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Purification and Characterization of a New Plant Endopeptidase Isolated from Latex of *Asclepias fruticosa* L. (*Asclepiadaceae*)

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Asclepias fruticosa L. is a small shrub containing latex with proteolytic activity. The crude extract (latex diluted 1:250 and ultracentrifuged) contained 276 μg of protein/mL and the proteolytic activity reached 1.2 caseinolytic U/mL. This enzyme preparation was very stable even after 2 hours at 45°C, but was quickly inactivated after 5 minutes at 80°C. Chromatographic purification was achieved by FPLC using a cation exchanger (SP-Sepharose FF). Thus, a unique proteolitically active fraction could be isolated, being homogeneous by bidimensional electrophoresis and mass spectrometry (M_r = 23,652). The optimum pH range was achieved at 8.5–10.5. The enzyme activity was completely inhibited by specific cysteine peptidases inhibitors. Isoelectric focusing followed by zymogram showed the enzyme had a pI greater than 9.3. The N-terminus sequence (LPDSVDWREKGVVFPIRNQGK) shows a great deal of similarity to those of the other cysteine endopeptidases isolated from latices of *Asclepiadaceae* even when a high degree of homology could be observed with other plant cysteine endopeptidases.

KEY WORDS: *Asclepias fruticosa; Asclepiadaceae;* latex; milkweed; plant endopeptidases.

1. INTRODUCTION

The presence of proteolytic enzymes in the latex of members of the *Asclepiadaceae* plant family was first reported by Winnick *et al.* (1940), who isolated a protease from *Asclepias speciosa,* which they named "asclepain." This name was almost immediately modified by the authors (Greenberg and Winnick, 1940) to "asclepain s" to distinguish the former from the protease isolated of *A. mexicana,* which was then named "asclepain m." Four decades later, two groups of peptidases from the latex of *A. syriaca* were purified and characterized (Brockbank and Lynn, 1979; Lynn *et al.,* 1980a) and the N-terminal sequences were compared to that of papain (Lynn *et al.,* 1980b). Later, multiple forms of a new peptidase (asclepain g) were isolated from the latex of *A. glaucescens* (Barragán *et al.,* 1985; Tablero *et al.,* 1991).

Calotropis was the second genus of *Asclepiadaceae* searched for protease content. Bose and Madhavakrishna (1958) isolated and characterized a new protease from

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[3] 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; SP-Sepharose, sulphopropyl-Sepharose; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA, ethylendiaminetetraacetic acid; IEF, Isoelectric focusing; MALDI-MS, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry; MOPS, 3-(N-morpholino) propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TAPS, N-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid.

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madar plants (*C. gigantea*), which they named "calotropain." The authors also estimated the amino acid composition of crystalline calotropain as well as the N- and C- terminal amino acids (Madhavakrishna and Bose, 1960). Much later (Abraham and Joshi, 1979a), two proteinases containing a carbohydrate moiety (called calotropain-FI and calotropain-FII) were purified from *C. gigantea* latex and some physicochemical properties of those proteinases were determinated (Abraham and Joshi, 1979b). Almost simultaneously, Pal and Sinha (1980) purified two other cysteine proteases present in the latex of *C. gigantea,* called calotropins DI and DII, which were devoid of carbohydrates. These peptidases were compared immunologically and their C-terminal sequence determined (Sengupta *et al.,* 1984).

Recently, three cysteine endopeptidases (araujiain h I, II, and III) have been isolated, purified, and characterized in our laboratory from the latex of *Araujia hortorum* Fourn. (Priolo *et al.,* 2000; Obregón *et al.,* 2001) and the study of their esterase activities using partial least-squares (PLS) modeling has also been made (Priolo *et al.,* 2001). Additionally, two cysteine endopeptidases from latices of *Morrenia brachystephana* Griseb. and *M. odorata* (Hook *et* Arn.) Lindley (morreniain b and o, respectively) have been also studied (Arribére *et al.,* 1998; Vairo Cavalli *et al.,* 2001).

From a very recent point of view (Endress and Bruyns, 2000), *Asclepiadaceae* should be subsumed as a subfamily (*Asclepiadaceae*) within the more complex family *Asclepiadaceae*, but because this taxonomical rearrangement is still controversial, we decided to maintain the family hierarchy for the aforementioned taxon.

In the present paper, we report the purification and characterization of *asclepain f,* a new cysteine endopeptidase isolated from the latex of *A. fruticosa* L.

2. MATERIALS AND METHODS

2.1. Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, OH). AMPSO,[3] CAPS, carboxypeptidase B, cysteine, E-64, EDTA, IEF standards, glycine, iodoacetic acid, MOPS, p-nitrophenyl esters of N-α-carbobenzoxy-L-amino acids, pepstatin A, 1,10-phenanthroline, PMSF, 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). SP-Sepharose Fast Flow and Pharmalyte 3-10 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

Fruits (follicles) of *A. fruticosa* L. were collected in Villa Elisa, Province of Buenos Aires, Argentina. The species is a small shrub with opposite, lanceolate and acuminate leaves, white flowers, and light green, inflated, pubescent, latex-containing follicles.

