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Isolation and Characterization of a Cysteine Protease from the Latex of *Araujia hortorum* Fruits

Nora Priolo,[1,2] Susana Morcelle del Valle,[1] M. Cecilia Arribére,[1] Laura López,[1] and Néstor Caffini[1]

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A new protease (*araujiain h I*) was purified to mass spectroscopy homogeneity from the latex of *Araujia hortorum* Fourn. (*Asclepiadaceae*) fruits by ultracentrifugation and ion exchange chromatography. The enzyme has a molecular mass of 24,031 (mass spectrometry) and an isoelectric point higher than 9.3. The optimum pH range for casein hydrolysis was 8.0–9.5. The enzyme showed remarkable caseinolytic activity at high temperatures, although its thermal stability decayed rapidly. The proteinase was activated by thiol compounds and inhibited by common thiol-blocking reagents, particularly E-64 and $HgCl_2$, suggesting the enzyme belongs to the cysteine protease family. The concentration of active sites as determined by titration with E-64 was 3.3 μM. When assayed on N-α-CBZ-amino acid-*p*-nitrophenyl esters, the enzyme showed higher preference for the glutamine derivative, followed by those of alanine, asparagine, glycine, and leucine, in decreasing order. Partial homology (36–48%) with other plant cysteine proteinases was observed in an internal fragment obtained by Protease V8 treatment.

KEY WORDS: *Araujia hortorum; Asclepiadaceae;* latex; milkweed; plant proteases.

1. INTRODUCTION

Five catalytic types of proteases can now be recognized, in which serine, threonine, cysteine, aspartic, or metallo groups play primary roles in enzyme catalysis. The serine, threonine, and cysteine proteases are catalytically very different from the aspartic and metalloproteases in that the nucleophile of the catalytic site is part of an amino acid, whereas it is an activated water molecule in the other two groups. In cysteine proteases the nucleophile is the sulfhydril group of a Cys residue and the catalytic mechanism is similar to that of serine-type proteases in that the proton donor is a His residue. Although there is evidence in some cysteine peptidases that a third residue is required to orient the imidazolium ring of the His, there are a number of families in which only a catalytic dyad is necessary. Forty-one families of cysteine peptidases are recognized (a family is a group in which every member shows a statistically significant relationship of amino acid sequence to at least one other member of the family in the part of the molecule that is responsible for peptidase activity). Most plant cysteine peptidases belong to the papain family, including those of *Asclepiadaceae,* the milkweed family (Barrett *et al.,* 1998).

Proteases play a prominent role in plant physiology, being the catalysts of important processes like hydrolysis of storage proteins during seed germination, activa-

[1] Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, C.C. 711, 1900 La Plata, Argentina.

[2] To whom correspondence should be addressed; e-mail: priolo@biol.unlp.edu.ar.

[3] **Abbreviations:** AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid; BLAST, Basic Local Alignment Search Tool; CAPS, 3-(ciclohexylamino)-l-propanesulfonic acid; CBZ, carbobenzoxy; DEAE-Sepharose, diethylaminoethyl-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; PVDF, polyvinylidenedifluoride; RAP, redissolved acetone precipitate; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

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tion of proenzymes, degradation of defective proteins, etc. (Rudenskaya *et al.,* 1998), but the presence of a high concentration of proteolytic enzymes in some tissues is more difficult to explain. Many plants exude a latex containing a high amount of digestive enzymes, mainly cysteine and serine proteinases. This would suggest genetic resources involved in convergent evolutionary processes related to the overproduction and accumulation of proteases in different plant families (Boller, 1986).

Proteolytic enzymes are tools which have been used in medicine as well as in industry for hundreds of years. However, in recent years, their utility has become of vital importance due to the availability of standard, potent, and active preparations with good solubility, stability and odor. Proteolytic enzymes of plant origin have received special attention in the field of medicine and industry due to their property of being active over a very wide range of temperature and pH. Proteases, which firmly maintain first place in the world market of enzymes, play an important role in biotechnology, given that proteolysis changes the chemical, physical, biological, and immunological properties of proteins. Enzymatic hydrolysis is strongly preferred over chemical methods because it yields hydrolyzates containing well-defined peptide mixtures and avoids the destruction of L-amino acids and the formation of toxic substances like lysino-alanine (Lahl and Brown, 1994; Mahmoud, 1994).

In spite of the fact that existing commercially available proteases have a high degree of proteolytic activity and are abundantly and cheaply available, there is a need for discovering new plant sources of potent, more active, and more specific proteolytic enzymes. Proteases are frequently present in the latex of *Asclepiadaceae* (milkweed family). Nevertheless, studies of these proteases are limited to a few species belonging to the genera *Asclepias* (Winnick *et al.,* 1940; Greenberg and Winnick, 1940; Carpenter and Lovelace, 1943; Brockbank and Lynn, 1979; Lynn *et al.,* 1980*a;* Barragán *et al.,* 1985; Tablero *et al.,* 1991) and *Calotropis* (Abraham and Joshi, 1979*a,* 1979*b;* Pal and Sinha, 1980; Sengupta *et al.,* 1984). In a recent paper (Arribére *et al.,* 1998) we reported the partial characterization of proteases isolated from the latex of five species of *Asclepiadaceae* grown in Argentina, as well as the purification and characterization of the proteases of one of them, *Morrenia brachystephana* Griseb. A further communication reported the presence of proteases in the latex of *Morrenia odorata* (Hook. et Arn.) Lindley (Arribére *et al.,* 1999).

In this paper we report the characterization of a protease isolated from the latex of *Araujia hortorum* fruits. This species is a South American climbing plant that grows in the south of Brazil, Paraguay, Uruguay, and Argentina (Burkart, 1979). The latex has been used in folk medicine as a local application to warts (Watt and Breyer-Brandwijk, 1962).

