Acta Biochim Biophys Sin (2009): 154–162 | © The Author 2009. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmn018.

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 c-II) has been isolated and characterized from the latex extracted from petioles of *Asclepias curassavica* L. (*Asclepiadaceae*). Asclepain c-II was the minor proteolytic component in the latex, but showed higher specific activity than asclepain c-I, 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 c-II 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 c-II 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

Received: October 23, 2008 Accepted: November 19, 2008

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 c-I, 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 c-I. 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 subshrub 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 ethylendiaminetetraacetic 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 c-II

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 (2*S*,3*S*)-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 c-II 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 c-II 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 c-II were determined by 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% 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 c-II. 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 c-I and c-II, according to nomenclature recommendations for proteases obtained from latex of species of the *Asclepiadaceae* family [36,37].

Asclepain c-I was the main fraction [25]. Asclepain c-II 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 c-II 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 c-I.

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

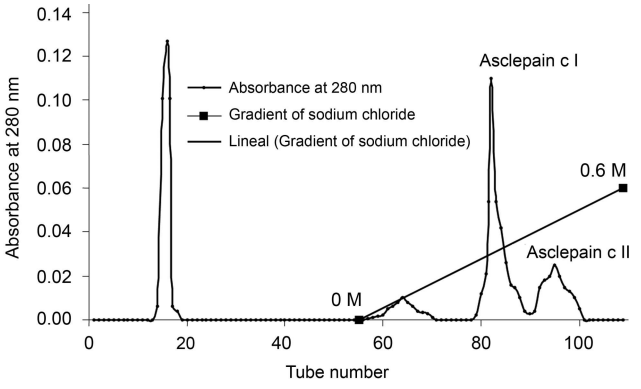

Fig. 1 Cation exchange chromatography Elution buffer: 0.05 M Tris–HCl (pH 8.25). Gradient: sodium chloride 0–0.6 M. Flow rate: 0.75 ml/min. Fraction volume: 2.0 ml. SP-Sepharose Fast Flow, column Pharmacia Biotech XK 16.

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 c-I	7.5	0.0243	0.182	0.2649	1.980	10.877	10.27	26.80
Asclepain c-II	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.

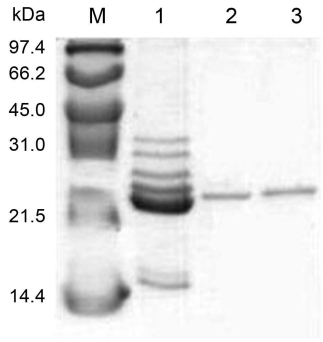

Fig. 2 SDS-PAGE M, Bio Rad molecular weight standards: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovoalbumin (45.0 kDa), carbonicanhydrase bovine (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); 1, crude extract; 2, asclepain c-I; 3, asclepain c-II.

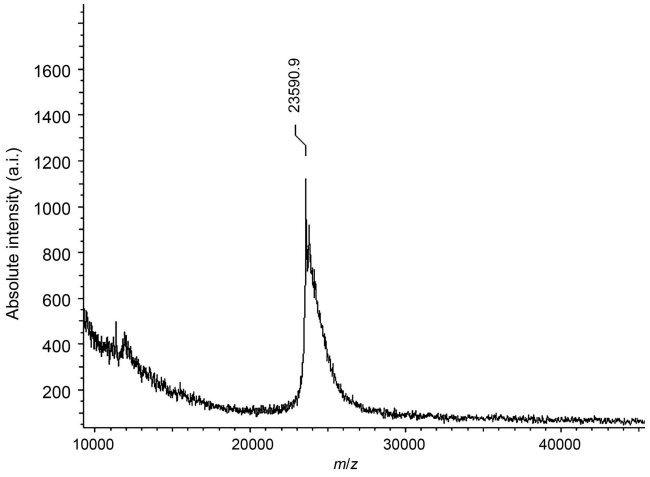

Fig. 3 Mass spectroscopy of asclepain c-II MALDI-MS/TOF was used for the determination of purity degree as well as of molecular weight of asclepain c-II. Sample was mixed with sinapinic acid (matrix) dissolved in 0.1% trifluoroacetic acid. Bovine trypsinogen was used for internal calibration.

protein, which focused at pI higher than 9.3 (**Fig. 4**). The pI value of asclepain c-II is comparable with those of other plant proteases from the *Asclepiadaceae* family (**Table 2**).