2.3. Preparation of the Crude Extract

Latex (1 mL) obtained by superficial incisions of fruits, received on 250 mL of 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA and cysteine, was first centrifuged at 16,000 × *g* for 30 minutes at 4°C. Gums and other insoluble materials were discarded, and the supernatant was ultracentrifuged at 100,000 × *g* for 60 minutes at 4°C. This new supernatant (248 mL) containing soluble proteins—the "crude extract"—was fractionated and conserved at −20°C for further assays.

2.4. Purification of the Crude Extract

The crude extract (9 mL) was applied onto a column (Pharmacia XK 16/40, with AK16 adaptors) packed with SP-Sepharose Fast Flow equilibrated with 50 mM Tris-HCl buffer (pH 8.2). Anion exchange chromatography (FPLC, Pharmacia) was developed by adding 30 mL of the starting buffer (50 mM Tris-HCl, pH 8.2), followed by 100 mL of a sodium chloride linear gradient (0.13−0.28 M) prepared in the same buffer.

2.5. Activity Assays with Protein Substrates

Most assays were performed with casein. The reaction mixture contained 1.1 mL of 1% casein solution and 0.1 mL of enzyme solution, both in 0.1 M glycine–NaOH buffer (pH 9.5) containing 20 mM cysteine. The reaction was carried out at 37°C and stopped by the addition of 5% trichloroacetic acid (1.8 mL), then each test tube was centrifuged at 4000 × *g* for 20 minutes and the absorbance of the supernatant was read at 280 nm. An arbitrary enzyme unit ("caseinolytic unit," U_{cas}), was defined as the amount of enzyme that produces an increase of one absorbance unit (1 cm light-path) per minute in the assay conditions.

Azocasein (López, 1995) was the substrate used in inhibition assays with 1,10-phenanthroline and for the active site titration. The reaction was carried out at 37°C in a microfuge tube containing 0.25 mL of 2% azocasein and 0.15 mL of enzyme solution, both in 0.1 M glycine-NaOH buffer (pH 9.5) containing 20 mM cysteine. The reaction was stopped by the addition of 1.2 mL of 10% trichloroacetic acid, then centrifuged at 4000 × *g* for 20 minutes. The supernatant (1.2 mL) was transferred to a test tube containing 1.4 mL of 1 M NaOH and the absorbance was read at 440 nm. One unit of protease activity is defined to be the amount of enzyme required to produce an absorbance change of one unit (1 cm light-path) per minute for the conditions under which the assay was carried out.

To calculate specific activity values, the protein content was determined by the Bradford's (1976) method.

2.6. Activity Assays with Synthetic Substrates

The activity of the protease on the *p*-nitrophenyl esters of the following N-α-carbobenzoxy (CBZ) amino acids was tested: N-α-CBZ-L-alanine, L-asparagine, β-benzyl-L-aspartic acid, L-glutamine, glycine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-tryptophan, L-tyrosine, and L-valine. The reaction mixture contained 2.0 mL of 0.1 M Tris-HCl buffer (pH 8.0), 50 μL of substrate solution (1 mM in acetonitrile), and 50 μL of enzyme solution. The reaction was carried out at 37°C and changes in the absorbance were measured at 405 nm during 3 minutes. In this case, one enzyme unit (U_{cbz}) was defined as the amount of enzyme that releases 1 micromole of *p*-nitrophenolate per minute at 37°C and pH 8.0.

2.7. Inhibition Assays

The effect of specific inhibitors (Salvesen and Nagase, 1989) on proteolytic activity was determined by pre-incubating the protease preparation with each inhibitor at 37°C for 30 minutes and the residual activity estimated on casein or azocasein at pH 9.5. E-64 (10–100 μM), iodoacetic acid (0.1–1 mM), mercuric chloride (0.1–1 mM), pepstatin A (0.1–0.5 mM), 1,10-phenanthroline (0.1–1 mM), and phenylmethylsulfonyl fluoride (0.1–1 mM) were the chemicals assayed. Controls were prepared by pre-incubating the protease preparation with the appropriate solvent used to dissolve the inhibitors.

2.8. pH-Dependence of Enzyme Activity

Proteolytic activity of enzyme solutions was measured on casein (range: pH 6.0–11.0) using 0.025 M sodium salts of the following "Good" buffers: MES, MOPS, TAPS, AMPSO and CAPS (Good and Izawa, 1972).

2.9. Thermal Stability

To determine the effect of heating on the stability of protease preparations at the optimum pH, enzyme solutions were kept at pH 9.5 for 0, 15, 30, 60, 90, and 120 minutes at 37°C, 45°C, 55°C, and 60°C, and for 0, 5, 10, 15, and 30 minutes at 80°C, and then the residual caseinolytic activity was measured as described.

2.10. Effect of Ionic Strength on Proteolytic Activity

The effect of ionic strength on enzyme activity was determined on casein in the presence of increasing quantities of sodium chloride (0.1–0.5 M).