2.1. Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, OH). AMPSO,[3] CAPS, carboxypeptidase B, cysteine, E-64, EDTA, iodoacetic acid, MOPS, *p*-nitrophenyl esters of N-α-carbobenzoxy-L-amino acids, pepstatine A, 1,10-phenanthroline, PMSF, sinapinic acid, TAPS, Tris, and glycine were purchased from Sigma Chemical Company (St. Louis, Mo). Coomassie brilliant blue R-250, acrylamide, bisacrylamide and low-molecular-weight markers were obtained from Bio-Rad (Hercules, CA). DEAE-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 of *Araujia hortorum* Fourn. were obtained from plants grown in Ringuelet, Province of Buenos Aires, Argentina (argentinean folk names: *tasi, doca*). The plant is a vine, with egg-shaped, oblong leaves, 5–10 cm long, whitish and pubescent on its abaxial face; fruits are ovoid, green, smooth, 8–12 cm long (Dimitri, 1972). Voucher specimens were deposited at the LPE herbarium (Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina).

2.3. Preparation of the Crude Extract

Latex obtained by superficial incisions of fruits, received on 0.1 M citrate–phosphate buffer (pH 6.5) containing 5 mM EDTA and cysteine, was first centrifuged at 16,000 × *g* for 30 min at 4°C. Gums and other insoluble materials were discarded, and the supernatant was ultracentrifuged at 100,000 × *g* for 60 min at 4°C. This new supernatant ("crude extract"), containing soluble proteins, was fractionated and conserved at −20°C for further studies.

2.4. Proteolytic (Caseinolytic) Activity Assays

Proteolytic assays were made using casein (Hammarsten type; Research Organics, Cleveland, OH) as

substrate. The reaction mixture was prepared by mixing 0.1 ml of enzyme extract with 1.1 ml of 1% casein containing 12 mM cysteine, in a 0.1 M Tris-HCl buffer (pH 8.0). The reaction was carried out at 45°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 30 min and the absorbance of the supernatant measured at 280 nm. An arbitrary enzyme unit (caseinolytic unit, U_{cas}) was defined as the amount of protease which produces an increment of one absorbance unit per min in the assay conditions (Priolo *et al.,* 1991).

2.5. Protein Determination

Proteins present in the crude extract were determined by Bradford's method (Bradford, 1976) using bovine albumin (Sigma Chemical Co., St Louis, MO) as standard. During chromatographic separation, the protein content of eluates was estimated by measuring the absorbance at 280 nm. Protein content of the active fractions was determined using Lowry's method (Lowry *et al.,* 1951), with bovine albumin as standard.

2.6. pH Profile of the Proteolytic Activity

The effect of pH on enzyme activity of both the crude extract and the main purified proteolytic fraction was measured with casein (pH range 6.4–10.5) using 10 mM sodium salts of the following "Good" buffers (Good and Izawa, 1972): MES, MOPS, TAPS, AMPSO, and CAPS (Sigma Chemical Co., St Louis, MO).

2.7. Inhibitor Effect

The action of different inhibitors of cysteine proteases was evaluated by incubating the crude enzyme preparation for 10 and 30 min at 45°C with mercuric chloride and E-64. The residual caseinolytic activity after each incubation assay was measured as indicated above.

2.8. Heat Inactivation

Progress curves for different temperatures (37, 45, 60, and 70°C) were made by measuring the caseinolytic activity versus the time (2, 5, 10, 15, 20, and 30 min) for both the crude extract and the purified protease (Dixon and Webb, 1979).

2.9. Purification of *araujiain h I*

The purification of the main proteolytic component (*araujiain h I*) was carried out by cation exchange chromatography (CM Sepharose CL-6B Fast Flow). Fourteen milliliters of the crude extract containing 75 mg of protein was loaded onto the column (Pharmacia K 15/30). The column was washed with 60 ml of 55 mM citrate–phosphate buffer (pH 6.4) and the bound material eluted with a linear gradient of sodium chloride (0–0.6 M) in the same buffer.

2.10. Characterization of *araujiain h I*

2.10.1. Thermal Stability

Thermal behavior of the purified protease was evaluated by measuring the residual caseinolytic activity at 45°C (pH 8.5) during 2 min after incubation of samples for 2.5, 5, 10, 20, and 30 min at 37, 45, and 60°C.

2.10.2. Isoelectric Focusing (IEF)

IEF was developed on immobilized pH gradient gels of polyacrylamide (10%) in the pH range from 3 to 10 (Biolyte 3–10 carrier ampholytes, Bio-Rad, Hercules, CA) in a Mini IEF Cell (Model 111, Bio-Rad). Samples were concentrated by acetone precipitation and further centrifugation at 11,000 × *g* during 20 min. Deionization was performed by redissolving the precipitates in deionized water and repeating the whole treatment twice. Isoelectric focusing of proteases was carried out according to the conditions 100 V for 15 min, 200 V for the following 15 min, and 450 V for the last 60 min. Then, gels were fixed and stained with Coomassie brilliant blue R-250.

2.10.3. Native Electrophoresis

The crude extract as well as the active chromatography fractions were analyzed on a 12.5% polyacrylamide gel in a Miniprotean II Cell (Bio-Rad, Hercules, CA). Samples were precipitated with 5 volumes of acetone, redissolved in the sample buffer [62.5 mM Tris-HCl, pH 6.8, with 10% (v/v) glycerol and 0.25% (w/v) bromophenol blue], and centrifuged at 16,000 × *g* for 10 min. Samples were loaded on the gel, and the electrophoresis performed by changing the polarity of electrodes during 2 h at 35 mA. Protein bands were detected by Coomassie brilliant blue R-250.

