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Two New Cysteine Endopeptidases Obtained from the Latex of *Araujia hortorum* Fruits

Walter D. Obregón,[1] Ma. Cecilia Arribére,[1] Susana Morcelle del Valle,[1] Constanza Liggieri,[1] Néstor Caffini,[1] and Nora Priolo[1,2]

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Two new endopeptidases were purified to homogeneity from the latex of *Araujia hortorum* fruits by a simple purification procedure involving ultracentrifugation and ion exchange chromatography. Molecular weights of araujiain h II and araujiain h III were 23,718 and 23546 (mass spectrometry), respectively. The isoelectric point of araujiain h II was 8.9, whereas araujiain h III had a pI higher than 9.3. Maximum proteolytic activity on caseine was reached at pH 8.0–9.0 for both endopeptidases, which were irreversibly inhibited by iodoacetate and E-64, suggesting they belong to the cysteine protease family. Esterolytic activity was determined on N-α-CBZ-amino acid-*p*-nitrophenyl esters, and the highest k_{cat}/K_m values for the both enzymes were obtained with the glutamine derivative. The N-terminal sequences of araujiain h II and araujiain h III showed a high degree of homology with other plant cysteine endopeptidases.

KEY WORDS: Araujiain; cysteine endopeptidases; latex; milkweed family; protein purification.

1. INTRODUCTION

Peptidases are essential for many aspects of plant physiology and developement; they serve as catalysts of processes like hydrolysis of storage proteins during seed germination, activation of proenzymes, and degradation of defective proteins, among other processes (Rudenskaya *et al.,* 1998). However, the presence of a high concentration of proteolytic enzymes in some plant tissues is much more difficult to explain. In latices obtained from plants of diverse families, proteolytic enzymes account for more than 50% of the total proteins and for 10% or more of the fresh matter (Feller, 1986). Apparently, there are various genetic resources involved in an evolutionarily convergent process related to the overproduction and accumulation of proteases in different plant families (Boller, 1986). Peptidases are grouped into families on the basis of statistically significant similarities between the protein sequences in the part named the *peptidase unit,* which is most directly responsible for enzyme activity. The term *family* describes a group of peptidases in which each member is homologous to the type example either by direct comparison or through a transitive relationship that must exist at least in the peptidase unit. The cysteine peptidase group is one of the five catalytic types now recognized and is composed of 41 families. Most plant cysteine peptidases belong to the papain family, including those of Asclepiadaceae, the milkweed family (Barrett *et al.,* 1998).

Proteolytic enzymes are a very important group of biological catalysts in medical research and biotechnology.

[1] Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CC 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; CM-Sepharose, carboxymethyl-Sepharose; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA, ethylendiaminetetraacetic acid; IEF, isoelectric focusing; MALDI TOF–MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; MOPS, 3-(N-morpholino) propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidenedifluoride; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

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From the commercial point of view, their preparations are the most important of the currently produced enzymes. Such preparations are commonly employed to produce many foods in which enzymes can replace potentially carcinogenic or otherwise harmful chemicals (Uhlig, 1998).

Proteases are frequently present in the latex of Asclepiadaceae, but studies of these proteases are limited to a few species belonging to the genera *Asclepias, Calotropis,* and *Morrenia.* In a previous paper (Priolo *et al.,* 2000), we reported the isolation and characterization of araujiain h I, a novel plant protease normally present in the latex exuded from fruits of *Araujia hortorum* Fourn. (Asclepiadaceae). In this article, the purification and characterization of two new cysteine peptidases from the same source is reported. The species is a South American climbing plant that grows in the south of Brazil, Paraguay, Uruguay, and Argentina (Burkart, 1979), and has been used in traditional medicine as a local application to warts (Watt and Breyer-Brandwijk, 1962), probably due to its high proteolytic activity.

2. MATERIALS AND METHODS

2.1. Chemicals

AMPSO,[3] CAPS, carboxypeptidase B, Coomassie brilliant blue R-250, cysteine, E-64, EDTA, iodoacetic acid, MOPS, *p*-nitrophenyl esters of N-α-CBZ-L-amino acids, TAPS, Tris, and glycine were purchased from Sigma Chemical Company (St. Louis, MO). Acrylamide, bisacrylamide, Biolyte 3–10 carrier ampholytes, and low-molecular weight markers were obtained from Bio-Rad (Hercules, CA). CM-Sepharose Fast Flow and Pharmalyte 8.5–10 was purchased from Pharmacia Biotech (Uppsala, Sweden). Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, OH).