2.11. SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Miniprotean II Cell (Bio-Rad) according to Laemmli (1970). Current was kept constant at 40 mA during stacking and then increased to 60 mA and kept constant for 40 minutes. Silver staining or Coomassie brilliant blue R-250 were used for staining gels (12.5% polyacrylamide).

2.12. Native Electrophoresis

Samples were precipitated by the addition of acetone (3 volumes) and the precipitate redissolved with sample buffer (62.5 mM Tris HCl [pH 8.8] containing 10% glycerol and bromophenol blue). Gels (5%–20% gradient) were prepared in 3M Tris-HCl buffer (pH 8.8). Polyacrylamide gel electrophoresis was performed in a Miniprotean II Dual Slab Cell (Bio-Rad) using 50 mM Tris-HCl (pH 8.3) as reservoir buffer. Current was kept constant at 50 mA for 80 minutes before changing the electrodes polarity. Gels were silver stained.

2.13. Bidimensional Electrophoresis

Native electrophoresis as described above was used as first dimension and SDS-PAGE as second dimension (12.5% polyacrylamide gel). The corresponding non-

stained lanes from the native electrophoresis were cut and frozen (−20°C) in 10% glycerol and treated according Utsumi *et al.* (1984) before application onto the denaturing gel.

2.14. Isoelectric Focusing (IEF)

A Mini IEF Cell (Model 111, Bio-Rad) was employed to carry out isoelectric focusing. Samples (1 mL) were treated with 5 volumes of acetone and the precipitates redissolved with 100 μL of redistilled water. Five percent polyacrylamide gels were used, containing broad pH-range ampholytes (Pharmalyte 3–10, Pharmacia). Focusing was carried out under constant voltage conditions in a stepped mode: 100 V for 15 minutes, 200 V for the next 15 minutes, and 450 V for the remaining 60 minutes. Gels were fixed and then stained using Coomassie brilliant blue R-250.

2.15. Zymograms

Unstained IEF gels were contacted for 10 minutes in an oven at 60°C with an agarose gel imbibed with a 1% casein solution (Westergaar *et al.,* 1980). After incubation, the agarose gel was dehydrated and stained using Coomassie brilliant blue R-250. Unstained bands evidenced proteolytic activity.

2.16. Titration of Active Sites with E-64

Titration of the active site was performed as described by Barrett and Kirschke (1981), with some modifications. The enzyme (3μM) was preincubated with activator buffer (50 mM Tris-HCl [pH 8.2] containing 20 mM cysteine). Fractions (75 μL) were incubated with 25 μL of different concentrations (0–10 μM) of E-64 solutions for 30 minutes at 37°C. Then, the residual activity was measured on azocasein as previously described. The enzyme concentration was established by determining both protein content (Bradford, 1976) and molecular mass value (mass spectrometry).

2.17. N-Terminal Sequence

The N-terminal sequence was determined by Edman's automated degradation using an Applied Biosystems (model 476) peptide sequencer. Protein homology searches were performed using the BLAST network service (Altschul *et al.,* 1997).

2.18. Mass Spectrometry

Matrix–Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI–MS) was used to determine the degree of purity as well as molecular weight of chromatographic fractions. Samples were mixed with sinapinic acid (matrix) dissolved in 0.1% trifluoroacetic acid and processed in a MALDI-MS Tof Bruker equipment (model Biflex) using carboxypeptidase B as standard.

3. RESULTS AND DISCUSSION

Crude extract (a colorless dilution of latex devoided of gums and other insoluble materials) of *A. fruticosa* L. (*Asclepiadaceae*) contained 27.6 mg of proteins/100 mL and 122 U_{cas}/100 mL showing highest proteolytic activity when assayed on casein in the presence of 20 mM cysteine (three-fold with respect to the same assay without cysteine).

IEF of crude extracts followed by zymograms on casein imbibed agarose gels revealed the presence of a unique band with proteolytic activity, corresponding to the more basic protein, which focused at pI > 9.3 (Fig. 1). The basic nature of the enzyme is a common characteristic shown by the endopeptidases from latices of species belonging to the family *Asclepiadaceae* (Tablero *et al.,* 1991).

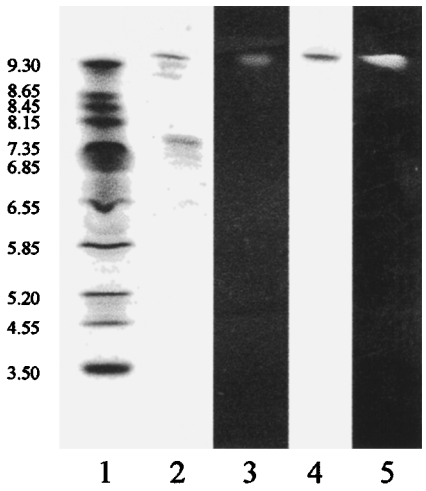

Fig. 1. Isoelectric focusing and zymogram. Lane 1, pI markers: amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β-lactoglobulin a (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85 and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45 and 8.65) and trypsinogen (pI 9.30); lane 2, IEF of crude extract; lane 3, zymogram of crude extract; lane 4, IEF of asclepain f; lane 5, zymogram of *asclepain f.*