2.10.4. SDS–Polyacrylamide Gel Electrophoresis

SDS–PAGE was carried out according to Laemmli (1970). Samples were precipitated with acetone as described above and redissolved in sample buffer containing 10 μM E-64 to prevent autodigestion and boiled for 5 min; then, β-mercaptoethanol was added and boiled again. Samples were then loaded on a polyacrylamide 14% separating gel overlaid by a 5% stacking gel. Electrophoresis was run in a vertical apparatus starting at 25 mA. When the samples left the stacking gel, the current was set at 50 mA until the bromophenol blue dye marker left the separating gel. The gels were stained with Coomassie brilliant blue R-250 and scanned for evaluation of the molecular masses using the Scion Image software.

2.10.5. Zymogram

To confirm the proteolytic activity of the bands, native electrophoresis unstained gels were contacted for 15 min at 56°C with an agarose gel imbibed in 1% casein solution (Westergaar *et al.,* 1980) and then stained with Coomassie brilliant blue R-250.

2.10.6. Titration of Active Sites with E-64

A dose–response relationship was studied between the purified enzyme and increasing concentrations of E-64, a well-known cysteine proteinase inhibitor. The method was an adaptation of that developed by Barrett *et al.* (1982). Fractions (25 μl) of *araujiain h I* eluted from the ion exchange column were incubated with 25 μl of different concentrations (0–10 μM) of E-64 solutions for 30 min at room temperature. Then, the residual caseinolytic activity was assayed as follows: 55 μl of 1% casein solution with 12 mM cysteine (pH 8.35) was added to the mixture and kept for 8 min at 45°C; the reaction was stopped by the addition of 900 μl of 5% TCA and the absorbance of soluble peptides read at 280 nm.

2.10.7. Esterolytic Activity Determination

Measurement of endoesterolytic activity (Silverstein, 1974) was performed with N-α-carbobenzoxy-*p*-nitrophenyl esters of some amino acids (Gln, Ala, Asp, Phe, Asn, Tyr, Trp, Gly, Leu, Val, and Pro). The synthetic substrates were obtained from Sigma Chemical Co. (St. Louis, MO). Assays were made at 40°C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 mM EDTA and 25 μM cysteine in the reaction mixture. Absorbance was measured at 405 nm every 10 sec for the first minute, and then every 15 sec. Both crude extract and *araujiain h I* were used for this assay. An arbitrary enzyme activity unit (U_{cbz}) was defined as the amount of protease that released 1.0 μM of *p*-nitrophenolate per min in the assay conditions. To determine the micromoles of *p*-nitrophenolate produced during the reaction, a standard curve (*p*-nitrophenol 15–70 μM) was carried out.

2.10.8. Protein Sequence Analysis

Alkylated *araujiain h I* was digested with Protease V8 (endopeptidase Glu-C) using a 1:25 enzyme protein ratio (w/w). This serine endopeptidase cleaves peptide bonds -COOH terminal to Glu residues at a rate that is about 3000-fold faster than that for Asp residues (Sprensen *et al.,* 1991). Peptides were separated by reverse-phase HPLC using a Nova Pack C18 column (Waters) and a 60-min linear gradient of 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA in acetonitrile at 0.1 ml/min and were analyzed by mass spectrometry. A sample of the main peptide was adsorbed on 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), indicating the specific residues which are identical ("identities"), as well as those which are nonidentical, but nevertheless have positive alignment scores ("positives").

2.10.9. Mass Spectrometry

Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS) was used for the determination of the molecular mass, as well as the degree of purity of active chromatographic fractions. MALDI/TOF mass spectra were acquired on a Bruker Biflex spectrometer equipped with a pulsed nitrogen laser (337 nm), in linear positive-ion mode, using a 19-kV acceleration voltage. Samples were prepared by mixing equal volumes of a saturated solution of the matrix (3,5-dimethoxy-4-hydroxycinnamic acid–sinapic acid) in 0.1% TFA in water/acetonitrile 2:1, and a 1–10 μM protein solution. From this mixture, 1 μl was spotted on the sample slide and allowed to evaporate to dryness. Proteins of known molecular mass were used as standards for mass calibration.

3. RESULTS AND DISCUSSION

Proteases contained in the latex of *Araujia hortorum* Fourn. fruits were studied. The crude enzyme extract (supernatant of 100,000 × *g*) showed higher caseinolytic activity in the presence of 12 mM cysteine. Highest activity (fourfold) was recovered when the crude extract was obtained with 5 mM EDTA and 5 mM cysteine.

Cation exchange chromatography of the crude extract yielded two main proteolytic peaks (Fig. 1): fraction I (*araujiain h I*), purified to mass spectroscopy homogeneity, and fraction II. The name *araujiain h I* is proposed for the new protease, according to previous recommendations (Barragán *et al.,* 1985; Tablero *et al.,* 1991). Specific activity was higher for the former, which was chosen for further analysis. When the crude extract was previously inactivated by treatment with $HgCl_2$, the chromatographic pattern observed was identical to that of the untreated enzyme (data not shown). Table I shows the purification scheme: a sixfold purification degree (yield: 12%) was obtained for *araujiain h I.*

A slight shift in the optimum pH range (Fig. 2) was observed for *araujiain h I* (pH 8.0–9.5) in comparison with the crude preparation (pH 7.5–8.5). The enzyme exhibited a pI value higher than 9.3, as can be seen in Fig. 3; this basic nature was also observed in the