2.2. Crude Extract Preparation

Fresh latex, obtained by superficial incisions of *Araujia hortorum* fruits, was gathered on 0.1 M citrate–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. This procedure was performed for discarding gums and other insoluble materials. The supernatant was then ultracentrifuged at 100,000 × *g* for 60 min at 4°C. This new supernatant, called *crude extract,* containing soluble proteins, was fractionated and conserved at −20°C for further studies.

2.3. Cation Exchange Chromatography

The crude extract was purified by cation exchange chromatography in a CM Sepharose CL-6B Fast Flow column, equilibrated and washed with 55 mM citrate–phosphate buffer (pH 6.4). Elution was performed with a linear gradient of sodium chloride (0−0.6 M) in the same starting buffer. The first assays were carried out using a diluted crude extract, resulting in the separation of two well-defined proteolytic fractions and a third peak with a very low protein content and low proteolytic activity eluting between both fractions (Priolo *et al.,* 2000). To study this minor component, concentrated crude extract was loaded onto the column under the same conditions, then a higher fraction was collected and rechromatographed in the same column with a narrower linear gradient of sodium chloride (0.25–0.5 M) in the same buffer.

2.4. Analytical Procedures and Measurement of Enzymatic Activity

Protein concentration was usually determined by Bradford's method (Bradford, 1976), but in chromatographic fractions the protein content was estimated by measuring the absorbance at 280 nm. Proteolytic activity was measured on casein as substrate, according to the method described by Arribére *et al.* (1998). One proteolytic unit was defined as the amount of protease which produces an increment of one absorbance unit per minute in the assay conditions (Priolo *et al.,* 1991). The effect of cysteine protease inhibitors was also tested: the final inhibitor concentration in the assay mixture for E-64 and iodoacetate was 50 μM and 25 mM, respectively.

The effect of pH on enzyme activity in the two purified proteases was measured on casein using Good buffers (Good and Izawa, 1972), pH range 6.4–11.5, prepared with 10 mM sodium salts of MES, MOPS, TAPS, AMPSO, and CAPS.

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

2.5. Electrophoresis (SDS–PAGE)

Samples containing proteases were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 14% (w/v) acrylamide (Laemmli, 1970). Samples were treated with E-64 before

PAGE to avoid autohydrolysis artifacts. Gels were stained with Coomassie brilliant blue R-250.

2.6. Mass Spectrometry

MALDI–TOF MS was used for the determination of molecular masses 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 a linear positive-ion mode, using 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 masses were used as standards for mass calibration.

2.7. Isoelectric Focusing

Two sorts of isoelectric focusing (IEF) gels were developed on immobilized pH gradient gels of polyacrylamide (10%), one in the pH range 3–10 and the other in the pH range 8.5–10, 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 following 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.

2.8. Measurement of Endoesterolytic Activity

These assays were carried out by the Silverstein's (1974) method modified to reach optimal conditions of each enzyme. The activity was studied using N-α-carbobenzoxy-*p*-nitrophenyl esters of some amino acids (Gln, Ala, Asp, Phe, Asn, Tyr, Trp, Gly, Leu, Val, and Pro) as substrates. 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. The continuous liberation of *p*-nitrophenol was followed spectrophotometrically at 405 nm in a Beckmann DU 640 equipped with a chamber termostatized at 37°C. An arbitrary enzyme activity unit (U_{CBZ}) was defined as the amount of protease that released 1 μmol of *p*-nitrophenolate per minute in the assay conditions.

2.9. Kinetic Parameters

N-α-CBZ-*p*-nitrophenyl ester of glutamine was employed to determine K_m and k_{cat} of the purified enzymes. Estimation of such parameters was performed following the method previously described for these substrates. The initial reaction rate was determined by monitoring the absorbance at 405 nm every 10 sec during 3 min. Both K_m and k_{cat} were calculated by regression analysis using the nonlinearized form of the Michaelis–Menten equation.