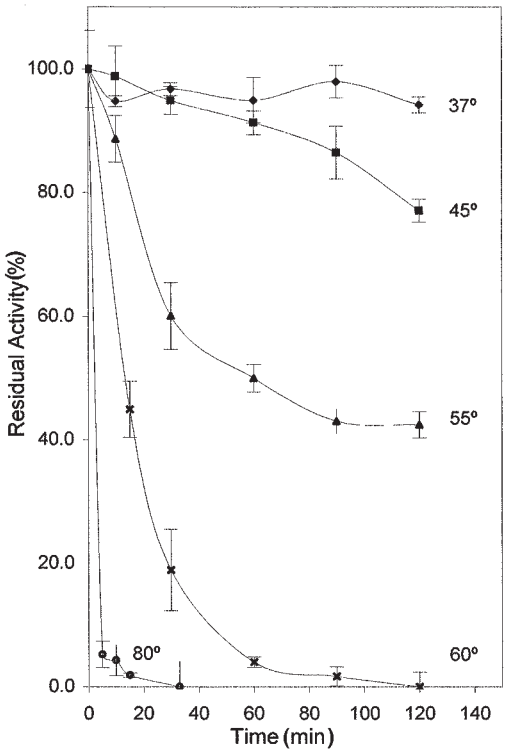

Fig. 2. Thermal stability. Activity on casein at pH 9.5 after 5 minutes at 37°C was taken as 100%. Data points represent the mean value of five determinations and each experiment was repeated twice.

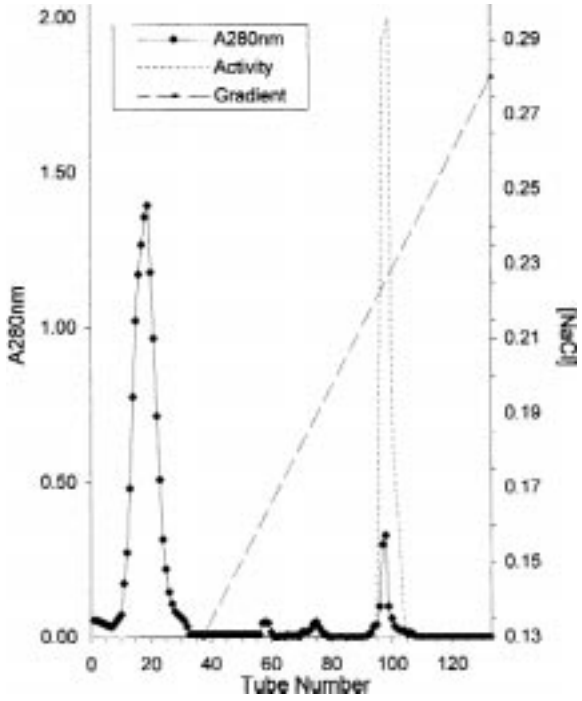

Fig. 3. Cation exchange chromatography. Flow rate, 0.5 mL/min^{-1}. Fractions of 1 mL were collected.

As shown in Fig. 2, the enzyme crude extract showed a remarkable thermal stability in the pH value of maximum activity: caseinolytic activity remains practically unchanged after 2 hours at 37°C, and the residual activity is notably high even after 2 hours at 45°C (80%) and is still high at 55°C (50% of remaining activity after 1 hour), but was inactivated after 5 minutes at 80°C. The preparation was also very stable at moderate ionic strength values, as residual activity decreased only 13% when NaCl raised 0.5 M concentration (data not shown).

Taking into account the pI value of the only proteolytic component (higher than 9.3), the crude extract was purified by FPLC using a cation exchanger (SP-Sepharose FF). To improve resolution, narrow saline gradients and different pH values were tested: best conditions were obtained with Tris-HCl 50 mM (pH 8.2) and a 0.13–0.28 M sodium chloride gradient. The elution pattern is shown in Fig. 3. A unique proteolytic fraction was obtained, which was pooled and used for subsequent studies. The existence of a unique active protein species is quite unusual in latices from *Asclepiadaceae*.

