

Purification and Biochemical Characterization of Asclepain c I from the Latex of *Asclepias curassavica* L.

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In this work we report the isolation, purification and characterization of a new protease from latex of *Asclepias curassavica* L. Crude extract (CE) was obtained by gathering latex on 0.1 M citric-phosphate buffer with EDTA and cysteine with subsequent ultracentrifugation. Proteolytic assays were made on casein or azocasein as substrates. Caseinolytic activity was completely inhibited by E-64. Stability at different temperatures, optimum pH and ionic strength were evaluated by measuring the residual caseinolytic activity at different times after the incubation. CE showed the highest caseinolytic activity at pH 8.5 in the presence of 12 mM cysteine. CE was purified by cation exchange chromatography (FPLC). Two active fractions, homogeneous by SDS-PAGE, were isolated. The major purified protease (asclepain cI) showed a molecular mass of 23.2 kDa by mass spectrometry and a pI higher than 9.3. The N-terminal sequence showed a high similarity with those of other plant cysteine proteinases. When assayed on *N*- α -CBZ-aminoacid-*p*-nitrophenyl esters, the enzyme showed higher preference for the glutamine derivative. Determinations of kinetic parameter (k_m and K_{cat}) were performed with PFLNA.

KEY WORDS: *Asclepias curassavica*; *Asclepiadaceae*; cysteine proteinase; latex; laticifers.

1. INTRODUCTION

The presence of proteolytic enzymes in latices from plants of diverse families has been known for many years. The function of these proteases, which come from different genetic resources, have not been elucidated. One possible function is the degradation of proteins during laticifer development or promotion of coagulation. Some plants immediately secrete latex when the leaves, stems and fruits are injured. The latex bleeding proceeds for a few minutes until a clot form around the wounded area. The coagulation process is vital for plant defense against possible patho-

gen attack (Pereira *et al.*, 2001). Latex itself may act to shield the cambial meristem and the contents of the sieve tubes from predators, or to ward off parasites or pathogens. Therefore, it seems reasonable to assume that the substances and enzymes needed for such purposes are present in latex (Boller, 1986).

⁴ Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid; BLAST, Basic Local Alignment Search Tool; CAPS, 3-(ciclohexylamino)-1-propanesulfonic acid; CBZ, carbobenzoxy; CE, Crude extract; CM-Sepharese, carboxymethyl-Sepharese; DMSO, dimethyl sulfoxide; DTT, DL-Dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDTA, ethylenediaminetetraacetic acid; MALDI-MS, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry; MES, 2-(*N*-Morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; PFLNA, L-pyroglyutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidenedifluoride; TAPS, *N*-tris (hydroxymethyl)methyl-3-aminopropanesulfonic acid; U_{cas} , arbitrary units of caseinolytic activity; U_{cbz} , arbitrary units of enzymatic activity with *N*- α -carbobenzoxy-*p*-nitrophenyl esters as substrates; U_{PFLNA} , arbitrary units of amidolytic activity.

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Proteolytic enzymes are still very used in different pharmaceutical preparations (Caffini *et al.*, 1988). Plant proteases have received special attention in the field of medicine and biotechnology due to their property of being active at a very wide range of temperature and pH (Dubey and Jannadham, 2003). The best known plant proteolytic enzymes with commercial value are papain from *Carica papaya*, ficin from *Ficus* spp. and bromelain from *Ananas comosus* (pineapple), which have been called “biological scalpels” because of their specific action on chronic and acute suppurating lesions and in debridement following burns without causing any deleterious action on healthy tissues. *Asclepiadaceae* (milkweed family) is a plant family whose members produce latex usually containing proteases with pharmaceutical applications; for instance, the latex of *Ervatamia coronaria* is cooling and has anti-inflammatory effect on wounds (Sundd *et al.*, 1998). On the other hand, *Calotropis procera* is a popular medicine plant of Asia and Africa and the ethanolic extract of this plant is reported to have antimicrobial, anti-inflammatory, antipyretic and anticancerous activities (Dubey and Jagannadham, 2003).