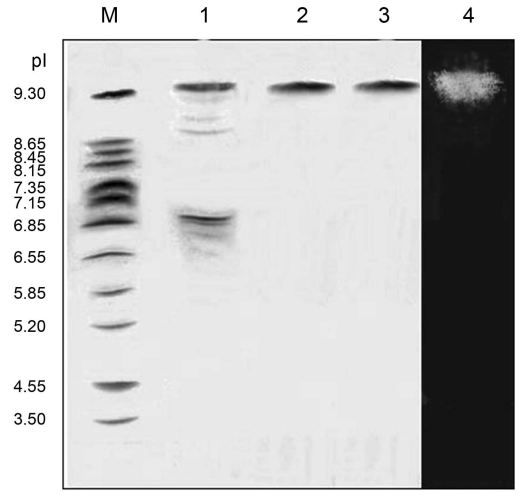

Fig. 4 isoelectric focusing and corresponding zymogram M, pI markers, amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β-lactoglobulin a (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85, 7.15, and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45, and 8.65), and trypsinogen (pI 9.30); 1, crude extract; 2, asclepain c-I; 3, asclepain c-II; 4, zymogram.

Dependence of enzyme activity on pH

Asclepain c-II 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 c-I [25], and similar to that of funastrain c-II [22]. On the other hand, asclepain c-II 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 c-II 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 3**). Both, asclepain c-I and c-II, 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 c-I [25].

Measurement of amidolytic activity

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

Kinetic parameters

K_m, k_{cat}, and k_{cat}/K_m ratio of asclepain c-II 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 c-II is the minor fraction, it showed higher specific activity (12.8 U_{cas}/mg) than asclepain c-I (10.8 U_{cas}/mg). The molecular mass of this new enzyme is very close to that of asclepain c-I [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 c-II 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 c-II 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	LPDSVDWREKGVVFPIRNQGK	80 (17/21)	[21]
Funastrain cII	LPNSVDWRQKGVVSAIRNQGKCGSCWAFSA	80 (24/30)	[22]
Asclepain a	LPNSIDWRQKNVVFPIKNQG	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	IPEYVDWRQKGAVTPVKNQGSCGSCWAFSA	70 (21/30)	[48]
Arabidopsis thaliana (cysteine protease)	LPVSVDWRKKGAVTPIKNQGSCGCCW	70 (21/30)	[49]
Morrenain o II	LPDSVDWRKKNLVFPVRNQGKXGSXWTFSA	70 (21/30)	[15]
Brassica oleracea (cysteine protease)	LPDEVDWRAKGAVFPVRNQGSCGSCWTFSA	66 (20/30)	[50]
Araujiain h III	LPESVDWRKKNLVFPVRNQGQXGSXXAFSA	66 (20/30)	[19]
Morrenain b II	LPDSVDWRKKNLVFPVRNQGKKG	65 (15/23)	[15]
Zea mays (cysteine protease)	LPETKDWREDGIVSPVKNQGHCGSCWTFSA	63 (19/30)	[51]
Solanum tuberosum	LPESVDWRDKGVLVGVKDQGSCGSCWAFSA	63 (19/30)	[52]
Lycopersicon esculentum	LPESIDWREKGVLVGVKDQGSCGSCWAFSA	60 (18/30)	[53]
Araujiain h II	VPDSIDWREKDAVLPIRNQGQXGSIWAFX	60 (18/30)	[19]
Morrenain b I	VPDKIDYREKGAVLDIRNQGQDGXIWAFLA	53 (16/30)	[16]

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

Table 4 Asclepain c-II'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 c-II 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 c-II 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 c-II for the *N*-α-Cbz-glutamine derivative was also observed in proteases isolated from *Araujia hortorum* (araujiain h-I, h-II, and h-III) and *Araujia angustifolia* (araujiain a-II) [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 c-I and c-II, two proteases isolated from the same species, was also exhibited by hieronymain-I and -II, isolated in our laboratory from *Bromelia hieronymi* fruits [42].