The purification scheme is presented in Table I. Because of the purification strategy adopted, consisting of a one-step chromatographic procedure, a notably yield (75.8%) was obtained. The purification factor of the active fraction is very low but this is a common fact in plant organs with high proteolytic activity, where proteases represent the bulk of protein content of crude

Table I. Purification Scheme of *Asclepain f*

Step	Activity (U_{cas})	Protein (mg)	Specific activity (U_{cas}/mg)	Purification (n-fold)	Yield (%)
Crude	305.6	68.5	4.5	1.0	100.0
Asclepain f	231.5	33.1	7.0	1.6	75.8

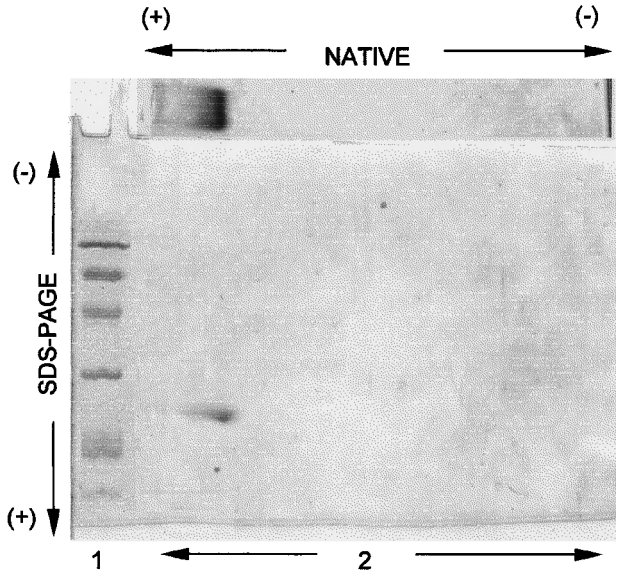

Fig. 4. Bidimensional electrophoresis of *asclepain f.* Lane 1, low range molecular weight standards (Bio-Rad): phosphorylase B (97.4 kDa), serum albumin bovine (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase bovine (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane 2, native electrophoresis of *asclepain f.*

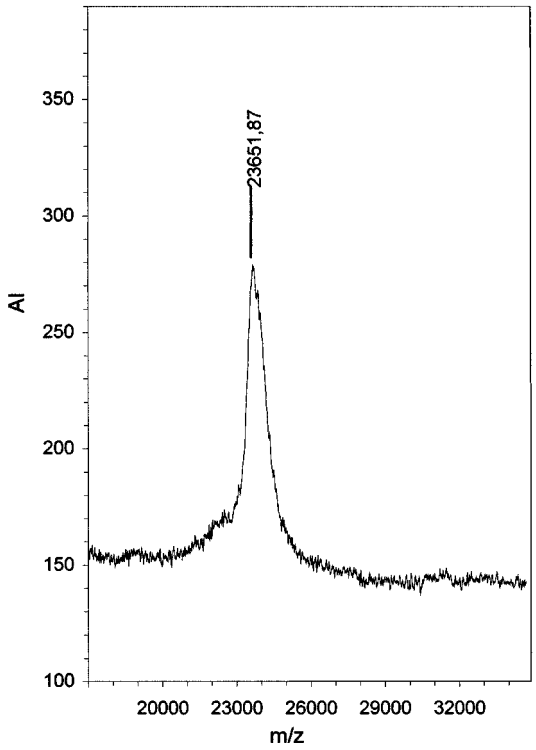

Fig. 5. Mass spectrometry of *asclepain f.*

extracts (López *et al.,* 2000). The new endopeptidase was named *asclepain f,* following the current nomenclature adopted for proteases obtained from latex of species of the *Asclepiadaceae* family (Rodríguez-Romero and Hernández-Arana, 1998).

Purity of *asclepain f* was checked by both bidimensional (native-denaturing) electrophoresis and IEF-zymogram: the enzyme appears as a single polypeptide chain of 25 kDa (Fig. 4) and pI $>$ 9.3 (see Fig. 1). Mass spectrometry confirmed the homogeneity of the enzyme; molecular mass obtained by this technique was 23,652 Da (Fig. 5), a very close value to those of other peptidases from species belonging to the *Asclepiadaceae* family (Rodríguez-Romero and Hernández-Arana, 1998), which, on the other hand, is of the same order of most plant cysteine proteinases.

Asclepain f (Table II) was inhibited by E-64, mercuric chloride, and iodoacetic acid (cysteine proteinases inhibitors) and notably stimulated by cysteine (3-fold), but proteolytic activity was not affected by the addition of characteristic inhibitors of other types of proteinases (PMSF, pepstatin A, and 1,10-phenantroline). Consequently, the new endopeptidase should be included in the cysteine group, like all the other studied proteases isolated from plants belonging to the family *Asclepiadaceae*.

As far as is known, E-64 and cysteine endopeptidases have been found to react with each other on an equimolecular basis, so this inhibitor can be used for titration of the enzyme active site. In the titration assay, the residual enzyme activity gave a straight line (residual activity versus E-64 concentration) that intersected the abscissa at 2.78 μM E-64 (data not shown), corresponding to 92.7% of active enzyme.