In view of its potential applications, it is important to look into biochemical constituents of the *Asclepiadaceae* plant latex. In the course of screening of proteolytic enzymes of different species of the *Asclepiadaceae* family, our laboratory have reported the purification and characterization of proteases present in lattices of *Morrenia brachystephana* (Arribère *et al.*, 1998; Vairo Cavalli *et al.*, 2001; Vairo Cavalli *et al.*, 2003), *M. odorata* (Arribère *et al.*, 1999; Vairo Cavalli *et al.*, 2001), *Araujia hortorum* (Obregon *et al.*, 2001; Priolo *et al.*, 2000), and *Asclepias fruticosa* (Trejo *et al.*, 2001). In the current paper we report the characterization of the crude extract obtained from latex of *A. curassavica* and the isolation and purification of the proteases present in it. Furthermore, we determined the biochemical properties of the major purified protease, which according to the nomenclature proposed for latex cysteine proteases (Barragán *et al.*, 1985; Tablero *et al.*, 1991) was named asclepain cI.

2. MATERIAL AND METHODS

2.1. Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, OH). AMPSO,

CAPS, cysteine, E-64, EDTA, MOPS, 1,10-phenanthroline, pI ampholytes (3.0–10.0), PMSF, *p*-nitrophenyl esters of *N*-carbobenzoxy-*L*-amino acids, sinapinic acid, TAPS, and Tris were purchased from Sigma Chemical Company (St. Louis, MO). Coomassie brilliant blue R-250, acrylamide, bisacrylamide, and low range molecular weight standards were obtained from Bio-Rad (Hercules, CA). PVDF membrane was provided by Millipore (Bedford, MA). CM-Sepharose Fast Flow were purchased from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were obtained from commercial sources and were of the highest purity available.

2.2. Plant Material

A. curassavica L. “scarlet milkweed” (Asclepiadaceae) is an erect, evergreen perennial subshrub with woody base. Like most milkweeds, it has opposite leaves and milky sap. The leaves are about 5–15 cm long, narrowly elliptic, and pointed at both ends. The flowers are arranged in umbels with reflexed, five parted corolla brilliant red-purple, exposing the crown of five orange horned hoods. It blooms continuously from spring until autumn. The fruits are spindle shaped pods, 7–10 cm long, that eventually split open to release little flat seeds that drift away on silky parachutes (Dimitri, 1972). Scarlet milkweed is native of South America but has become a naturalized weed in tropical and subtropical pastures, fields and distributed areas throughout the world.

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).

2.3. Crude Enzyme Preparation

Superficial incisions of petioles provided milk-like latex, which was received on cold 0.1 *M* citric-phosphate buffer (pH 6.5) containing 5 mM EDTA and cysteine. This suspension was first centrifuged at 16,000 × *g* for 30 min at 4°C. Gums and cell debris were discarded, and the supernatant was ultracentrifuged at 100,000 × *g* during 1 h at 4°C. The new clear supernatant containing soluble proteins, named crude enzyme extract, was frozen for further studies.

2.4. Protein Content and Proteolytic Activity Assays

Proteins were measured according to Bradford (1976), using bovine serum albumin as standard. Routinely, the proteolytic activity of the enzymes was monitored using casein. The reaction mixture contained 1.1 ml of 1% casein solution in adequate buffer and 0.1 ml enzyme solution. The reaction was carried out at 42°C and stopped by the addition of 1.8 ml 5% trichloroacetic acid, then test tubes were centrifuged at $3000 \times g$ for 20 min and the absorbance of amino acids and soluble peptides was measured at 280 nm. The control assay was performed without enzyme in the reaction mixture and used as reference. An arbitrary enzyme unit (Ucas) was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the assay conditions.

Azocasein was the substrate used in inhibition assays with 1,10-phenantroline. CE obtained without EDTA (0.15 ml) was added to 0.25 ml of 1% (w/v) azocasein in 0.1 M Tris-HCl buffer pH 8.5 containing 12 mM cysteine, at 42°C. After incubation for 5 min, the reaction was stopped by the addition 1 ml of 10% trichloroacetic acid, then centrifuged at $5000 \times g$ for 15 min, the supernatant (0.9 ml) was transferred to a test tube containing 1 ml of 1 M NaOH and the absorbance was read at 440 nm (Trejo *et al.*, 2001).

2.5. pH Profile

The activity of crude enzyme preparation was measured at different values of pH (pH 6.0–10.0) on casein using 0.01 M sodium salts of the “Good” buffers (Good and Izawa, 1972) prepared with 10 mM sodium salts of MES, MOPS, TAPS, AM-PSO and CAPS. At each pH a control assay was done without enzyme and used as blank.