2.10. Protein Sequence Analysis

Purified proteases (araujiain h II and araujiain h III) were adsorbed onto a PVDF membrane (Millipore) and washed several times with deionized water. The N-terminal sequences were determined by Edmans automated degradation using a Beckman LF3000 protein sequencer equipped with a PTH-amino acid analyzer System Gold (Beckman). 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

3.1. Purification of the Crude Extract

Centrifugation followed by ultracentrifugation of the latex suspension allowed the elimination of insoluble particles and gums present in the latex, producing the *crude extract*. This crude extract (7.5 ml, containing 17.3 mg of protein) was loaded onto a CM Sepharose column and eluted by the application of a linear gradient of sodium chloride, the eluted fractions being recorded at 280 nm. Six protein fractions were eluted, three of which showed proteolytic activity. The homogeneity of the second fraction was confirmed by rechromatography (data not shown). Three endopetidases were obtained, named araujiain h I (Priolo *et al.,* 2000), araujiain h II, and araujiain h III (Fig. 1). The purification scheme is shown in Table I.

3.2. Characterization of Araujiain h II and Araujiain h III

3.2.1. General Enzymatic Parameters

The effect of pH and specific inhibitors on the proteolytic activity was studied as indicated in Section 2. The highest activity was obtained at pH 8.0–9.0 for the two

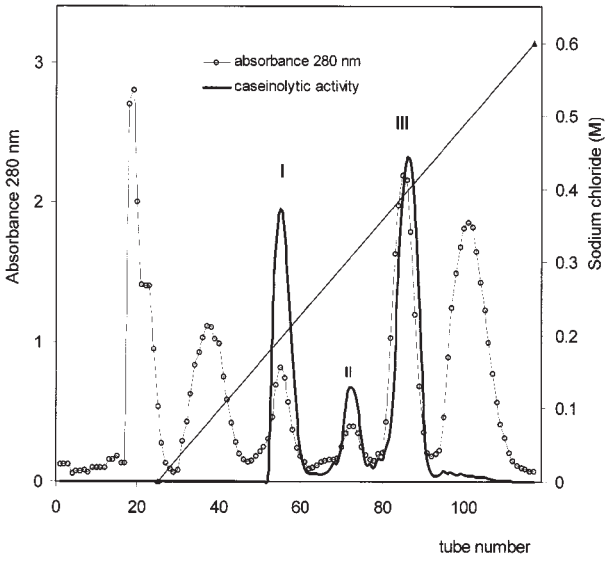

Fig. 1. Cation exchange chromatography. CM-Sepharose CL-6B Fast Flow, column Pharmacia K 15/30. Elution buffer: 55 mM citrate–phosphate (pH 6.4). Gradient: sodium chloride 0–0.6 M. Flow rate: 17 cm hr^{-1}. Fraction volume: 1.6 ml.

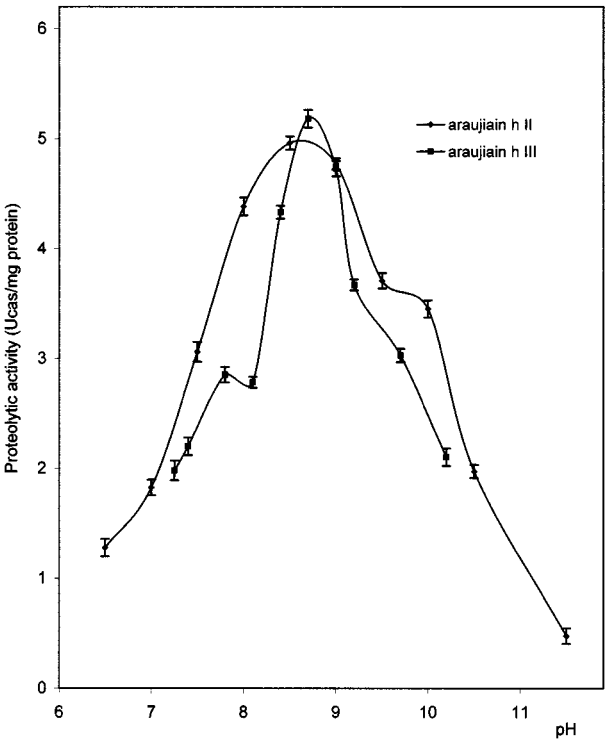

Fig. 2. Effect of pH on proteolytic activity of araujiain h II and araujiain h III.

peptidases (Fig. 2) in the presence of 12 mM cysteine. Iodoacetate and E-64 totally and irreversibly inhibited enzyme activity in all cases.