Table II. Effect of Inhibitors on Proteolytic Activity of *Asclepain f*

Inhibitors	Inhibitory concentration (mM)	Residual activity (%)
None		100.0
$HgCl_2$	1.0	0.0
$HgCl_2$ + 20 mM Cys	1.0	96.7
E-64	0.1	0.0
Iodoacetic acid	1.0	5.5
PMSF	1.0	100.0
Pepstatin A	0.5	100.0
1-10 phenantroline	1.0	89.0

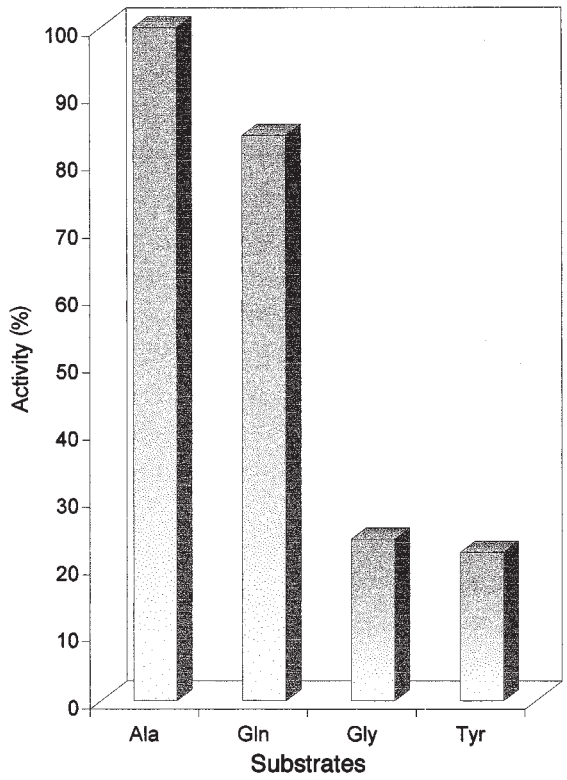

Fig. 6. Comparative esterolytic activity of *asclepain f* on N–α–carbobenzoxy–L–amino acids. Bars represent the mean value of three determinations and each experiment was repeated twice. Only substrates with detectable activity are shown.

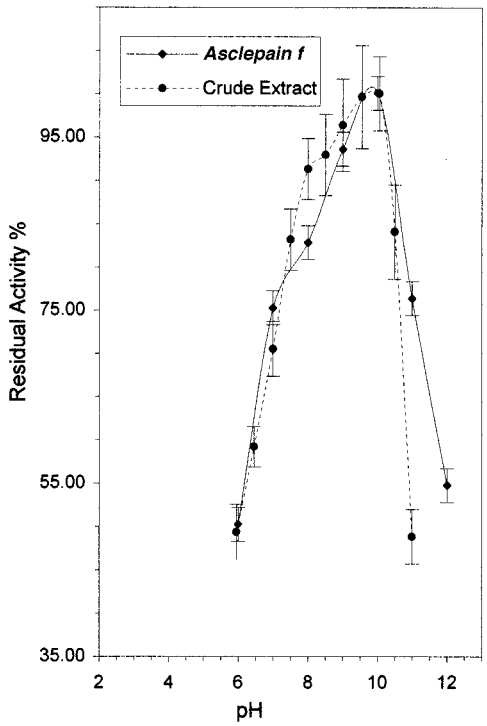

Fig. 7. Effect of pH on proteolytic activity. Maximal activity on casein was taken as 100%. Data points represent the mean value of five determinations and each experiment was repeated three times.

When the enzyme was assayed on *p*-nitrophenyl esters of N-α-carbobenzoxy amino acid derivatives (Fig. 6), the highest relative esterolytic activities were obtained for the alanine and glutamine derivatives, respectively.

Asclepain f exhibits an optimum pH range (90% of maximum activity) between 8.5 and 10.5, which is similar to that obtained with the crude extract (Fig. 7).

The N-terminal sequence (21 amino acids) of *asclepain f* was compared with those of 25 other high-homology plant cysteine proteinases. As shown in Table III, some motifs are notably conserved: DWR and QG are present in all sequences, as well as the proline residue located in position 2. Amino acids located in some positions are particularly conserved: Arg10, Val13, and Pro15 are present in the 80%, 88%, and 96% of the sequences compared, respectively.

The N-terminus shows a great deal of sequence similarity to those of the other cysteine endopeptidases isolated from latices of *Asclepiadaceae* even when a high degree of homology could be observed with other plant cysteine endopeptidases. This fact is in agreement with the proposal of Rowan and Buttle (1994) for the *Bromeliaceae* endopeptidases, in the sense that enzymes isolated from species of the same botanical family are closely more related to each other than to other members belonging to the papain family.

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Table III. Comparison of N-terminal Amino Acid Sequences of *Asclepain f* and Other Cysteine Plant Endopeptidases (Shared Amino Acids Are Shown as White Characters on a Black Background)

