugation at $4000\,g$ for 15 min, 0.9 ml of the supernatant obtained was added to 1 ml of 1 M NaOH, and the absorbance was measured at 440 nm. In this case, one unit of proteolytic activity (U_{azocas}) was defined as the amount of enzyme (g) that produced an absorbance increase of one unit per minute under the assay conditions.

Controls were prepared by preincubating the enzyme with the appropriate solvent used to dissolve the inhibitors [30]. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%.

Electrophoresis

Purified samples of asclepain cII were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Tris—glycine cathodic buffer in 10% polyacrylamide gels [31]. Voltage was kept constant at 40 mV for the stacking gel and at 150 mV for the resolution gel. The gels were stained with coomassie brilliant blue R-250 and the molecular weight of asclepain cII was estimated by using the Scion Image software. Protein molecular weight markers (SDS-PAGE Molecular Weight Standards, Low Range; Bio Rad, Hercules, USA) were used as standards to generate the calibration curve.

Isoelectric focusing and zymogram

Isoelectric focusing (IEF) was performed in a mini IEF cell (Model III, Bio-Rad). The sample was concentrated and deionized with five volumes of cold acetone and further centrifugation at $3000\,g$ for 15 min. The precipitate obtained was redissolved with deionized water and the treatment repeated twice. Polyacrylamide gels containing broad pH range ampholytes $(3.0-10.0)$ were used. 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. Gel was fixed and then stained with Coomassie brilliant blue R-250.

In order to ascertain if the protein fraction had proteolytic activity, the unstained gel was contacted for 10 min at $50°C$ with an agarose gel imbibed for 20 min in 1% casein solution (0.1 M Tris—HCl buffer, pH 8.3) with 12 mM cysteine and washed twice with distilled water. Then, the agarose gel was stained by Coomassie brilliant blue R-250. Clear bands on the stained agarose gels evidence the presence of proteolytic activity [32].

Mass spectrometry

The molecular weight and purity of asclepain cII were determined by Matrix assisted laser desorption ionisation mass spectrometry/time-of-flight (MALDI-MS/TOF). Mass spectrum was acquired on a Bruker Daltonics® model Ultraflex spectrometer equipped with a pulsed nitrogen laser (337 nm), in a linear positive ion mode, using a 20 kV acceleration voltage. Samples were prepared by mixing equal volumes of a saturated solution of the matrix (sinapic acid) in 0.1% Trifluoroacetic acid

(TFA) (aq.): acetonitrile (2:1), and protein solution. From this mixture, 1 μl was spotted on the sample slide and allowed to evaporate to dryness. Bovine trypsinogen was used for internal calibration.

N-terminal sequence

The N-terminal sequence was determined by Edman's automated degradation using a Beckman LF3000 protein sequencer equipped with a System Gold (Beckman, Fullerton, USA) PTH-amino acid analyzer. The Basic Local Alignment Search Tool (BLAST) network service [33] was used to perform protein homology studies but considering only those specific residues that are identical ('identities').

Measurement of endoesterolytic activity

These assays were carried out by the Silverstein's [34] method modified to reach optimal conditions of the enzyme. The activity was studied using N-α-Cbz-L-Gln-p-nitrophenyl esters of some L-amino acids (Ala, Asn, Gln, Gly, Ile, Leu, Trp, Pro, and Val) as substrates. Assays were made at 37°C in 0.1 M Tris–HCl buffer (pH 8.0) containing 2 mM EDTA, 25 μM cysteine and 1 mM of each substrate in the reaction mixture. Liberation of p-nitrophenol was followed spectrophotometrically at 405 nm in an Agilent 8453 E UV-visible spectroscopy system (Santa Clara, CA, USA) equipped with a chamber termostatized at 37°C. An arbitrary enzyme activity unit (U_{cbz}) was defined as the amount of protease (g) that released one micromol of p-nitrophenolate per min in the assay conditions. To determine the micromoles of p-nitrophenolate produced during the reaction, a standard curve with p-nitrophenol (5–50 μM) in 0.1 M Tris–HCl buffer pH 8.0 containing 5% acetonitrile was carried out.