Progress curves for different temperatures (37°C, 45°C, 60°C, and 70°C) were made by measuring the caseinolytic activity versus time (2, 5, 10, 15, 20, and 30 min) for the two purified proteases (Figs. 3A and 3B) at their optimum pH values.

The electrophoretic (Fig. 4) and isoelectric focusing data (Fig. 5) confirmed the homogeneity of the two purified proteases. Araujiain h III had a pI higher than 10.5, whereas the araujiain h II pI was estimated to be 8.9.

The M_r of the enzymes, according to mass spectrometry (MS), was araujiain h II, 23718 Da, and araujiain h III, 23546 Da (Fig. 6).

3.2.2. Kinetic Parameters

To calculate kinetic parameters, esterolytic activity was measured for each protease, using N-α-CBZ-*p*-nitrophenyl esters of different amino acids as substrates. Amino acid preferences were estimated on a U_{CBZ} basis (Table II). The glutamine derivative was the best substrate to determine kinetic parameters (K_m and k_{cat}) of the studied endopeptidases because of their highest preference for this derivative (Table III).

3.2.3. Sequence Analysis

The results obtained from matching the N-terminal amino acid sequences of araujiain h II and araujiain h III

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	7.5	2.3	17.3	22.8	171.0	9.9	1	100
Araujiain h I	19	0.1	1.9	1.8	34.2	18.0	1.8	11
Araujiain h II	16.5	0.07	1.1	0.2	3.3	3.0	0.3	10
Araujiain h III	21	0.2	4.2	2.5	52.5	12.5	1.3	24

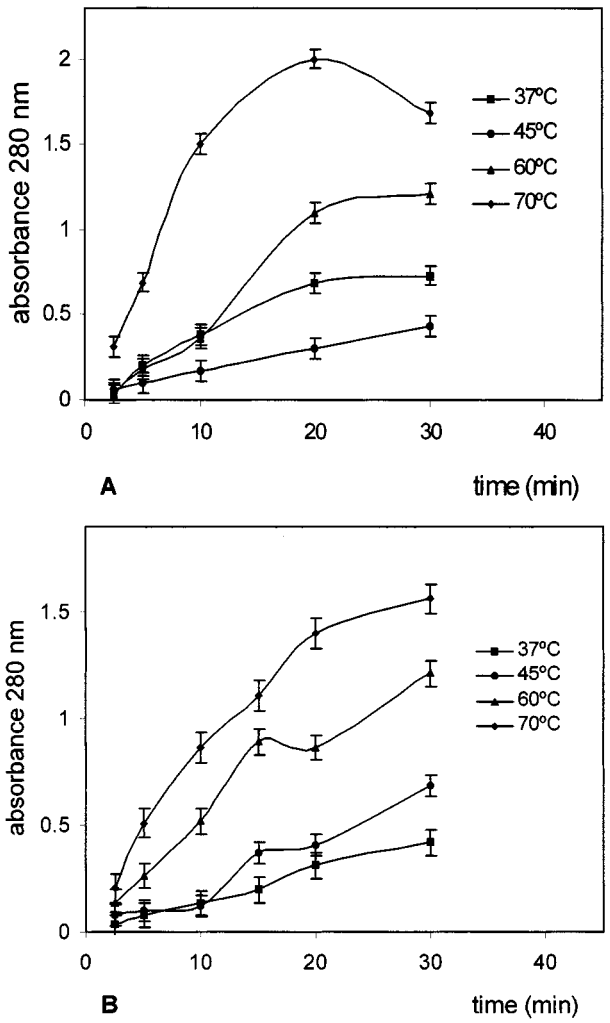

Fig. 3. Enzyme activity of (A) araujiain h II and (B) araujiain h III as a function of temperature.