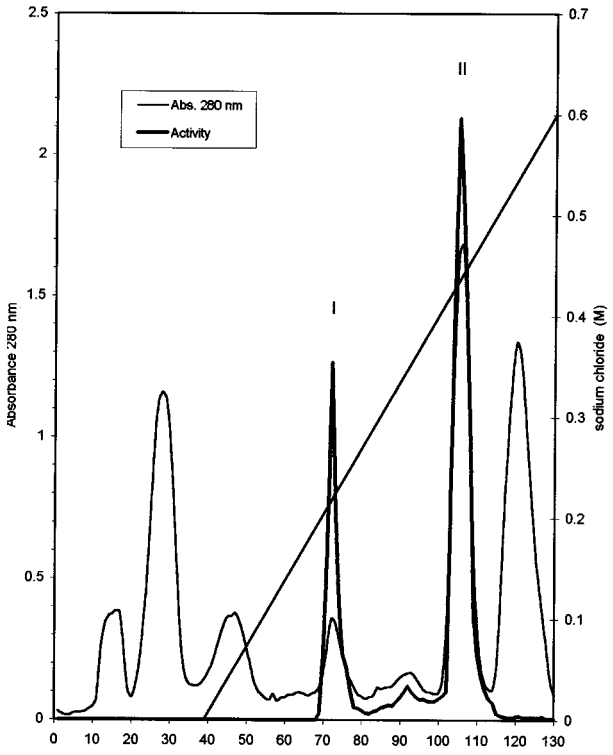

Fig. 1. Cation exchange chromatography (CM-Sepharose CL-6B Fast Flow, column Pharmacia K 15/30). Elution buffer: 55 mM citrate–phosphate (pH 6.4). Gradient: sodium chloride 0–0.6 M. Flow rate: 17 cm h^{-1}. Fraction volume: 1.6 ml.

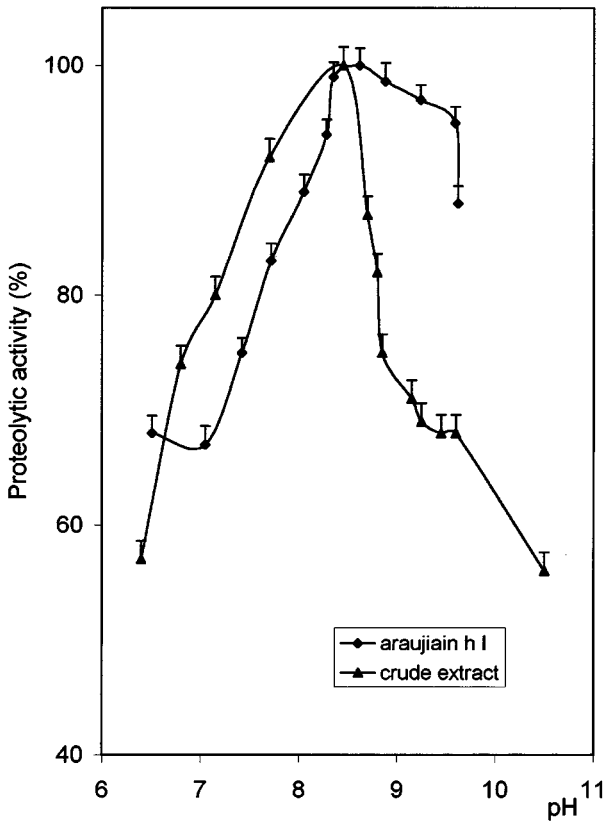

Fig. 2. Effect of pH on proteolytic activity of crude extracts and *araujiain h I.*

Table I. Purification of the Proteolytic Components Present in the Latex of *Araujia hortorum*

Sample	Volume (ml)	Protein (mg/ml)	Total proteins	UCAS/ml	Total UCAS	Specific activity UCAS/mg	Purification (fold)	Yield (%)
Crude extract	120	5.4	642.7	68.4	8220	12.8	—	100
Araujiain h I	18.5	0.08	1.46	6.15	113.78	77.9	6	12

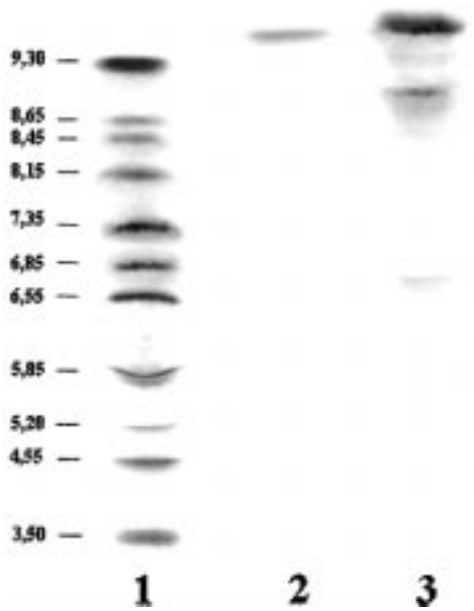

Fig. 3. Isoelectric focusing. **Lane 1:** IEF Bio Rad markers; **lane 2:** *araujiain h I.* **lane 3:** crude extract.

Table II. Effect of Inhibitors on Proteolytic Activity of Crude Extract

Inhibitor	Inhibitory concentration (mM)	Percentage activity
None		100
$HgCl_2$ (10 min)	0.1	0
$HgCl_2$ (10 min) + 12 mM cys	0.1	96.25
E-64 (30 min)	0.01	0

proteases isolated from the latex of *Asclepias glaucescens* (Barragán *et al.,* 1985; Tablero *et al.* 1991), *Morrenia brachystephana* (Arribére *et al.,* 1998), and *Morrenia odorata* (Arribére *et al.,* 1999).

Inactivation assays with $HgCl_2$ and E-64 suggested the possible cysteinic nature of the proteases present in the crude extract. When $HgCl_2$ was added, proteolytic activity almost completely reverted by adding cysteine to the incubation mixture. When E-64 was added, the inhibition obtained for crude extract was complete and irreversible (Table II). The activation, inhibition, and reactivating results would indicate dependence of the proteolytic activity upon the presence of active -SH groups in the enzymes present in the crude extract. This behavior was previously shown by proteases from other *Asclepiadaceae* species (Lynn *et al.,* 1980*a;* Abraham and Joshi, 1979*b;* Tablero *et al.,* 1991; Arribére *et al.,* 1998, 1999).