Plant species	N-terminus sequence	Reference	% homology
Asclepias fruticosa	LPDSVDWREKGVVFPIRNQGK		100
Morrenia brachystephana	LPDSVDWRKKNLVFPVRNQGK	Priolo *et al.* (2001)	81.0
Morrenia odorata	LPDSVDWRKKNLVFPVRNQGK	Priolo *et al.* (2001)	81.0
Asclepias syriaca (asclepain B)	LPNFVDWRKNGVVFPIRNQGG	Lynn *et al.* (1980b)	76.2
Araujia hortorum (araujian h II)	VPDSIDWREKDAVLPIRNQGQ	Obregón *et al.* (2001)	71.4
Asclepias syriaca (asclepain A)	LPNSIDWRQKNVVFPIKNQGG	Lynn *et al.* (1980b)	71.4
Arabidopsis thaliana	LPVSVDWRKKGAVTPIKNQGG	Gan and Amasino (1995)	71.4
Oryza sativa	LPESVDWREKGAVAPVKNQGQ	Watanabe *et al.* (1991)	71.4
Brassica napus	LPIAVDWRKKGAVTPIKNQGS	Noh and Amasino (1999)	66.7
Zingiber officinale	LPDSIDWRENGAVVPVKNQGS	Choi *et al.* (1999)	66.7
Zinnia elegans	LPKSVDWRKKGAVSPVKNQGQ	Ye and Varner (1996)	66.7
Araujia hortorum (araujiain h III)	VPESDDWRKKNLVFPVRNQGQ	Obregón *et al.* (2001)	61.9
Carica papaya (papaya peptidase omega)	LPENVDWRKKGAVTPVRHQGS	Dubois *et al.* (1988)	61.9
Carica papaya (papaya peptidase IV)	LPESVDWRAKGAVTPVKHQGY	Ritonja *et al.* (1989)	61.9
Petunia x hybrida	LPETKDWREAGIVSPVKNQGK	Tournaire *et al.* (1996)	61.9
Nicotiana tabacum	LPETKDWREAGIVSPVKNQGK	Ueda *et al.* (2000)	61.9
Pseudotsuga mentziesii	LPESIDWREKGAVTAVKNQGS	Tranbarger and Misra (1996)	61.9
Carica papaya (chymopapain)	YPQSIDWRAKGAVTPVKNQGA	Watson *et al.* (1990)	57.1
Carica candamarcensis	YPESIDWRKKGAVTPVKNQGS	Jaziri *et al.* (1994)	57.1
Carica papaya (papaya proteinase I)	IPEYVDWRQKGAVTPVKNQGS	Cohen *et al.* (1986)	57.1
Lycopersicon esculentum	LPESIDWREKGVLVGVKDQGS	Schaffer and Fischer (1988)	57.1
Pisum sativum	LPEDFDWREKGAVTPVKDQGS	Guerrero *et al.* (1990)	57.1
Vicia faba	LPEDFDWREKGAVTPVKDQGS	Yu and Greenwood (1994)	57.1
Zea mays	LPETKDWREDGIVSPVKNQGH	Griffiths *et al.* (1997)	57.1
Mesembryanthemum crystallinum	VPRSIDWRVKGAVTPVKNQGR	Firsthoefel *et al.* (1998)	57.1
Dianthus caryophyllus	LPESVDWRKKGAVSHVKDQGQ	Jones *et al.* (1995)	57.1

REFERENCES

Abraham, K. I. and Joshi, P. N. (1979a). *Biochim. Biophys. Acta* **568**, 111–119.

Abraham, K. I. and Joshi, P. N. (1979b). *Biochim. Biophys. Acta* **568**, 120–126.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). *Nucleic Acids Res.* **25**, 3389–3402.

Arribére, M. C., Cortadi, A. A., Gattuso, M. A., Bettiol, M. P., Priolo, N. S., and Caffini, N. O. (1998). *Phytochem. Anal.* **9**, 267–273.

Barragán, B. E., Hernández-Arana, A., Oliver, M., Castaneda-Agulló, M., and Del Castillo, L. M. (1985). *Rev. Latinoamer. Quim.* **16**, 158–160.

Barrett, A. J. and Kirschke, H. (1981). *Meth. Enzymol.* **80**, 535–561.

Bose, S. M. and Madhavakrishna, W. (1958). *Enzymologia* **19**, 186–200.

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.

Brockbank, W. J. and Lynn, K. R. (1979). *Biochim. Biophys. Acta* **578**, 13–22.

Choi, K. H., Laursen, R. A., and Allen, K. N. (1999). *Biochemistry* **38**, 11624–11633.

Cohen, L. W., Coghlan, V. M., and Dihel, L. C. (1986). *Gene* **48**, 219–227.

Dubois, T., Kleinschmidt, T., Schnek, A. G., Looze, Y., and Braunitzer, G. (1988). *Biol. Chem. Hoppe-Seyler* **369**, 741–754.

Endress, M. E. and Bruyns, P. V. (2000). *Bot. Rev.* **66**, 1–56.

Firsthoefel, N. R., Cushman, M. A. F., Ostrem, J. A., and Cushman, J. C. (1998). *Plant Sci.* **136**, 195–206.

Gan, S. and Amasino, R. M. (1995). *Science* **270**, 1986–1988.

Good, N. E. and Izawa, S. (1972). *Meth. Enzymol.* **24**, 53–68.

Greenberg, D. M. and Winnick, T. (1940). *J. Biol. Chem.* **135**, 761–787.

Griffiths, C. M., Hosken, S. E., Oliver, D., Chojecki, J., and Thomas, H. (1997). *Plant Mol. Biol.* **34**, 815–821.