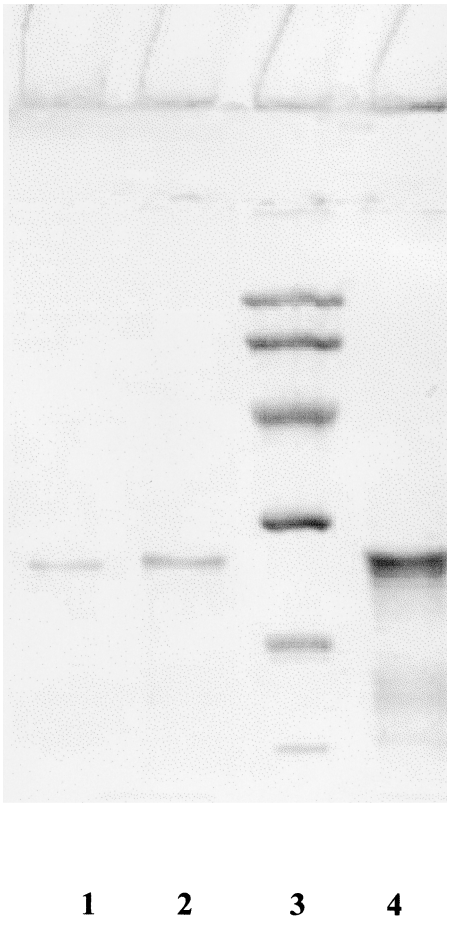

Fig. 4. SDS–PAGE. Lane 1, Araujiain h II; lane 2, araujiain h III; lane 3, molecular weight standards; lane 4, crude extract.

with those of other cysteine peptidases are shown in Tables IV and V.

4. DISCUSSION

The present paper describes the isolation, purification, and characterization of two new proteases, araujiain h II and araujiain h III, from the latex of *Araujia hortorum* fruits. Both were compared with araujiain h I, which we characterized and reported in a previous paper (Priolo *et al.,* 2000). The names araujiain h II and araujiain h III are proposed for the new proteases, according to nomenclature recommendations (Barragán *et al.,* 1985; Tablero *et al.,* 1991). Although araujiain h III showed the highest yield (24% of the total protein, 58% of the protease fraction), araujiain h I (11% of the total protein, 38% of the protease fraction) showed the highest specific activity.

As for araujiain h I, the optimum pH range for the two new proteases was 8.0–9.0 (casein); inhibition assays clearly demonstrated that they belong to the cysteine proteinase family. In general, heat inactivation curves showed that proteolytic activity increased with temperature up to 70°C. This behavior has been attributed to the protective role played by the substrate, which would avoid self-digestion of the studied proteases (Priolo *et al.,* 2000). The enzymes exhibited pI values higher than 8.8; this basic nature had been previously observed in 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). SDS–PAGE revealed that araujiain h II and araujiain h III appeared as unique bands, confirming their purity.