As can be seen in Fig. 4, *araujiain h I* had a poor stability at high temperatures, but the caseinolytic activity increased with temperature: maximum proteolytic activity was obtained at 70°C for the crude extract (Fig. 5a) and at 60°C for *araujiain h I* (Fig. 5b). These results suggested a protective role played by the substrate and, in the case of the crude extract, probably by other proteins.

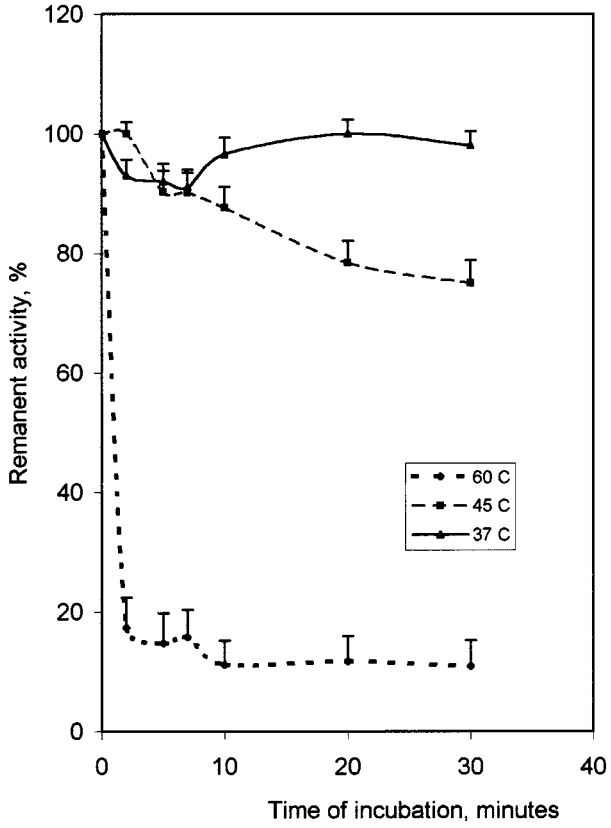

Fig. 4. Thermal stability of *araujiain h I.* The determinations of activity were made on casein at pH 8.5.

Native electrophoresis of the crude extract showed two fractions, one of them with higher proteolytic activity (*araujiain h I*), as evidenced by the corresponding zymogram (Fig. 6). SDS–PAGE revealed that *araujiain h I* appeared as a unique band (Fig. 7), with a relative mass of about 25.5 kDa. In the titration of active sites with E-64 the residual enzyme activity gave a curve going downward with linearity at E-64 concentrations up to 5 μM. Extrapolation of the linear curve crossed the *X* axis at the E-64 concentration of 3.3 μM (Fig. 8), which matches with the protein concentration in the eluted fraction obtained by Lowry determination. Thus, the E-64 and the enzyme were found to react with each other on an equimolecular basis. By combining both methods, we calculated the molecular mass of *araujiain h I* to be about 24 kDa, in good agreement with the value obtained by mass spectroscopy (24,031), as shown in Fig. 9. As reported