2.6. pH Stability

To measured pH stability, 2 ml of the enzymatic preparation was precipitated with 3 volumes of acetone, centrifuged at $3000 \times g$ for 20 min, and then the precipitated was redissolved in the Good buffer adjusted to pH 8.5. Samples (0.1 ml) were incubated at 20°C for 1–3 h and the residual activity was assayed according to the method used for proteases described above.

2.7. Thermal Stability

After incubation the crude preparation (0.1 ml) for 30, 60, 90, and 120 min at 40, 50, 60, and 70°C, the residual proteolytic activity was determined as indicated above.

2.8. Ion Strength Stability

The ability of the CE to retain its activity under growing ionic strength, was tested by exposing it at different concentrations of sodium chloride (0.1–1.0 M) during 1 h at 40°C. The remaining proteolytic activity was measured at 42°C with casein as substrate as previously described.

2.9. Effect of Inhibitors on Proteolytic Activity

Inhibition of the hydrolysis of casein or azocasein (López, 1995) by the CE was investigated using E-64, PMSF and 1,10-phenantroline. The enzyme preparation (0.99 ml) was incubated with 0.01 ml of the one following inhibitors: 10 mM E-64, 100 mM PMSF and 100 mM 1,10-phenanthroline during 30 and 60 min at 30°C. The residual proteolytic activity was determined as indicated above. Controls were prepared by preincubating the CE with the appropriate solvent used to dissolve the inhibitors (Dunn, 2001). A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%.

2.10. Effect of Cysteine

The effect of cysteine preincubation on the enzyme preparation was evaluated by incubating it with different concentrations of cysteine (10, 20, 30, and 40 mM) as well as without cysteine during 1 h at 0°C. Residual caseinolytic activity was measured as previously indicated.

2.11. Isoelectric Focusing (IEF) and Zymogram

IEF was performed in a Mini IEF Cell (Model 111, Bio-Rad). The CE was concentrated and deionized by acetone precipitation and further centrifugation at $3000 \times g$ for 15 min: the precipitate 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 which protein fractions have proteolytic activity, unstained gel was contacted for 10 min at 50°C with an agarose gel imbibed during 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, agarose gel was stained by Coomassie Brilliant Blue R-250. Proteolytic activity became visible as clear bands on the stained agarose gels (Westergaard *et al.*, 1980).

2.12. Cation Exchange Chromatography

The CE was purified by cation exchange chromatography in a SP Sepharose Fast Flow column, equilibrated and washed with 0.05 M Tris-HCl buffer (pH 8.25). Chromatography was developed in a FPLC equipment (Pharmacia) by washing with the equilibrating buffer and further elution of the bound material with a sodium chloride linear gradient (0–0.6 M) in the same starting buffer at a flow rate of 0.75 ml/min. Cation exchange chromatography was spectrophotometrically monitored by measuring absorption at 280 nm. Caseinolytic activity was tested on the eluted fractions, and those showing proteolytic activity were pooled and stored at –20°C for further studies.

2.13. Electrophoresis (SDS-PAGE)

Samples containing proteases were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (w/v) polyacrylamide gels (Laemmli, 1970). Current was kept constant at 40 mA during stacking and then increased to 60 mA and kept constant for 40 min. Gels were stained with Coomassie Brilliant Blue R-250. Finally, protein purity was verified by the silver staining procedure (Blum *et al.*, 1987).

2.14. Mass Spectrometry

MALDI-MS/TOF was used for the determination of molecular masses, as well as to ascertain the degree of purity of active chromatographic

fractions. Mass spectra were 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.

2.15. Measurement of Endoesterolytic Activity

The esterolytic activity of asclepain cI was determined by the Silverstein's method (1974), modified according to optimal conditions of the enzyme. The activity was studied using *N*- α -carboxy-*p*-nitrophenyl esters of some 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 equipped with a chamber thermostated at 37°C. An arbitrary enzyme activity unit (U_{cbz}) was defined as the amount of protease 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.

2.16. Measurement of Amidolytic Activity

Amidolytic activity was determined by hydrolysis of PFLNA according to Filippova *et al.* (1984). This assay was performed using a solution of 1 mM PFLNA in DMSO. The reaction mixture contained 1.5 ml 0.1 M phosphate buffer pH 6.5, 0.3 M KCl, 10^{-4} M EDTA, 0.003 M 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 that released one micromol of *p*-nitroaniline per min in the assay conditions.