The values of kinetic parameters of asclepain c-II were of the same order as those obtained with araujiain h-II and h-III isolated from *Araujia hortorum* [19].

As most cysteine proteases obtained from other plant latex sources, asclepain c-II, 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].

Funding

This work was supported by the grants from ANPCyT (PICT 9-9916), CONICET (PIP 2813) and the University of La Plata, Argentina, as well as for CYTED IV.22. C.L. is member of the CIC Support Professional Career Program. W.D.O. is CONICET fellowship holder.

References

1 Grudkowska M and Zagdañska B. Multifunctional role of plant cysteine proteinases. Acta Bichimica Polonica 2004, 51: 609−624.
2 Rao M, Tanksale A, Ghatge M and Deshpande V. Microbiol Mol Biol Rev 1998, 62: 597−635.
3 Bell A. Biochemical mechanisms of disease resistance. Annu Rev Plant Physiol 1981, 32: 21−81.
4 Alvan der Hogrn R and Jones JDG. The plant proteolytic machinery and its role in defence. Curr Opin Plant Biol 2004, 7: 400−407.
5 Boller T. Roles of proteolytic in interactions of plant with other organisms. In: Dalling MJ ed. Plant Proteolytic Enzymes. Boca Raton: CRC Press, 1986.
6 Campbell DA and Szardenings AK. Functional profiling the proteome with affinity labels. Curr Opin Chem Biol 2003, 7: 296−303.
7 Baker EN and Drenth J. The cysteine proteinases structure and mechanism. In: Journal F and Mc Pherson A. eds. Biological Macromolecules and Assemblies. New York: Willey & Sons, 1987.
8 Dubey VK and Jagannadham MV. Procerain, a stable cysteine protease from the latex of *Calotropis procera*. Phytochemistry 2003, 62: 1057−1071.
9 Caffini NO, López LMI, Natalucci CL and Priolo NS. Proteasas de plantas superiores. Características generales, rol fisiológico y aplicaciones. Acta Farm Bonaerense 1988, 7: 195−213.
10 Raffo L. 2000. http://www.tracker.com.ar/docs/Enzimas.htm.
11 Uhlig H. Industrial Enzymes and their Applications. New York: Willey & Sons 1998, p1−11, 146−179.
12 Walsh G. Proteins Biochemistry and Biotechnology. New York: Wiley & Sons, 2004.
13 Vierstra RD. Proteolysis in plants: mechanisms and functions. Plant Mol Biol 1996, 32: 275.
14 Arribére MC, Cortadi A, Gattuso M, Bettiol M, Priolo NS and Caffini NO. Comparison of *Asclepiadaceae* latex proteases and characterization of *Morrenia brachystephana* Griseb. cysteine peptidases. Phytochem Anal 1998, 9: 267−273.
15 Vairo Cavalli SE, Cortadi A, Arribére MC, Conforti P, Caffini NO and Priolo NS. Comparison of two cysteine endopeptidases from latices of *Morrenia brachystephana* Griseb. and *Morrenia odorata* (Hook et Arn.) Lindley (*Asclepiadaceae*). Biol Chem 2001, 382: 879−883.