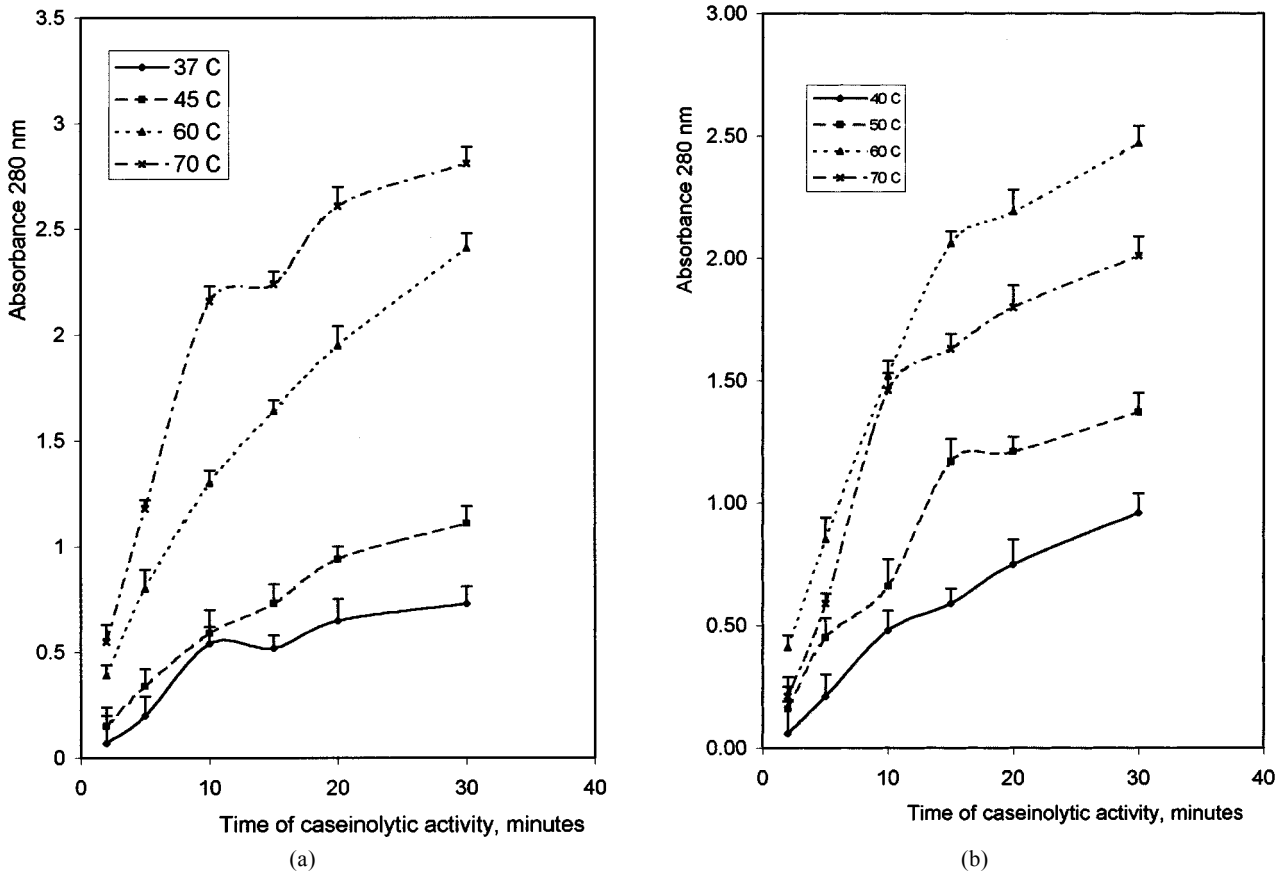

Fig. 5. (a) Enzyme activity of crude extract as a function of temperature; (b) enzyme activity of *araujiain h I* as a function of temperature. In both cases the determinations of activity were made on casein at pH 8.5.

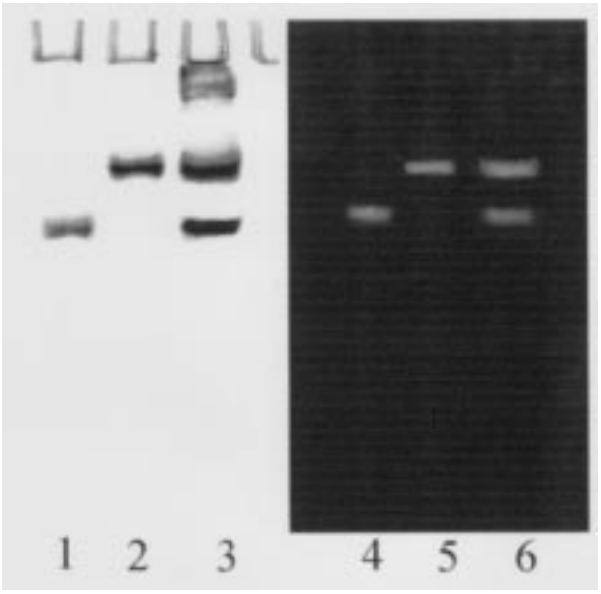

Fig. 6. Native electrophoresis (PAGE) and corresponding zymograms. **Lane 1:** fraction II; **lane 2:** fraction I (*araujiain, h I*); **lane 3:** crude extract; **lanes 4, 5,** and **6:** zymograms of fraction II, fraction I, and crude extract, respectively.

for other plant proteases (Harrach *et al.,* 1998), molecular masses determined by SDS–PAGE are higher than those obtained by other methods. The results are almost coincident with those obtained for other proteases from *Asclepiadaceae: M_r* values of *Asclepias syriaca* proteases are 21 and 23 kDa (Brockbank and Lynn, 1979; Lynn *et al.,* 1980*a*), proteases from *Asclepias glaucescens* have M_r of 23 kDa (Barragán *et al.,* 1985; Tablero *et al.,* 1991), the four calotropins isolated from *Calotropis gigantea* show molecular masses ranging from 23 to 27 kDa (Abraham and Joshi, 1979*a,* 1979*b;* Pal and Sinha, 1980), two proteases obtained from *Morrenia brachystephana* present molecular masses of 25.5 and 26 kDa (Arribére *et al.,* 1998) and the proteases isolated from *Morrenia odorata* show M_r of 24.2 and 25.8 (Arribére. *et al.,* 1999).

Endoesterolytic activity of the crude extract and *araujiain h I,* determined on N-α-CBZ-amino acid-*p*-nitrophenyl esters, exhibited different patterns: both showed higher preference for the glutamine derivative, but in the crude extract it is followed by those of

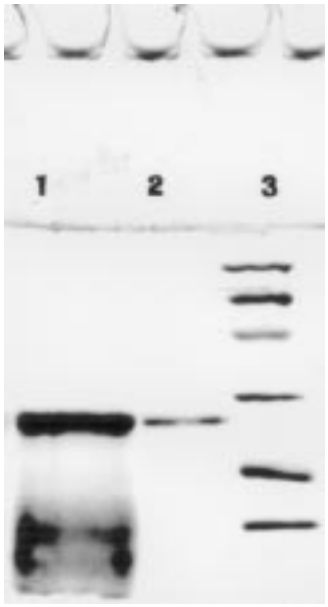

Fig. 7. SDS–PAGE. **Lane 1:** crude extract; **lane 2:** *araujiain h I;* **lane 3:** molecular weight Sigma markers: α-lactalbumin, bovine milk (14.2 kDa); trypsin inhibitor, soybean (20.0 kDa); trypsinogen, bovine pancreas (24.0 kDa); carbonic anhydrase, bovine erythrocytes (29.0 kDa); glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (36.0 kDa); ovalbumin, chicken egg (45.0 kDa); albumin, bovine serum (66.0 kDa).

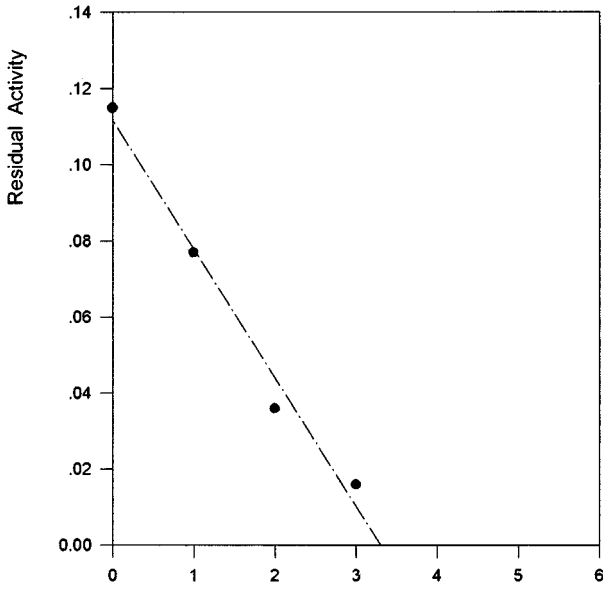

Fig. 8. Titration of active sites with E-64.