2.17. Kinetic parameters

The kinetic parameters V_m , K_m , and k_{cat} of asclepain cI were determined using PFLNA as substrate. Estimation of such parameters was performed as indicated above for concentrations of PFLNA ranging from 0.1 to 0.5 mM in the reaction mixture. The initial reaction rate was determined by monitoring the absorbance at 410 nm every 5 s during 2 min. V_m , K_m and k_{cat} were calculated using linear regression analysis by means of Lineweaver-Burk, Wolff-Hanes and Eadie-Hofstee plots.

2.18. Protein Sequence Analysis

The purified protease, asclepain cI, was adsorbed onto a PVDF membrane (Millipore) and washed several times with deionized water. The N-terminal sequence was determined by Edman's automated degradation using a Beckman LF3000 protein sequencer equipped with a System Gold (Beckman) PTH-amino acid analyzer. Protein homology searches were performed using the BLAST network service (Altschul *et al.*, 1997), considering only those specific residues that are identical ("identities").

3. RESULTS AND DISCUSSION

A. curassavica L. belongs to the *Asclepiadaceae* family, whose members usually develop secretory tissues (laticifers) which frequently include proteolytic enzymes. The presence of proteolytic activity was detected by *in situ* histochemical assays (Arri  re *et al.*, 1998) in longitudinal sections of young stems laticifers.

The crude extract, supernatant of $100,000 \times g$, contained 933 μg of proteins/ml and 0.988 Ucas/ml and showed the highest proteolytic activity when assayed on casein in presence of 12 mM cysteine.

For industrial applications, enzyme purity is usually of secondary importance to cost (Harris, 1989; Illanes, 1994). For these reason, the effect of pH and thermal stability of the enzyme were assayed on the CE before starting with the purification procedure.

The enzyme preparation showed high caseinolytic activity within a pH range from 7.5 to 9.0, while maximum activity was around pH 8.5 (Fig. 1). Caseinolytic activity remained practically

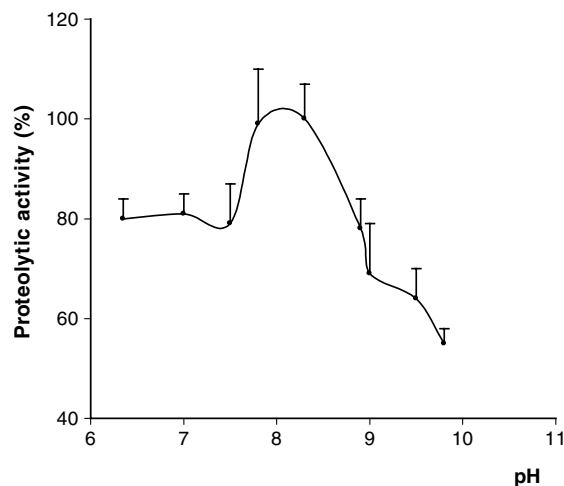


Fig. 1. Effect of pH on proteolytic activity of CE measured on casein (range pH 6.0–10.0) using 0.01 M sodium salts of the following "Good" buffers: MES, MOPS, TAPS, AMPSO and CAPS.

unalterable when the CE was incubated during 2 h at pH 8.5 at 37°C, still maintaining 70% of residual activity after 4 h of incubation. (Fig. 2).

In order to establish to which mechanistic class belonged the proteases present in the CE, different proteolytic enzyme inhibitors were tested. The data shown in Fig. 3 reveal that the enzymatic preparation was immediately inhibited by 10 mM E-64. On the contrary, the enzyme activity was notably enhanced by the addition of 12 mM cysteine to the reaction mixture. Incubation with 100 mM PMSF for 30 min depressed activity to 28% of the initial value, but inhibition was partially reverted when the reaction was carried out in presence of both, 100 mM PMSF and 12 mM cysteine. These results allow supposing that -SH groups could be involved

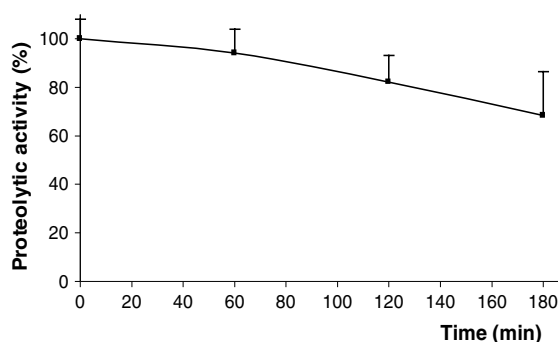


Fig. 2. Stability at pH optimum on the proteolytic activity of CE, using "Good" buffers adjusted to pH 8.5 following 1–3 h incubation at 20°C.