Measurement of amidolytic activity

Amidolytic activity was determined by hydrolysis of L-pyroglutamil-L-phenylalanyl-L-leucine-p-nitroanilide (PFLNA) according to Filippova *et al.* [35]. This assay was performed using a solution of 1 mM PFLNA in dimethyl sulfoxide (DMSO). The reaction mixture contained 1.5 ml of 0.1 M phosphate buffer, pH 6.5, 0.3 M KCl, 10^{-4} M EDTA, 0.003 M dithiothreitol (DTT), 0.18 ml substrate, and 0.12 ml enzyme. The p-nitroaniline released at 37°C was detected spectrophotometrically at 410 nm. An arbitrary enzyme activity unit (U_{PFLNA}) was defined as the amount of protease (g) that released one micromol of p-nitroaniline per min in the assay conditions.

Kinetic parameters

N-α-CBZ-L-Gln-p-nitrophenyl ester was used to determine V_m, K_m, k_{cat}, and k_{cat}/K_m of asclepain cII. Estimation of kinetic parameters was performed following the method described previously for this substrate [34]. Substrate final concentration ranged from 125×10^{-6} to 2×10^{-3}. K_m, k_{cat}, V_m, and k_{cat}/K_m values were calculated by hyperbolic regression analysis using the nonlinearized form of the Michaelis–Menten equation.

Results

Purification

Cation exchange chromatography allowed the separation of three fractions (**Fig. 1**). No proteolytic activity was found in the former fraction eluted, but the two other fractions eluted with the linear salt gradient used were active. The active fractions were named asclepain cI and cII, according to nomenclature recommendations for proteases obtained from latex of species of the *Asclepiadaceae* family [36,37].

Asclepain cI was the main fraction [25]. Asclepain cII was purified with 12.1-fold, the percent recovery in terms of total activity was 9.8% and the specific activity was 12.8 U_{cas}/mg (**Table 1**).

Homogeneity of the enzyme

Purity of asclepain cII was checked both by SDS-PAGE and mass spectrometry: 23,500 and 23,590 Da, respectively (**Figs. 2** and **3**). The presence of a single band both in SDS-PAGE and IEF (**Fig. 4**) show that the enzyme is homogenous, like asclepain cI.

As it has already been mentioned before, IEF and zymogram confirmed the presence of a unique band with proteolytic activity, corresponding to a basic
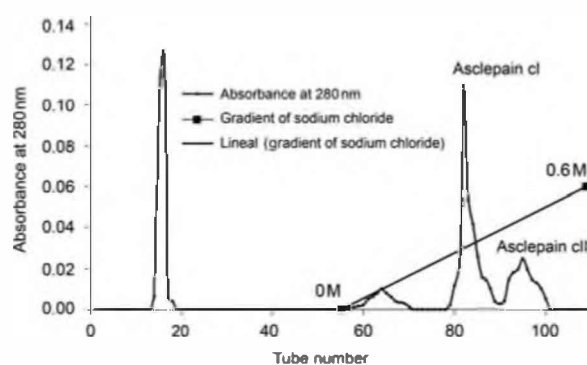

Fig. 1 Cation exchange chromatography Elution buffer: 0.05 M Tris–HCl (pH 8.25). Gradient: sodium chloride 0–0.6 M. Flow rate: 0.75 ml/min. Fraction volume: 2.0 ml. SP-Sepharose Fast Flow, column Pharmacia Biotech XK 16.

Table 1 Purification of the proteolytic components present in the latex of *Asclepias curassavica* L

Sample	Volume (ml)	Protein (mg/ml)	Total proteins	Arbitrary enzyme unit U_{cas} (ml^{-1})	Total U_{cas}	Specific activity (U_{cas}/mg)	Purification (fold)	Yield (%)
CE	1.5	0.9333	1.399	0.9880	1.480	1.0586	1	100
Asclepain cI	7.5	0.0243	0.182	0.2649	1.980	10.877	10.27	26.80
Asclepain cII	10.5	0.0075	0.078	0.0968	1.016	12.836	12.125	9.80