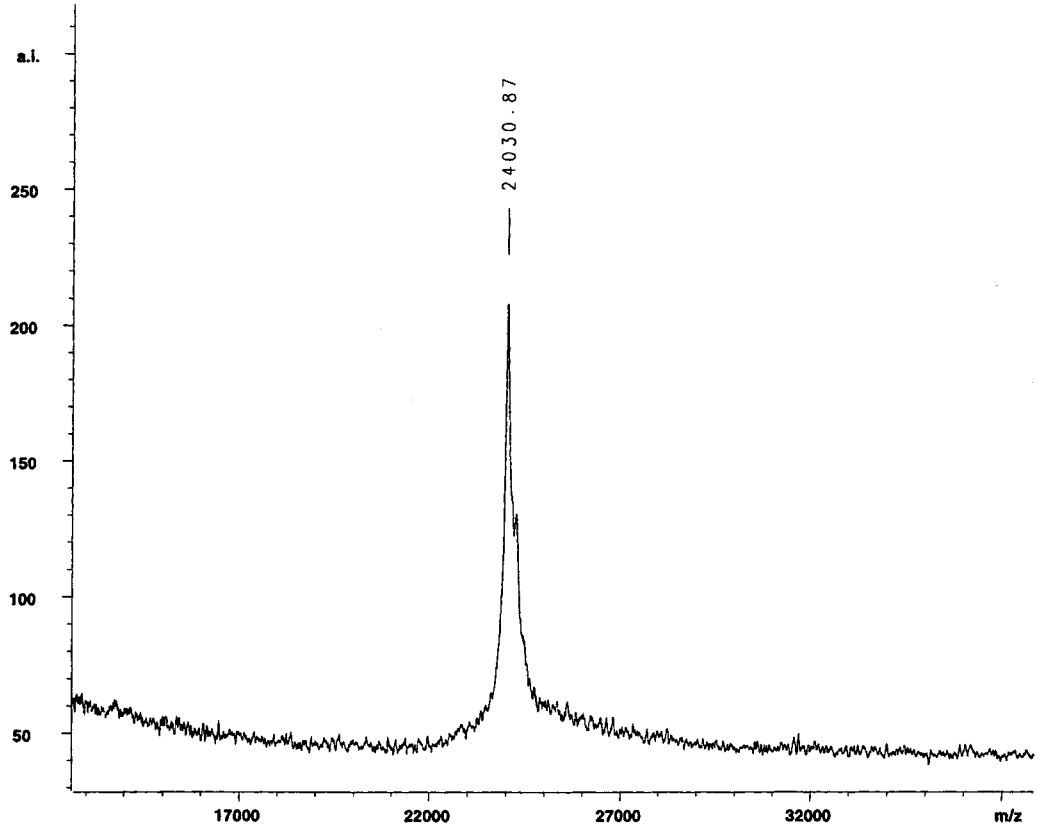

Fig. 9. Mass spectroscopy of *araujiain h I.*

Table IV. Sequence Analysis

Plant source	Sequence	Identities	Positives	Reference
Araujia hortorum	AF TYVAK NG ITSRDK YPY RGQQ GQC YQLQ KVV RISGYQS VP	41/41 (100%)	—	
Hemerocallis sp.	AF ++ KNGIT+ D YPY Q G C VV I G+Q VP	21/44 (48%)	25/44 (57%)	Valpuesta *et al.* (1995)
	AF EFIQK NG ITTEDS YPY AEQD GTC ASNLLNSP VV SIDGHQD VP			
Carica papaya	A YVAKNGI R KYPY+ +QG C Q +V+ SG V	21/44 (48%)	25/44 (57%)	Revel *et al.* (1993)
(papaya proteina omega)	A LEYVAK NG IHLRSK YPY KAKQ GQC RAKQVGGPI V KTSGVR V Q			
Brassica napus	AF ++ KNG + +K YPY G G+C L K VV I GY+ VP	21/45 (47%)	27/45 (60%)	Dietrich *et al.* (1989)
	AF QFIMK NG GLNTEKD YPY HGTN GQC NSLLKNSR VV TIDGYED VP			
Arabidopsis thaliana	AF ++ KNG + DK YPY+G G C Q++ KVV I Y+ VP	21/45 (47%)	28/45 (62%)	Koizumi *et al.* (1993)
	AF EFIIK NG GIDTDKD YPY KGVD GQC DQIRKNA KVV TIDSYED VP			
Zingiber officinale	AF ++ NG I S + PYYRGQ G C VV I Y++VP	20/44 (45%)	25/44 (57%)	Choi *et al.* (1999)
	AF QFIVN NG GINSEET YPY RGQD GQC NSTVNAP VV SIDSYEN VP			
Phaseolus vulgaris	AF ++ +NG I + + YPY+G G C Q + KVV+I GY+ VP	20/45 (44%)	29/45 (64%)	Rotari *et al.* (1997)
	AF QFIIQ NG GIDTEED YPY QGID GQC DQTKKKT KVV QIDGYED VP			
Zinnia elegans	AF YV +NG+ ++YPY +G C ++ +KV ISGY VP	19/44 (43%)	27/44 (61%)	Ye and Varner (1993)
	AF AYVTR NG LHKEEE YPY IMSE GQC DEKRDASE KV TISGYHD VP			
Phaseolus vulgaris	AF ++ +NG I + + YPY+G G C + + KVV+I GY+ VP	19/45 (42%)	29/45 (64%)	Sohlberg and Sussex (1997)
	AF QFIIQ NG GIDTEED YPY QGID GQC DETKKKT KVV QIDGYED VP			
Oryza sativa	AF ++ KNG I + D YPY+ G+C + KVV I G++ VP	19/45 (42%)	27/45 (60%)	Watanabe *et al.* (1991)
	AF DFIIK NG GIDTEDD YPY KAVD GQC DINRENA KVV SIDGFED VP			
Carica papaya	YVA NG+ + YPY+ +Q +C KV+I+GY+ VP	17/41 (42%)	25/41 (61%)	Watson *et al.* (1990)
(quimopapaina)	YVAN NG VHTSKV YPY QAKQYK C RATDKPGP KV KITGYKR VP			
Pisum sativum	A+ ++ +NG + S+ YPY G+Q Q + KVV I+GY++V	18/44 (41%)	28/44 (64%)	Kardailsky and Brewin (1996)
	A YRFIVE NG GLDSQID YPY LGRQST C NQAKKNT KVV SINGYKN V			
Vicia sativa	AF ++ +NGIT+ YPY + G C + K V I G+++VP	17/43 (40%)	26/43 (61%)	Becker *et al.* (1997)
	A EFIKO NG ITTESN YPY AAKD GTC DVEKED KA V SIDGHEN VP			
Cicer arietinum	AF ++ +NG D+ YPY G + +C + KVV I GY+ VP	18/45 (40%)	26/45 (58%)	Cervantes *et al.* (1994)
	AF EFIIR NG GIDTDQD YPY NGFERK C DPTKKNA KVV SIDGYED VP			
Zea mays	AF ++ NG I + YPY+G G+C + KVV I Y+ VP	18/45 (40%)	25/45 (56%)	Pechan *et al.* (1999)
	AF EFIIN NG GIDTEKD YPY KGTD GRC DVNRKNA KVV TIDSYED VP			
Pisum sativum	AF ++ +NGIT+ YPY + G C + + + V I GY++VP	17/44 (39%)	27/44 (61%)	Cercos *et al.* (1999)
	AF EFIKQ NG ITTESN YPY AAKD GTC DLKKEDKAE V SIDGYEN VP			