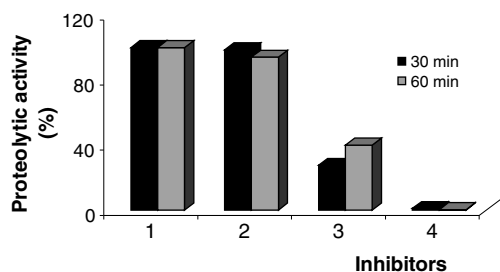


Fig. 3. Effect of inhibitors on the proteolytic activity of CE incubated on each inhibitor solution during 30 and 60 min at 30°C. 1: CE and distilled water; 2: CE and phenantroline; 3: CE and PMSF; 4: CE and E-64.

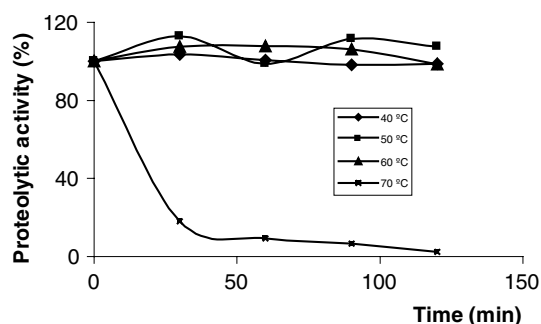


Fig. 4. Thermal stability on the proteolytic activity of CE. Determinations of activity were made on casein at pH 8.5.

in the catalytic mechanism of the enzymes, suggesting that the proteases present in the latex of *A. curassavica* stems belong to the cysteine type.

As can be seen in Fig. 4, the enzyme preparation showed a remarkable thermal stability. After 2 h at 40, 50, and 60 °C, the caseinolytic activity remained practically constant, but the enzyme was almost completely inactivated by heating for 40 min at 70 °C.

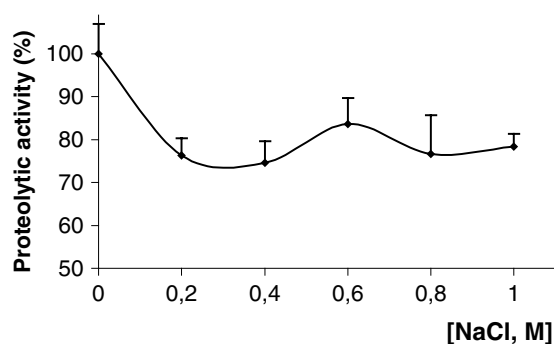


Fig. 5. Effect of ionic strength on the proteolytic activity of CE using different concentrations of sodium chloride (0.0–1.0 M) during 1 h at 40°C.

The enzyme preparation was also stable enough at moderate ionic strength values, as residual activity declined only 30% when NaCl raised to 1 M concentration (Fig. 5).

IEF of the CE (Fig. 6) showed that most protein fractions were located near the cathode, evidencing their basic nature, and the corresponding zymogram corroborated their proteolytic behavior ($pI > 9.3$).

FPLC cation exchange chromatography allowed the separation of three fractions (Fig. 7). No proteo-

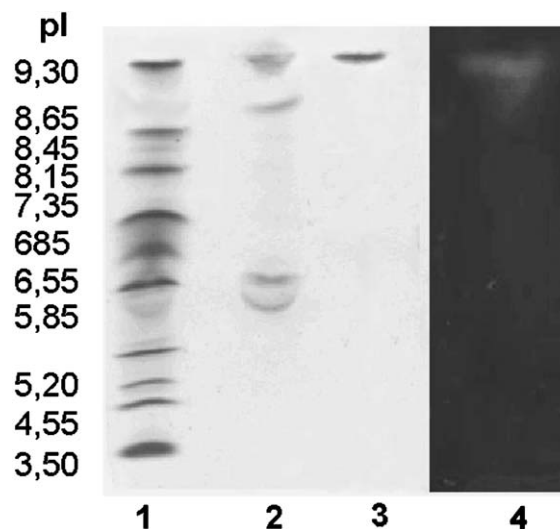


Fig. 6. Isoelectric focusing and corresponding zymogram. Lane 1, pI markers: amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β -lactoglobulin a (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85 and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45 and 8.65) and trypsinogen (pI 9.30); Lane 2, CE; Lane 3, asclepain cI; Lane 4, zymogram of asclepain cI.

