

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 (LPSFVDWRQKGVVFPINRQGGCGSCWTFSA) 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

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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, senescence, 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 biomedical [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 latices 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 antigonorrhoeic [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 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 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-phenanthroline 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

(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	LP SPFVDWRQKGVVFFIRNQQCGSCWTF SA	100 (30/30)	This study
Asclepain c I	LP NSVDWRQKGVVFFIR DQ K CG SCWTF SA	86 (26/30)	[25]
Asclepain b	LP N F VDWRK NG VVFFIRN Q Q	85 (18/21)	[39]
Asclepain f	LP D S VDWREK GV VFFIRN Q GK	80 (17/21)	[21]
Funastrain cII	LP NSVDWRQKG VS ATRN Q GK CG SCW A F SA	80 (24/30)	[22]
Asclepain a	LP NSIDWRQ KN VVFFIR KN Q G	75 (15/20)	[39]
<i>Oryza sativa</i>	LP ASVDWR CK GA LV EV KN Q CG SCW A F SA	73 (22/30)	[45]
<i>Zinnia elegans</i>	LP KSVDWR KK GA LV SE VK Q CG SCW A F S	72 (21/29)	[46]
Papaya proteinase omega: PP III	LP ENVDWR KK GA LV EV RH Q CG SCW A F SA	70 (21/30)	[47]
Papaya proteinase: PP I	IP EYVDWRQ KG AV LV EV KN Q CG SCW A F SA	70 (21/30)	[48]
<i>Arabidopsis thaliana</i> (cysteine protease)	LP VSVDWR KK GA LV PI KN Q CG SC CC W	70 (21/30)	[49]
Morrenain o II	LP D S VDWR KK N L V FP VRN Q G K CG S X W TF SA	70 (21/30)	[15]
<i>Brassica oleracea</i> (cysteine protease)	LP D E VDWR AK GA V FPVRN Q G SC CG S W TF SA	66 (20/30)	[50]
Araujain h III	LP E S VDWR KK N L V FP VRN Q G Q X G S XX A F SA	66 (20/30)	[19]
Morrenain b II	LP D S VDWR KK N L V FP VRN Q G KK G	65 (15/23)	[15]
<i>Zea mays</i> (cysteine protease)	LP ET KD WRE D GI V SE VK Q CG SCW TF SA	63 (19/30)	[51]
<i>Solanum tuberosum</i>	LP E S VDWR DK GV LV GV KD Q CG SCW A F SA	63 (19/30)	[52]
<i>Lycopersicon esculentum</i>	LP E S IDWRE K GV LV GV KD Q CG SCW A F SA	60 (18/30)	[53]
Araujain h II	V P D SIDWRE K DA V LV PI RN Q G Q X G S L W A F X	60 (18/30)	[19]
Morrenain b I	V P D KIDWRE K GA V LD IR N Q G Q D C X L W A F L A	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

<i>N</i> - α -Cbz-L-amino acid <i>p</i> -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. curassavica*, 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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