An arbitrary enzyme unit (U_{cas}) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions.
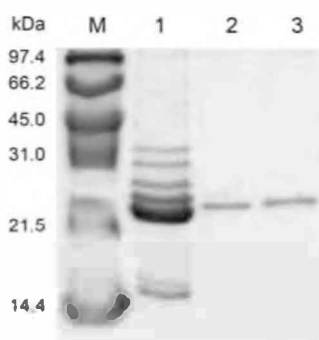

Fig. 2 SDS-PAGE M, Bio Rad molecular weight standards: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovoalbumin (45.0 kDa), carbonicanhydrase bovine (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); 1, crude extract; 2, asclepain cI; 3, asclepain cII.
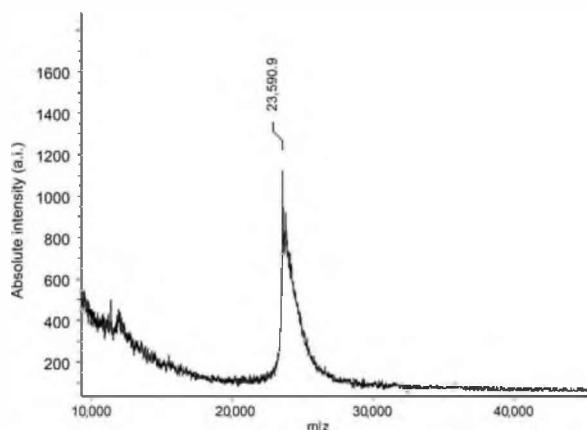
Fig. 3 Mass spectroscopy of asclepain cII MALDI-MS/TOF was used for the determination of purity degree as well as of molecular weight of asclepain cII. Sample was mixed with sinapinic acid (matrix) dissolved in 0.1% trifluoroacetic acid. Bovine trypsinogen was used for internal calibration.

protein, which focused at pI higher than 9.3 (**Fig. 4**). The pI value of asclepain cII is comparable with those of other plant proteases from the *Asclepiadaceae* family (**Table 2**).
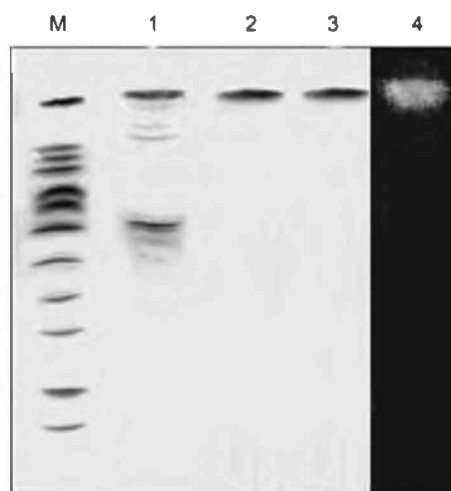
Fig. 4 isoelectric focusing and corresponding zymogram M, pI markers, amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β-lactoglobulin a (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85, 7.15, and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45, and 8.65), and trypsinogen (pI 9.30); 1, crude extract; 2, asclepain cI; 3, asclepain cII; 4, zymogram.

Dependence of enzyme activity on pH

Asclepain cII showed maximum activity within the range of pH 9.4 and 10.2 (casein), which is higher than that exhibited by the CE of *Asclepias curassavica* and asclepain cI [25], and similar to that of funastrain cII [22]. On the other hand, asclepain cII showed a high stability at pH 10 retaining about 95% of the residual activity after 1 h (**Fig. 5**).

Dependence of enzyme activity on temperature

Asclepain cII exhibited poor thermostability, as only 30% of its activity was retained after 2 h of incubation at 50°C (**Fig. 6**). At 60–70°C the lost of activity is rapid.