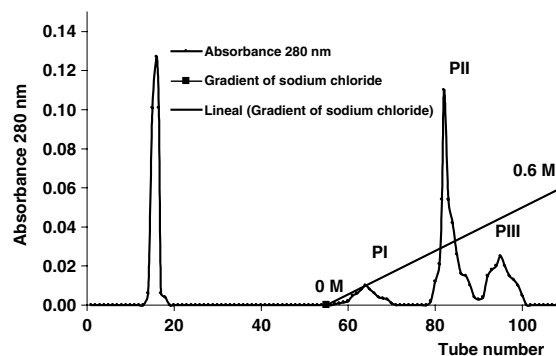


Fig. 7. Cation exchange chromatography (SP-Sepharose Fast Flow, column Pharmacia Biotech XK 16). Elution buffer: 0.05 M Tris-HCl (pH 8.25). Gradient: sodium chloride 0–0.6 M. Flow rate: 0.75 ml min⁻¹. Fraction volume: 2.0 ml.

Table 1. Purification of the Proteolytic Components Present in the Latex of *A. curassavica* L

Sample	mg/ml	UCAS/ml	Specific activity UCAS/mg	Purification (fold)	Yield (%)
Crude extract (CE)	0.9333	0.988	10.586	1	100
Asclepain cI	0.0243	0.2649	10.877	10.27	26.80
Asclepain cII	0.0075	0.0968	12.836	12.125	9.80

lytic activity was found in the former fraction eluted. The other two fractions eluted with the linear salt gradient were active and assigned as asclepain cI and asclepain cII, according to nomenclature recommendations for proteases obtained from latex of species of the *Asclepiadaceae* family (Barragán *et al.*, 1985; Tablero *et al.*, 1991). Activity measurements showed that asclepain cI is the most active fraction, and therefore was chosen for further analysis.

Asclepain cI from latex of *A. curassavica* was purified 10.3 fold, the percent recovery in terms of total activity was 26.8% and the specific activity 10.88 Ucas/mg (Table 1).

As can be observed in Fig. 6, asclepain cI exhibited a *pI* value higher than 9.3; this basic nature had been previously observed in proteases isolated from the latex of others members of the *Asclepiadaceae* family (Obregón *et al.*, 2001; Trejo *et al.*, 2001).

The SDS-PAGE analysis, the silver staining of gels (Fig. 8) and mass spectrometry results (Fig. 9) revealed the homogeneity of the purified proteases. Molecular mass of asclepain cI obtained by mass spectrometry was 23,200 Da. This value is in good agreement with the results obtained when other proteases from *Asclepiadaceae* family were studied and for most cysteine peptidases (Vairo Cavalli *et al.*, 2003; Trejo *et al.*, 2001).

Asclepain cI was tested against several *N*- α -carbobenzoxy-*p*-nitrophenyl esters of different L-amino acids. The preferred substrate was the Gln derivative and, in decreasing order, those of Lys, Ile, Tyr, Ala, Gly, Asp, Asn y Leu (Table 2). Some of this results are similar to those obtained for the proteases of *A. hortorum* Fourn (Obregón *et al.*, 2001).

The amidolytic activity was evaluated with PFLNA as substrate because it is a convenient chromogenic substrate for thiol proteinases such as papain, ficin and bromelain. The value of this activity was 2.38 U_{PFLNA}. Kinetic parameters (K_m , V_m , k_{cat} and k_{cat}/K_m) were determined for amidolytic

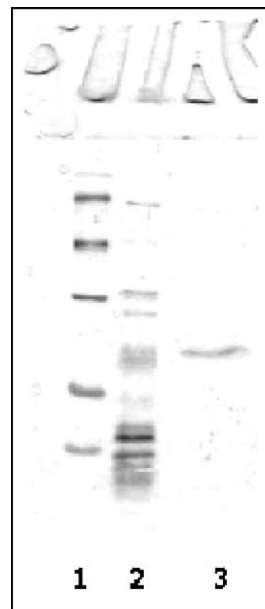


Fig. 8. SDS-Page. Lane 1: Bio Rad molecular weight standards: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase bovine (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); Lane 2: CE; Lane 3: asclepain cI.