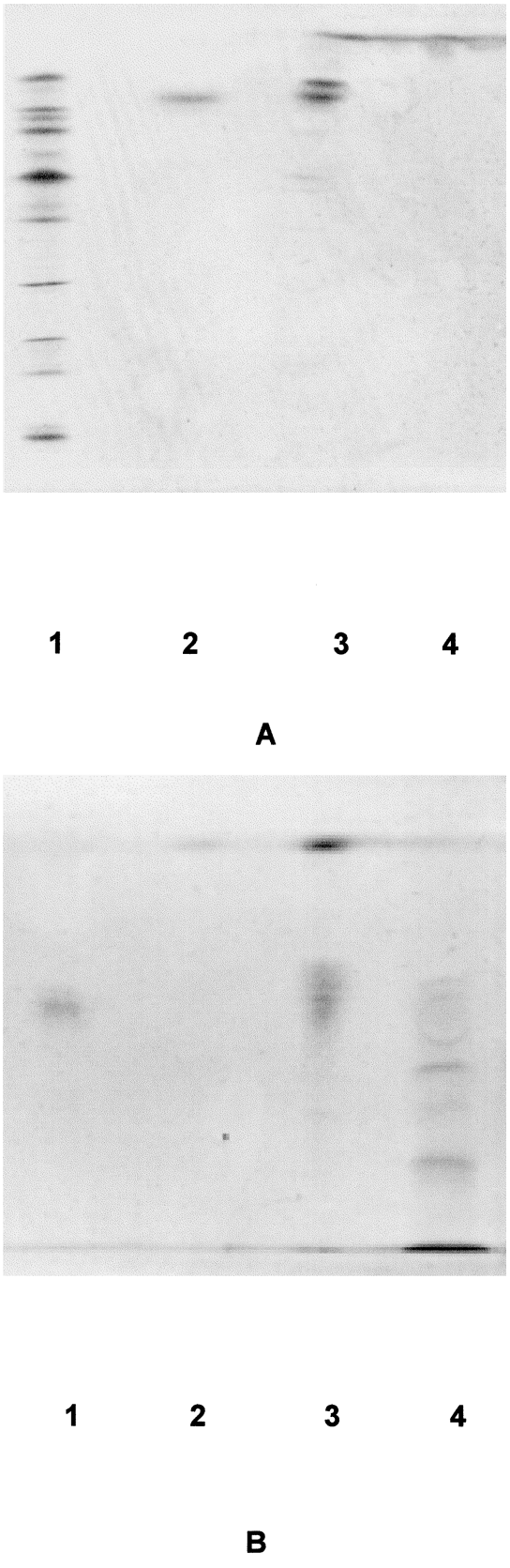

Fig. 5. (A) Isoelectric focusing, pH 3.0–10.0. Lane 1, pI standards (Bio Rad); lane 2, araujiain h II; lane 3, crude extract; lane 4, araujiain h III. (B) Isoelectric focusing, 8.0–10.5. Lane 1, Araujiain h II; lane 2, araujiain h III; lane 3, crude extract; lane 4, pI standards (Bio Rad).

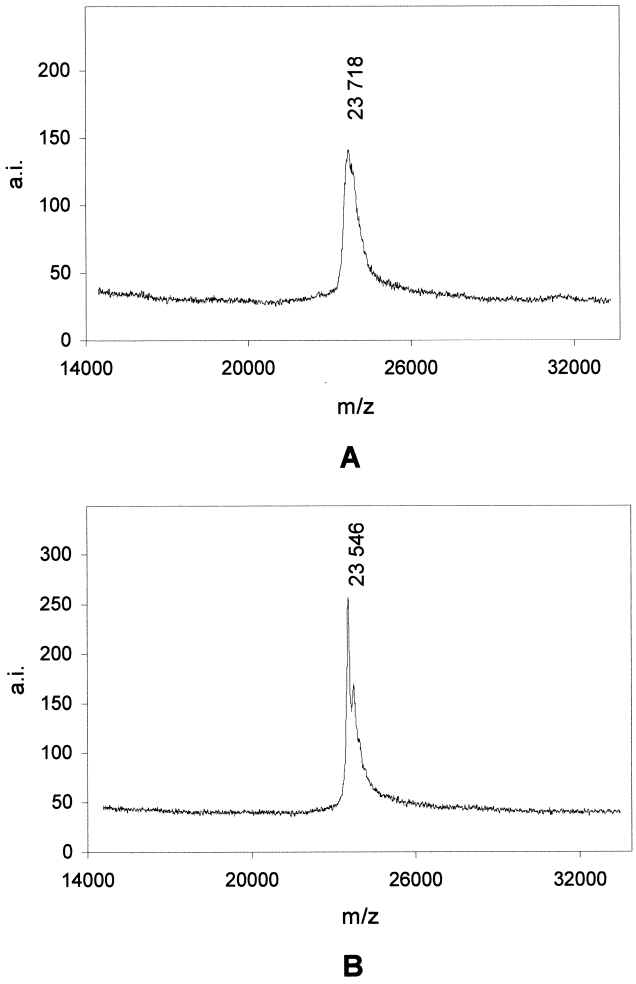

Fig. 6. Mass spectroscopy of (A) araujiain h II and (B) araujiain h III.

Table II. Comparative Endoesterolytic Activity (U_{CBZ}) of the Two Proteases

N-CBZ-amino acid p-nitrophenyl ester	Araujiain h-II		Araujiain h-III	
	U_{CBZ}	% preference	U_{CBZ}	%preference
Gln	6.06	100	72.38	100
Ala	2.84	57.89	29.45	41.66
Asp	2.84	57.89	71.61	98.96
Gly	1.08	31.58	56.34	78.12
Leu	1.47	36.84	25.62	36.46
Trp	0.70	26.32	29.45	41.66
Asn	1.08	31.58	21.78	31.25
Phe	1.08	31.58	25.62	36.46
Val	0.00	0.00	6.45	10.42
Pro	0.00	0.00	0.00	0.00
Ile	0.00	0.00	0.00	0.00