16 Vairo Cavalli S, Arribére MC, Cortadi A, Caffini NO and Priolo NS. Morrenain b I, a papain-like endopeptidase from the latex of *Morrenia brachystephana* Griseb. (*Asclepiadaceae*). J Protein Chem 2003, 22: 15–22.
17 Arribére MC, Vairo Cavalli SE, Priolo NS, Caffini NO, Gattuso M and Cortadi A. Proteolytic enzymes from the latex of *Morrenia odorata* (Hook. et Arn.) Lindley (*Asclepiadaceae*). Acta Horticulturae 1999, 501: 259–268.
18 Priolo N, Morcelle del Valle S, Arribére MC, López LMI and Caffini NO. Isolation and characterization of a cysteine portease from the latex of *Araujia hortorum* fruits. J Protein Chem 2000, 19: 39–49.
19 Obregón WD, Arribére MC, Morcelle del Valle S, Liggieri C, Caffini NO and Priolo NS. Two new cysteine endopeptidases obtained from the latex of *Araujia hortorum* fruits. J Protein Chem 2001, 20: 17–25.
20 Obregón WD, Curciarello R, Caffini N and Priolo N. Hydrolytic profile and isolation of the proteolytic components of latex from *Araujia angustifolia* fruits. Acta Farm Bonaerense 2006, 25: 206–212.
21 Trejo SA, López LMI, Cimino CV, Caffini NO and Natalucci CL. Purification and characterization of a new plant endopeptidase isolated from latex of *Asclepias fruticosa* L. (*Asclepiadaceae*). J Protein Chem 2001, 20: 445–453.
22 Morcelle del Valle SR, Trejo SA, Canals F, Avilés FX and Priolo NS. Funastrain c II, a cysteine endopeptidase purified from the latex of *Funastrum clausum*. Protein J 2004, 25: 205–215.
23 Roig JT. Plantas medicinales, aromáticas o venenosas de Cuba-La Habana, Cuba. Ed. Científico-Técnica, Tomo II-2da. ed., 3ra. reimpresión. 1945, 426, 428–1125.
24 Toursarkissian M. Plantas medicinales de la Argentina. Bs. As., Ed. H. Sur. 1980, 11–178.
25 Liggieri C, Arribére MC, Trejo SA, Canals F, Avilés F and Priolo N. Purification and biochemical characterization of asclepain c I from the latex *Asclepias curassavica* L. J Protein 2004, 23: 403–411.
26 Dimitri M. Enciclopedia Argentina de Agricultura y Jardinería. Acme SACI, Buenos Aires 1972, 774–780.
27 Bradford MM. A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976, 72: 248–254.
28 Good NE and Izawa S. Hydrogen ion buffers. Meth Enzymol 1972, 24: 53–68.
29 Morcelle del Valle S, Caffini NO and Priolo NS. Proteolytic properties of *Funastrum clausum* latex. Fitoterapia 2004, 75: 480–490.
30 Dunn BM. In: Beynon R and Bond JS eds. Determination of Protease Mechanism. Proteolytic Enzymes, a Practical Approach. Oxford: IRL Press, 2001.
31 Shägger H and Von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987, 166: 368–379.
32 Westergaard JL, Hackbarth C, Treuhaft MW and Roberts RCJ. Detection of proteinases in electrophoretograms of complex mixtures. Immul Meth 1980, 34: 167–175.
33 Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ. Nucleic Acids Res 1997, 25: 3389–3402.
34 Silverstein RM. The assay of the bromelains using *N*-CBZ-L-lysine p-nitrophenyl ester and *N*-CBZ-L-glicine p-nitrophenyl ester as substrates. Anal Biochem 1974, 62: 478–484.
35 Filippova IYu, Lysogorskaya EN, Oksenoit ES, Rudenskaya GN and Stepanov VM. L-pyroglutamyl-L-phenylalanyl-L-leucine-p-nitroanilide – a chromogenic substrate for thiol proteinase assay. Anal Biochem 1984, 143: 293–297.