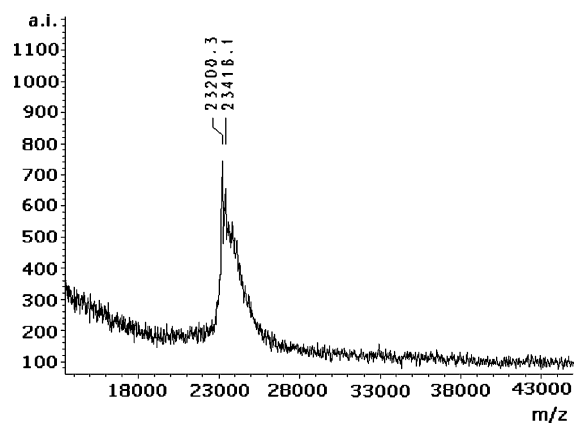


Fig. 9. Mass spectroscopy of asclepain cI. MALDI-MS/TOF was used for the determined of purity degree as well as of molecular weight of asclepain cI. Sample was mixed with sinapinic acid (matrix) dissolved in 0.1% trifluoroacetic acid. Bovine trypsinogen was used for internal calibration.

activity (Table 3). The comparison of PFLNA K_m value for asclepain cI with those obtained for papain, ficin and bromelain (Filipova *et al.*, 1984) indicated approximately twice lower affinity for this substrate.

Comparison of the *N*-terminus of asclepain cI with similar sequences of other proteases is shown

Table 2. Endoesterolytic Activity: U_{CBZ} and the Corresponding Percentage Preference. The Activity was Studied using *N*- α -carboxy *p*-nitrophenyl Esters of Aminoacids. Assays were Carried out at 37 °C and Changes in the Absorbance were Measured at 405 nm during 2 min. In this case one Enzyme unit (Ucbz) was defined as the Amount of Enzyme that Releases one Micro-mole of *p*-nitrophenol per min at 37 °C and pH 8.0

N-CBZ-amino acid <i>p</i> -nitrophenyl ester	Percentage preference	U _{CBZ}
Gln	100	11.49
Lys	78	145.98
Ile	59	194.64
Tyr	51	224.58
Ala	47	243.3
Gly	22	530.84
Asp	18	648.81
Asn	12	973.2
Leu	12	973.2
Val	0	0

Table 3. Kinetics Parameters of Asclepain cI using PFLNA as Substrate (range of concentrations 0.1–0.5 *M*). The Initial Reaction rate was Determined by Monitoring the Absorbance at 410 nm every 5 s during 2 min

	K _m (mM)	V _m (mM/seg)	K _{cat} (Seg-1)	K _{cat} /K _m (seg-1/mM)
A	0.7284	0.0001643	46.961	6.44
B	0.8175	0.0001879	53.701	6.57
C	0.8192	0.0001999	5.713	6.97

A: Lineweaver–Burk equation; B: Wolf–Hanes equation; C: Eddie–Hofstee equation.

in Table 4. Asclepain cI present a high identity with other plant cysteine peptidases, particularly with funastrain cII (87%) and asclepain *f* (86%). The presence of cysteine at position 25 is characteristic of the catalytic site of the cysteine peptidases

Table 4. Comparison of *N*-terminal Amino Acid Sequences of Asclepain cI and other Cysteine Plant Endopeptidases. Shared Amino Acids are Shown as White Characters on a Black Background