Table III. Kinetic Parameters of the Proteases

Sample	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
Araujiain h II	23.8×10^{-2}	223.08	9.37×10^5
Araujiain h III	9.9×10^{-2}	1132.08	1.15×10^2

Table IV. Sequence Analysis of Araujiain h II

Plant source/enzymes	N-Terminal sequence	Identities	References
Araulia hortorum	VPDS IDWREKDALP IRNQGQCGS IWAFXA IASVE		
araujiain h II		35/35 (100%)	
Carica papaya (chymopapain isoforms II, III, IV and V)	PQSIDWRAKGAVTPVKNQGACGSCWAFSTIATVE	23/34 (68%)	Taylor *et al.* (1999)
Carica candamacensis (cysteine proteinase III)	PESIDWRKKGAVTPVKNQGSCGSCWAFSTIATVE	23/34 (68%)	Walreavens *et al.* (1993)
Oryza sativa	LPESVDWREKGAVAPVKNQGQCGSCWAFSAVSTVE	23/35 (66%)	Watanabe *et al.* (1991)
Zingiber officinale	LPDSIDWRENGAVVPVKNQGGCGSCWAFSTVAAVE	23/35 (66%)	Choi *et al.* (1999)
Asclepias syriaca (asclepain a)	LPNSIDWPQKNVVEPIKNQG	13/20 (65%)	Lynn *et al.* (1980)
Carica candamarcensis syn. *Carica pubescens* (cysteine proteinase IV)	PESIDWRKKGAVTPVKNQGSCGSCWAFSTIVTVE	22/34 (65%)	Walreavens *et al*. (1993)
Pseudotsuga menziesii	LPESIDWREKGAVTAVKNQGSCGSCWAFSTVAAVE	22/35 (63%)	Tranbarger and Misra (1996)
Zinnia elegans	LPKSVDWRKKGAVSPVKNQGQCGSCWAFSTVAAVE	22/35 (63%)	Choi *et al.* (1999)
Ananas comosus	VPQSIDWRDSGAVTSVKNQGRCGSCWAFASIATVE	22/35 (63%)	Lee *et al.* (1997)
Carica papaya (papaya proteinase omega)	LPENVDWRKKGAVTPVRHQGSCGSCWAFSAVATVE	21/35 (60%)	Dubois *et al.* (1988)
Arabidopsis thaliana	LPKSVDWRKKGAVAPVKDQGQCGSCWAFSTVAAVE	21/35 (60%)	Zhao *et al.* (2000)
Araujia hortorum **(araujiain h III)**	LPESVDWRKKNLVFPVRNQGQCGSCXAFSAVAXI	20/34 (59%)	Present Paper
Asclepias syriaca (aslepain b)	LPNFVDWRKNGVVFPIRNQGQ	12/21 (57%)	Lynn *et al.* (1980)
Carica papaya (papaya proteinase I)	IPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIE	18/35 (51%)	Mitchel *et al.* (1970)

Mass spectroscopy showed that the molecular masses of both proteases were closely related (23546–23.718 kDa). Results are almost coincident with those for araujiain h I, with a molecular mass of 24.031 (Priolo *et al.,* 2000), as well as those obtained for other proteases from Asclepiadaceae: M_r of *Asclepias syriaca* proteases are 21.0 and 23.0 kDa (Brockbank and Lynn, 1979; Lynn *et al.,* 1980), proteases from *Asclepias glaucescens* have M_r of 23.0 kDa (Barragán *et al.,* 1985; Tablero *et al.,* 1991), the four calotropins isolated from *Calotropis gigantea* have molecular masses ranging from 23.0 to 27.0 kDa (Abraham and Joshi, 1979*a*, *b*; Pal and Sinha, 1980), two proteases isolated from *Morrenia brachystephana* have molecular masses of 25.5 and 26.0 kDa (Arribére *et al.,* 1998) and the proteases isolated from *Morrenia odorata* have M_r of 24.2 and 25.8 kDa (Arribére *et al.,* 1999).