Effect of inhibitors on proteolytic activity

Different inhibitors specific for distinctive classes of proteases were used to determine the class to which the purified protease belongs (**Fig. 7**). The enzyme was

Table 2 Comparison of biochemical characteristics of phytoproteases belonging to *Asclepiadaceae* family

Phytoproteases	Molecular mass (Da)	pI	pH optima	References
Asclepain cII	23,590	9.3	8.5	This study
Asclepain cI	23,200	9.3	8.5	[25]
Araujiain aI	23,464	9.3	8.00	Our previous work
Araujiain aII	23,528	9.3	8.25	Our previous work
Araujiain aIII	23,488	9.3	9.25	Our previous work
Araujiain hI	24,031	9.3	8.75	[19]
Araujiain hII	23,718	8.9	8.5	[19]
Araujiain hIII	23,446	10.5	8.5	[19]
Asclepain f	23,652	9.3	9.5	[21]
Funastrain cII	23,636	9.3	9.5	[22]
Morrenain bI	23,205	9.3	8.70	[15]
Morrenain bII	26,000	9.3	8.25	[17]
Morrenain oI	27,000	9.3	8.5	[17]
Morrenain oII	25,800	9.3	8.5	[17]
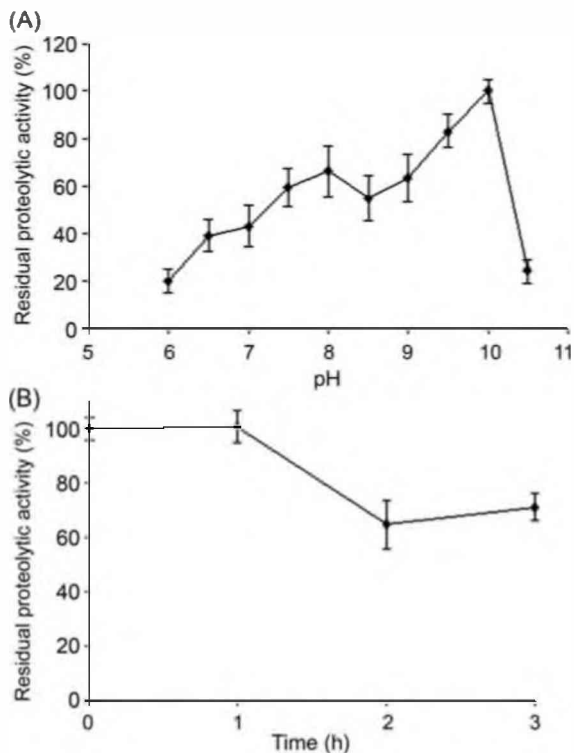
Fig. 5 Effect of pH (A) Proteolytic activity of asclepain cII measured on casein (range pH 6.0–11.0) using 0.01 M sodium salts of the following buffers: MES, MOPS, TAPS, AMPSO, and CAPS. (B) Stability at pH optimum on the proteolytic activity of asclepain cII, using buffers adjusted to pH 10.0 following 1–3 h incubation at 20°C.
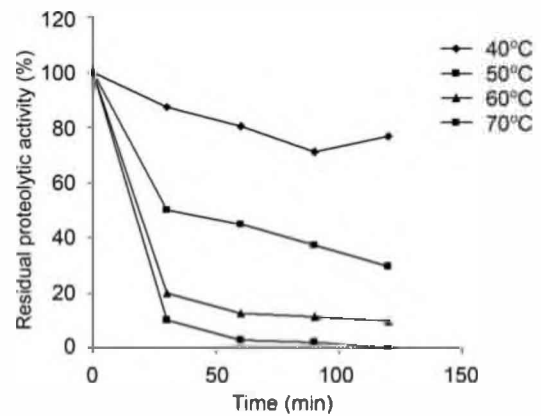
Fig. 6 Thermal stability on the proteolytic activity of the asclepain cII at 40°C, 50°C, 60°C, and 70°C following 30, 60, 90, and 120 min incubation.
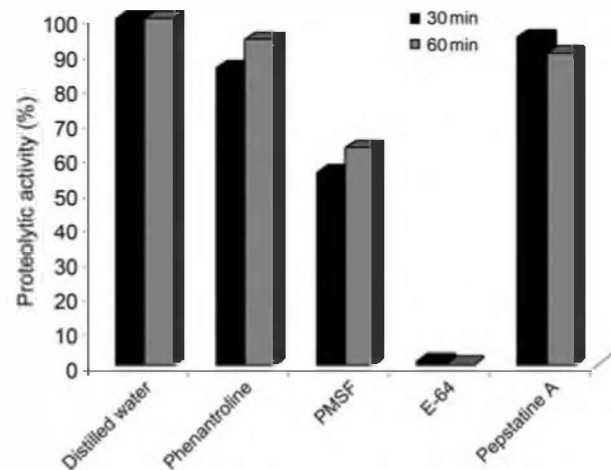
Fig. 7 Effect of inhibitors on the proteolytic activity of asclepain cII incubated on each inhibitor solution during 30 and 60 min at 30°C.