Plant source	Sequence	Identities	References
<i>Asclepain cI</i>	LPNSVDWRQKGVVFP IRDQGKCGSCWTFSAV	100% (31/31)	
<i>Funastrain cII</i>	LPNSVDWRQKGVV SAIRN QGKCGSCWAFSAV	87% (27/31)	Morcelle <i>et al.</i> (2004)
<i>Asclepain f</i>	LPD SYDWR EKG VVFP IRN QGK	86% (18/21)	Trejo <i>et al.</i> (2001)
<i>Carica candamarcensis</i> (Cysteine proteinase I)	SI DWRQK GA V TPV RN QGS CGSCWTFSSV	75% (21/28)	Walreavens <i>et al.</i> J (1993)
<i>Morrenain oII</i>	LP SVDWR K VF P R Q GK GS WTFSAV	74% (23/31)	Vairo Cavalli <i>et al.</i> (2001)
<i>Hordeum vulgare</i>	LP P SVDWRQKGA V T G V K D Q G K C G S C W A F S A V	74% (23/31)	Koehler and Ho (1990)
<i>Oryza sativa</i>	LP E SVDWR E K G A V A P V K N Q G Q C G S C W A F S A V	70% (22/31)	Watanabe <i>et al.</i> (1991)
<i>Carica papaya</i> (Papaya proteinase III)	LP E N VDWR K K G A V I P V R H Q G S C G S C W A F S A V	70% (22/31)	Dubois <i>et al.</i> (1988)
<i>Oryza sativa</i>	LP E SVDWR T I K G A V A E I K D Q G G C G S C W A F S A V	70% (22/31)	Watanabe <i>et al.</i> (1991)
<i>Carica candamarcensis</i> (Cysteine proteinase II)	P G SVDWRQKGA V I P V K D Q N P C G S C W A F S T V	70% (21/30)	Walreavens <i>et al.</i> (1993)
<i>Morrenain bII</i>	LP D SYDWR K K N L V F P V R N Q G K K G	69% (16/23)	Vairo Cavalli <i>et al.</i> (2001)
<i>Brassica napus</i> (Cysteine proteinase COT44)	V P V T VDWRQKGA V N A I K D Q G T C G S C W A F S	69% (20/29)	Dietrich <i>et al.</i> (1989)
<i>Carica papaya Papaya</i> (proteinase I)	I P E Y VDWRQKGA V I T P V K N Q G S C G S C W A F S A V	68% (21/31)	Dubois <i>et al.</i> (1988)
<i>Lycopersicon esculentum</i>	LP E S I D W R E K G V L V G V K D Q G S C G S C W A F S A V	68% (21/31)	Schaffer and Fischer (1988)
<i>Pisum sativum</i>	LP E D F D W R E K G A V I P V K D Q G S C G S C W A F S	65% (19/29)	Guerrero <i>et al.</i> (1990)
<i>Lycopersicon esculentum</i> (Cysteine proteinase III)	LP D W R G V P Q G K C G S C W T F S	65% (19/29)	Drake <i>et al.</i> (1996)
<i>Zea mays</i> (Cysteine proteinase II)	LP E T K D W R E D G I V S P V K N Q G H C G S C W T F S	65% (19/29)	Domoto <i>et al.</i> (1995)
<i>Arabidopsis thaliana</i>	LP D E F Y D W R A N G A V S V K D Q G N C G S C W A F S A V	64% (20/31)	Sato <i>et al.</i> (2000)
<i>Phaseolus vulgaris</i> (Cysteine proteinase EP-C1)	V P P SVDWR K K G A V I T D V K D Q G Q C G S C W A F S T V	64% (20/31)	Ogushi <i>et al.</i> (1992)
<i>Carica papaya</i> (Papaya proteinase IV Papaya peptidase B)	LP E SVDWR A K G A V I P V K H Q G Y C E S C W A F S T V	64% (20/31)	Ritonja <i>et al.</i> (1989)
<i>Araujain hIII</i>	LP E SVDWR K K N L V F P V R N Q G Q X G S X X A F S A V	64% (20/31)	Obregón <i>et al.</i> (2001)

belonging to the papain family. Asclepain cI shared two motifs (CGS) and (WTFS) surrounding the catalytic cysteine with most of the sequence analyzed. The Gln residue at position 19 is conserved throughout all cysteine peptidases, as it is part of the active site. On the other hand, asclepain cI and funastrain cII present at position 15 the residues Pro and Ala. Respectively, both of them presenting hydrophobic characteristics, a fact that allows to expect similar folding patterns at that site.

Notably, differences between asclepain cI and asclepain f are reduced to the amide form of acidic amino acids (Asn replaced by Asp at position 3 and 18; Gln replaced by Glu at position 9). As happen in most peptidases of the papain family, Pro2 showed a high degree of conservation, (Barret, 1998), consistent with the protective anti-aminopeptidase role attributed to it.

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