Endoesterolytic activity of araujiain h II and araujiain h III, determined on N-α-CBZ-amino acid-*p*-nitrophenyl esters, exhibited different patterns, but in both cases higher preference was shown for the glutamine derivative. In the case of araujiain h II, the preference for the glutamine derivative is followed by those of alanine and aspartic acid. Esterolytic behavior of araujiain h III is quite different, as the enzyme showed almost similar preferences for the glutamine and aspartic acid derivatives, followed by the glycine derivative. On the basis of these results, kinetic parameters were calculated with the glutamine N-α-CBZ derivative for both proteinases. The k_{cat}/K_m ratio is considered the best kinetic quantity to express catalytic efficiency; according to this relation, the esterolytic activity of araujiain h III is 8.1 times higher than that of araujiain h II.

The N-terminus of araujiain h II (35 amino acids) and araujiain h III (34 amino acids) showed a high similarity with proteases from species of the *Asclepias* genus and considerable homology with Caricaceae cysteine proteases. Papain, considered as the archetype of cysteine peptidases, showed a remarkably degree of homology (55% and 64%) with araujiain h II and araujiain h III, respectively. Both proteinases shared three motifs (DWR, QG, and CGS) with most of the sequences analyzed. Nevertheless, they presented a different N-terminal amino acid (valine and leucine, respectively) and had only 53% of homology in their N-terminus sequences, which is in good agreement with the differences observed in the pI values as well as in the endoesterolytic patterns and in the K_m/k_{cat} ratios. As both enzymes are located in the same histological compartment, specific functions could be expected for each of them.

Table V. Sequence Analysis of Araujiain h III

Plant source/enzymes	N-Terminal sequence	Identities	References
***Araujia hortorum* (araujiain h III)**	L P E S V D W R K K N L V F P V R N Q G Q C G S C X A F S A V A X I	34/34 (100%)	
Zinnia elegans	L P K S V D W R K K G A V S P V K N Q G Q C G S C W A F S T V A A V	25/34 (74%)	Ye and Varner (1996)
Carica papaya (papaya proteinase omega)	L P E N V D W R K K G A V T P V R H Q G S C G S C W A F S A V A T V	25/34 (74%)	Dubois *et al.* (1988)
Asclepias syriaca (asclepain b)	L P N F V D W R K N G V V F P I R N Q G Q	15/21 (71%)	Lynn *et al.* (1980)
Arabidopsis thaliana	L P K S V D W R K K G A V A P V K D Q G Q C G S C W A F S T V A A V	24/34 (71%)	Zhao *et al.* (2000)
Oryza sativa	L P E S V D W R E K G A V A P V K N Q G Q C G S C W A F S A V S T V	24/34 (71%)	Watanabe *et al.* (1991)
Brassica napus	L P V S V D W R K K G A V T P I K D Q G L C G S C W A F S A V A A I	23/34 (68%)	Walreavens *et al.* (1993)
Dianthus caryophyllus	L P E S V D W R K K G A V S H V K D Q G Q C G S C W A F S A I G A V	23/34 (68%)	Jones *et al.* (1995)
Carica candamarcensis syn. *Carica pubescens* (Cysteine proteinase III)	P E S I D W R K K G A V T P V K N Q G S C G S C W A F S T I A T V	22/33 (67%)	Walreavens *et al.* (1993)
Asclepias syriaca (asclepain a)	L P N S I D W R Q K N V V F P I K N Q G	13/20 (65%)	Lynn *et al.* (1980)
Carica papaya (papaya proteinase I)	I P E Y V D W R Q K G A V T P V K N Q G S C G S C W A F S A V V T T I	22/34 (65%)	Mitchel *et al.* (1970)
Pseudotsuga menziesii	L P E S I D W R E K G A V T A V K N Q G S C G S C W A F S T V A A V	22/34 (65%)	Tranbarger and Misra (1996)
Carica candamarcensis syn. *Carica pubescens* (cysteine proteinase IV)	P E S I D W R K K G A V T P V K N Q G S C G S C W A F S T I V T V	21/33 (64%)	Walreavens *et al.* (1993)
Zingiber officinale	L P D S I D W R E N G A V V P V K N Q G G C G S C W A F S T V A A V	21/34 (62%)	Choi *et al.* (1999)
Carica pubescens	P E S I D W R K K G A V T P V K N Q G S X G S X W A F S T I V T V	20/33 (61%)	Walreavens *et al.* (1993)
***Araujia hortorum* (araujiain h II)**	V P D S I D W R E K D A V L P I R N Q G Q C G S I W A F X A I A S V E	18/34 (53%)	Present paper

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