Guerrero, F. D., Jones, J. T., and Mullet, J. E. (1990). *Plant Mol. Biol.* **15**, 11–26.

Jaziri, M., Kleinschmidt, T., Walraevens, V., Schnek, A. G., and Looze, Y. (1994). *Biol. Chem. Hoppe-Seyler* **375**, 379–385.

Jones, M. L., Larsen, P. B., and Woodson, W. R. (1995). *Plant. Mol. Biol.* **28**, 505–512.

Laemmli, U. K. (1970). *Nature* **227**, 680–685.

López, L. M. I. (1995). "Aislamiento, purificación y caracterización de proteasas del látex de *Maclura pomifera* (Raf.) Schneid. (Moraceae)." Ph. D. thesis, Faculty of Exact Sciences, La Plata University, Argentina.

López, L. M. I., Sequeiros, C., Natalucci, C. L., Brullo, A., Maras, B., Barra, D., and Caffini, N. (2000). *Protein Express. Purif.* **18**, 133–140.

Lynn, K. R., Brockbank, W. J., and Clevette, N. A. (1980a). *Biochim. Biophys. Acta* **612**, 119–125.

Lynn, K. R., Yaguchi, M., and Roy, C. (1980b). *Biochim. Biophys. Acta* **624**, 579–580.

Madhavakrishna, W. and Bose, S. M. (1960). *Enzymologia* **22**, 251–261.

Noh, Y. S. and Amasino, R. M. (1999). *Plant Mol. Biol.* **41**, 195–206.

Obregón, W. D., Arribére, M. C., Morcelle del Valle, S., Liggieri, C., Caffini, N. O., and Priolo, N. S. (2001). *J. Protein Chem.* **20**, 317–325.

Pal, G. and Sinha, N. K. (1980). *Arch. Biochem. Biophys.* **202**, 321–329.

Priolo, N., Morcelle del Valle, S., Arribére, M. C., López, L. M. I., and Caffini, N. O. (2000). *J. Protein Chem.* **19**, 39–49.

Priolo, N., Arribére, M. C., Caffini, N., Barberis, S., Nieto Vázquez, R., and Luco, J. (2001). *J. Mol. Catal. B: Enzymatic* **635**, 1–13.

Ritonja, A., Buttle, D. J., Rawlings, N. D., Turk, V., and Barrett, A. J. (1989). *FEBS Lett.* **258**, 109–112.
Rodriquez-Romero, A. and Hernández-Arana, A. (1998). In *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds.), Academic Press, London, pp. 576–578.
Rowan, A. D. and Buttle, D. J. (1994). *Meth. Enzymol.* **244**, 555–568.
Salvesen, G. and Nagase, H. (1989). In *Proteolytic enzymes: A practical approach* (Beynon, R. J. and Bond, J. S., eds.), Oxford, United Kingdom: IRL Press, pp. 83–104.
Schaffer, M. A. and Fischer, R. L. (1988). *Plant Physiol.* **87**, 431–436.
Sengupta, A., Bhattacharya, D., Pal, G., and Sinha, N. K. (1984). *Arch. Biochem. Biophys.* **232**, 17–25.
Tablero, M., Arreguín, R., Arreguín, B., Soriano, M., Sánchez, R. I., Rodriguez-Romero, A., and Hernández-Arana, A. (1991). *Plant Sci.* **74**, 7–15.
Tournaire, C., Kushnir, S., Bauw, G., Inze, D., Teyssendier de la Serve, B., and Renaudin, J. P. (1996). *Plant Physiol.* **111**, 159–168.
Tranbarger, T. J. and Misra, S. (1996). *Gene* **172**, 221–226.
Ueda, T., Seo, S., Ohashi, Y., and Hashimoto, J. (2000). *Plant Mol. Biol.* **44**, 649–657.
Utsumi, S., Damodaran, S., and Kinsella, J. E. (1984). *J. Agric. Food Chem.* **32**, 1406–1412.
Vairo Cavalli, S. E., Cortadi, A., Arribére, M. C., Conforti, P., Caffini, N. O., and Priolo, N. S. (2001). *Biol. Chem. Hoppe-Seyler* **382**, 879–883.
Watanabe, H., Abe, K., Emori, Y., Hosoyama, H., and Arai, S. (1991). *J. Biol. Chem.* **266**, 16897–16902.
Watson, D. C., Yaguchi, M., and Lynn, K. R. (1990). *Biochem. J.* **266**, 75–81.
Westergaar, J. L., Hackbarth, C., Treuhaft, M. W., and Roberts, R. C. (1980). *J. Immunol. Meth.* **34**, 167–175.
Winnick, T., Davies, A. R., and Greenberg, D. M. (1940). *J. Gen. Physiol.* **23**, 275–308.
Ye, Z. H. and Varner, J. E. (1996). *Plant Mol. Biol.* **30**, 1233–1246.
Yu, W. J. and Greenwood, J. S. (1994). *Exp. Bot.* **45**, 261–268.