immediately and irreversibly inhibited by 10 mM E-64 (cysteine proteases inhibitor). Incubation with 100 mM PMSF (serine proteases inhibitor) for 30 min decreased activity to 63% of the initial value, but inhibition was partially reverted when 12 mM cysteine was added to the reaction mixture (data not shown). As 1,10-phenantroline (metaloproteases inhibitor) and pepstatine (aspartic proteases inhibitor) do not affect the activity of asclepain cII.

N-terminal protein sequence

The N-terminus sequence of asclepain cII (30 amino acids) was analyzed with the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). This sequence showed higher identity (86% and 80%, respectively) with proteases isolated from species of *Asclepias* and *Funastrum* genera

(**Table 3**). Both, asclepain cI and cII, shared the motifs surrounding the catalytic cysteine (CGS and WTFS), that also occur in most of the sequences compared. The DWR and QG motifs are notably conserved and are present in all the cases, as well as the proline residue located in position 2, to which is attributed a protective anti-aminopeptidase role [38].

Measurement of endoesterolytic activity

When the enzyme was assayed using *N*-α-Cbz-L-amino acid-*p*-nitrophenyl esters, the highest relative esterolytic activity was obtained for the glutamine derivative (**Table 4**), as happened with asclepain cI [25].

Measurement of amidolytic activity

Asclepain cII exhibited very low activity on PFLNA, a typical substrate for cysteine endopeptidases (data not shown), unlike asclepain cI, which showed an important activity on this substrate [25].

Kinetic parameters

K_m, k_{cat}, and k_{cat}/K_m ratio of asclepain cII were determined with the substrate for which the CE showed the greatest preference, that is *N*-α-Cbz-L-amino acid-*p*-nitrophenyl

ester. The obtained results were: $K_m = 0.1634$ mM; $V_m = 0.000974$ mM/s; $k_{cat} = 121.48$ s^{-1}; and $k_{cat}/K_m = 7.4 \times 10^5$ s^{-1}/mM. The k_{cat}/K_m values within a range from $10^5 - 10^6$ s^{-1}/mM revealed a high affinity of the enzyme for this substrate.

Discussion

Although asclepain cII is the minor fraction, it showed higher specific activity (12.8 U$_{cas}$/mg) than asclepain cI (10.8 U$_{cas}$/mg). The molecular mass of this new enzyme is very close to that of asclepain cI [25] and similar to those obtained for other species of the *Asclepias* genus, like *Asclepias syriaca*, that displays proteases of molecular weight 21,000 and 23,000 Da [39,40], *Asclepias glaucescens*, with a single protease of molecular weight 23,000 Da [36,37] and *Asclepias fruticosa*, also containing a single protease of molecular weight 23,652 Da [21], as well as those from other genera of the same family, within the interval from 20 to 35 kDa [16,18,22].

The important loss of proteolytic activity at 60–70°C of asclepain cII denotes a different behavior from other proteases studied in our laboratory [15,16,18,19].

Table 3 Comparison of N-terminal amino acid sequences of asclepain cII and other cysteine plant endopeptidases