36 Barragán BE, Cruz MT, del Castillo LM and Castañeda-Agulló M. Proteinases of mexican plants XI. Asclepain g from the látex of *Asclepias glaucescens*. Rev Latinoameri Quím 1985, 16: 117–119.
37 Tablero M, Arreguín R, Arreguín B, Soriano M, Sánchez RI, Rodríguez Romero A and Hernández-Arana A. H.B.K Purification and characterization of multiple forms of asclepain g from *Asclepias glaucescens*. Plant Sci 1991, 74: 7–15.
38 Barrett A, Rawlings ND and Woessner JF. In: Handbook of Proteolytic Enzymes. London: Academic Press, 2004.
39 Lynn KR, Brockbank WJ and Clevette NA. Multiple forms of the asclepains, cysteinyl proteases from milkweed. Biochim Biophys Acta 1980, 612: 119–125.
40 Brockbank WJ and Lynn KR. Purification and preliminary characterization of two asclepains from the latex of *Asclepias syriaca* L. (milkweed). Biochim Biophys Acta 1979, 578: 113–122.
41 Priolo NS, López LMI, Arribére MC, Natalucci CL and Caffini NO. New purified plant proteinases for the food industry. Acta Alimentaria 1991, 20: 189–196.
42 Bruno M, Trejo S, Avilés X, Caffini NO and López L. Isolation and characterization of hieronymain II, another peptidase isolated from fruits of *Bromelia hieronymi* Mez (*Bromeliaceae*). Protein J 2006, 25: 224–231.
43 Domsalla A and Melzig MF. Occurrence and properties of proteases in plant lattices. Planta Med 2008, 74: 699–711.
44 Salas CE, Gomes MTR, Hernandez M and Lopes MTP. Plant cysteine proteinases: evaluation of the pharmacological activity. Phytochemistry 2008, 69: 2263–2269.
45 Watanabe H, Abe K, Emori Y, Hosoyama H and Arai SJ. Molecular cloning and gibberellin-induced expression of multiple cysteine proteinases of rice seeds (oryzains). Biol Chem 1991, 266: 16897–16902.
46 Ye ZH and Varner JE. Induction of cysteine and serine proteases during phylogenesis in *Zinnia elegans*. Plant Mol Biol 1996, 30: 1233–1246.
47 Revell DF, Cummings NJ, Baker KC, Collins ME, Taylor MA, Sumner IG and Pickersgill RW, *et al*. Nucleotide sequence and expression in *Escherichia coli* of cDNAs encoding papaya proteinase omega from *Carica papaya*. Gene 1993, 127: 221–225.
48 Cohen LW, Coghlan VM and Dihel LC. Cloning and sequencing of papain-encoding cDNA. Gene 1986, 48: 219–227.
49 Asamizu E, Sato S, Kaneko T, Nakamura Y, Kotani H, Miyajima N and Tabata S. Structural analysis of *Arabidopsis thaliana* chromosome 5. VIII. Sequence features of the regions of 1,081,958 bp covered by seventeen physically assigned P1 and TAC clones. DNA Res 1998, 5: 379–391.
50 Coupe SA, Sinclair BK, Watson LM, Heyes JA and Eason JR. Identification of dehydration-responsive cysteine proteases during post-harvest senescence of broccoli florets. J Exp Bot 2003, 54: 1045–1056.
51 Domoto C, Watanate H, Abe K and Arai S. Isolation and characterization of two distinct cDNA clones encoding corn seed cysteine proteinases. Biochem Biophysis Acta 1995, 1263: 241–244.
52 Avrova AO, Stewart HE, De Jong WD, Heilbronn J, Lyon GD and Birch PR. A cysteine protease gene is expressed early in resistant potato interactions with *Phytophthora infestans.* Mol Plant Microbe Interact 1999, 12: 1114–1119.
53 Harrak H, Azelmat S, Baker EN and Tabaeizadeh Z. Isolation and characterization of a gene encoding a drought-induced cysteine protease in tomato (*Lycopersicon esculentum*). Genome 2001, 44: 368–374.