Glycine max	+F +V ++G I + D YPYR ++G C+ ++Q KV I GY+++	17/43 (40%)	30/43 (70%)	Kalinski *et al.* (1992)
	S F EWVLEH G GIATDDD YPY RAKE GRC KANKIQD KV TIDGYETL			
Carica candamarcensis	YV +G+ + +YPY +Q +C K +V+ISGY+ VP	16/41 (39%)	25/41 (61%)	Jaziri *et al.* (1994)
	YVVDH G VHTEKE YPY EEKQYK C RAKD K KPPI V KISGYKK VP			
Actinidia deliciosa	F ++ NG I + + YPY Q G+C + Q +K V I Y++VP	17/44 (39%)	26/44 (59%)	Podivinsky *et al.* (1989)
	F QFIIN NG GINTEEN YPY TAQD GEC NVELQNE K Y V TIDTYEN VP			
Actinidia chinensis	F ++ NG I + + YPY Q G+C Q +K V I Y +VP	17/44 (39%)	24/44 (55%)	Praekelt *et al.* (1988)
	F QFIIN NG GINTGEN YPY TAQD GEC NLDLQNE K Y V TIDTYGN VP			
Vigna mungo	AF ++ K GIT+ YPY+ Q+G C + + V I G+++ VP	17/45 (38%)	27/45 (60%)	Yamauchi *et al.* (1992)
	AF EFIKQK G GITTESN YPY KAQE GTC DESKVNDLA V SIDGHEN VP			
Alnus glutinosa	AFT++ N G+ S YPY+G G C ++ + I+G++ VP	16/45 (36%)	26/45 (58%)	Goetting-Minesky and Mullin (1994)
	AF TFIQH G H LASEAN YPY KGVD GTC NTNKQAIHAAEINGFED VP			

aspartic acid, asparagine, glycine, leucine, tryptophan, tyrosine, phenylalanine, and alanine in decreasing order, while in *araujiain h I* the preference order is as follows: glutamine, alanine, aspartic acid, leucine, and tryptophan (Table III).

The N-terminus of *araujiain h I* was found to be blocked, as was previously reported for calotropins DI and DII isolated from *Calotropis gigantea* (Sengupta *et al.,* 1984). Therefore, the sequence of an internal peptide obtained by hydrolysis with Protease V8 (endopeptidase Glu-C) was determined. Table IV shows the sequence of this fragment (41 residues) compared to those of 21 plant cysteine proteinases, none of which belongs to the *Asclepiadaceae* family, as only N-terminal sequences (Lynn *et al.,* 1980*b*) and C-terminal sequences (Sengupta *et al.,* 1984) of proteinases from species of this family have been reported. Despite this, a remarkable degree of homology was obtained (identities = 48–36%, positives = 70–55%) and several motifs are notably conserved (YPY in all cases; AF, NG, GXC, KVV or KVX or XVV, and VP, in most cases), suggesting that *araujiain h I* probably shares an ancestral gene with cysteine proteases obtained from taxonomically unrelated plant species.

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Table III. Kinetic Studies

	Crude extract		araujiain h I	
N-CBZ-amino acid *p*-nitrophenyl ester	U_{CBZ}	Percentage preference	U_{CBZ}	Percentage preference
Gln	53.31	100	59.02	100
Ala	2.67	7.14	27.50	47.61
Asp	24.95	48.00	8.50	16.13
Gly	16.44	32.40	5.10	10.45
Leu	14.80	29.40	4.85	10.06
Trp	11.57	23.44	2.57	6.28
Tyr	10.67	21.80	1.08	3.79
Asn	16.37	32.25	0.92	3.52
Phe	7.52	16.02	0.77	3.30
Val	1.81	5.55	0.32	2.54
Pro	0.00	0.00	0.00	0.00

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