Plant source	Sequence	Identities (%)	Reference
Asclepain c II	LPSFVDWRQKGVVFPIRNQGQCGSCWTFSA	100 (30/30)	This study
Asclepain c I	LPNSVDWRQKGVVFPIRDQGKCGSCWTFSA	86 (26/30)	[25]
Asclepain b	LPNFVDWRKNGVVFPIRNQGQ	85 (18/21)	[39]
Asclepain f	LPDSVDWRPKGVVFPIRNQGK	80 (17/21)	[21]
Funastrain cII	LPNSVDWRQKGVVSATRNQGKCGSCWAFSA	80 (24/30)	[22]
Asclepain a	LPNSIDWRQKNVVFPIRNQG	75 (15/20)	[39]
Oryza sativa	LPASVDWRGKGAVNPVKNQGQCGSCWAFSA	73 (22/30)	[45]
Zinnia elegans	LPKSVDWRKKGAVSPVKNQGQCGSCWAFS	72 (21/29)	[46]
Papaya proteinase omega: PP III	LPENVDWRKKGAVTPVRHQGSCGSCWAFSA	70 (21/30)	[47]
Papaya proteinase: PP I	IPEYVDWRQKGAVTPVRNQGSCGSCWAFSA	70 (21/30)	[48]
Arabidopsis thaliana (cysteine protease)	LPVSVDWRKKGAVTPIRNQGSCGCCW	70 (21/30)	[49]
Morrenain o II	LPDSVDWRKKNLVFPVRNQGKCGSXWTFSA	70 (21/30)	[15]
Brassica oleracea (cysteine protease)	LPDEVDWRAKGAVFPVRNQGSCGSCWTFSA	66 (20/30)	[50]
Araujiain h III	LPESVDWRKKNLVFPVRNQGKCGSXXAFSA	66 (20/30)	[19]
Morrenain b II	LPDSVDWRKKNLVFPVRNQGKKC	65 (15/23)	[15]
Zea mays (cysteine protease)	LPETKDWREDGIVSPVKNQGKCGSCWTFSA	63 (19/30)	[51]
Solanum tuberosum	LPESVDWRDKGVLVGVKDQGSCGSCWAFSA	63 (19/30)	[52]
Lycopersicon esculentum	LPESIDWREKGVLVGVKDQGSCGSCWAFSA	60 (18/30)	[53]
Araujiain h II	VPDSIDWREKDAVLPIIRNQGKCGSIMAFX	60 (18/30)	[19]
Morrenain b I	VPDKIDYREKGAVLLIRNOGQDGKTWAFLA	53 (16/30)	[16]

Shared amino acids are shown as white characters on a black background.

Table 4 Asclepain cII's endoesterolytic activity using *N*-α-Cbz-L-Gln *p*-nitrophenyl esters of some L-amino acids as substrates

N-α-Cbz-L-amino acid *p*-nitrophenyl ester	U_{cbz}
Gln	75.35
Asn	130.00
Ala	181.28
Gly	226.06
Tyr	361.88
Asp	904.26
Val	0
Leu	0
Ile	0
Lys	0

An arbitrary enzyme activity unit (U_{cbz}) was defined as the amount of protease (g) that released one micromol of *p*-nitrophenolate per minute in the assay conditions.

Thermal behavior of these enzymes is a useful property, since it could be easily inactivated when used in the food industry, so that active enzyme is not ingested [41].

As asclepain cII was efficiently inhibited by E-64, but not by other type-specific inhibitors, it can be assumed that −SH groups could be involved in the catalytic mechanism of the enzyme, suggesting that this new protease belongs to the cysteine type.

The analysis of the N-terminal protein sequence of asclepain cII confirms that the protein belongs to the cysteine peptidases (CPs) group, peptidase C1A subfamily (MEROPS database nomenclature). This group includes the mammalian CPs (cathepsins B, C, F, H, L, K, O, S, V, X, and W) and is represented by papain as the archetypical protease.

The preference of asclepain cII for the *N*-α-Cbz-glutamine derivative was also observed in proteases isolated from *Araujia hortorum* (araujiain hI, hII, and hIII) and *Araujia angustifolia* (araujiain aII) [19]. In the case of the protease isolated from *Asclepias fruticosa* (asclepain f), the enzyme showed greatest preference by the alanine derivative, but the glutamine derivative was the second in order of preference [19,21].

The differential behavior on PFLNA by asclepain cI and cII, two proteases isolated from the same species, was also exhibited by hieronymainI and II, isolated in our laboratory from *Bromelia hieronymi* fruits [42].

The values of kinetic parameters of asclepain cII were of the same order as those obtained with araujiain hII and hIII isolated from *Araujia hortorum* [19].

As most cysteine proteases obtained from other plant latex sources, asclepain cII, the new protease isolated and purified from the latex of *A. curasavica*, could be useful in pharmaceutical and biotechnology industries due to their wide ranges of activity over temperature and pH [43,44].

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