Biochemical and PMF MALDI-TOF Analyses of Two Novel Papain-Like Plant Proteinases

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Abstract: Two cysteine endopeptidases from latex of *Araujia angustifolia* (araujiain aI and araujiain aIII) were purified and characterized by means of conventional and proteomics techniques (MALDI-TOF). N–terminal sequences showed a high percentage of identity with cysteine proteinases belonging to the papain family. The peptide mass fingerprint analysis demonstrated a close homology among both proteinases.

Keywords: Plant proteinases, *Asclepiadaceae*, *Araujia angustifolia*, peptide mass fingerprint, proteomics techniques.

1. INTRODUCTION

Latex is widely distributed in plants: more than 35,000 species have been reported to contain it [1]. The presence of latex is one of the characteristic features of *Asclepiadaceae*, *Apocynaceae*, *Caricaceae, Euphorbiaceae,* and *Moraceae* families [2]. Experimental evidence has shown that latex generally contributes to protect the plants against predators in both mechanical (by wound coagulation) and chemical (by the presence of toxic substances) manner [3].

The presence of hydrolases in latex, like chitinases and proteases, suggests that it may help plants defend against pathogens, parasites and herbivores by attacking the invader once the plant cell is lysed [4]. The diverse roles of plant proteases in defense responses triggered by pathogens or pests are becoming clearer. Some proteases, such as papain in latex, execute the attack on the invading organism.

Plant cysteine proteinases are extensively used in many industrial processes. They have been exploited commercially in food, leather and textile industries [5]. Pharmaceutical applications of these enzymes include the debridement of necrotic tissue from ulcers and burn wounds [6, 7].

Studies on proteases from *Asclepiadaceae* species growing in Argentina have been previously reported by our group: *Araujia hortorum* [8, 9], *Asclepias fruticosa* [10], *Morrenia brachystephana* [11-13], *M. odorata* [14], *Philibertia gilliesi* [15], *Funastrum clausum* [16, 17] and *Asclepias curassavica* [18]. In this paper the isolation and biochemical characterization of two cysteine endopeptidases found in the latex of *Araujia angustifolia* (Hook. *et* Arn.) Decaisne fruits are reported.

2. EXPERIMENTAL

2.1. Abbreviations Used

AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy- propanesulfonic acid; CAPS, 3-(ciclohexylamino)-1-propanesulfonic acid; Cbz, carbobenzoxy; CE, crude extract; CPs, cysteine peptidases; DMSO, dimethyl sulfoxide; DTT, DL-Dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA, ethylendiaminetetraacetic acid; FPLC, Fast Protein Liquid Chromatography; 4-HCCA, α-cyano-4-hydroxy-cinnamic acid; MES, 2-(N-morpholino) ethanesulfonic acid; MOPS, 3-(N-morpholino) propanesulfonic acid; PAGE, Polyacrylamide Gel Electrophoresis; PFLNA, L-pyro-glutamil-L-phenylalanyl-L-leucine-*p*-nitroanilide; PMF, peptide mass fingerprint; SDS, sodium dodecyl sulfate; SP-Sepharose, Sulfo Propyl-shepharose, TAPS, N-tris(hydroxyl-methyl)methyl-3-aminopropanesulfonic acid.

2.2. Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc., Cleveland, Ohio. AMPSO, CAPS, cysteine, DTT, E-64, EDTA, iodoacetic acid, MES, MOPS, *p*-nitrophenyl esters of *N*-α-Cbz-L-amino acids, TAPS and Tris were purchased from Sigma Chemical Company, St. Louis. Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide and low molecular weight markers were obtained from Bio-Rad, Hercules, California. SP-Sepharose and Sulfo propyl-sheparose Fast Flow were purchased from Pharmacia Biotech, Uppsala. All other chemicals were obtained from commercial sources and were of the highest purity available.

2.3. Crude Extract Preparation, Protein and Activity Determination

Latex obtained of fruits *Araujia angustifolia* [19] was gathered on 50 mM citric-citrate buffer pH 4.5, containing 5 mM EDTA and 5 mM cysteine, was centrifuged at 100,000×g for 60 min at 4ºC. This supernatant, containing soluble proteins, was called crude extract (CE). Proteins present in the CE and the purified proteases were determined by Bradford´s method [20]. Proteolytic assays were made using casein as susbtrate, and caseinolytic units (Ucas) were used to express enzymatic activity [21].

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0929-8665/09 $55.00+.00

2.4. Purification of Endopeptidases

The purification of the proteolytic components was carried out by cation exchange chromatography. One ml of the crude extract containing 1.5 mg of protein was loaded onto a Pharmacia XK 16/40 column having AK16 adaptors, packed with SP-Sepharose Fast Flow and equilibrated with 0.055 M Tris-HCl buffer pH 7.4. The chromatography was developed in an FPLC equipment (Pharmacia), by washing first with the equilibrating buffer (flow rate: 1 ml/min) and then eluting the bounded material with a NaCl broken gradient (125 ml of 0-0.5 M, 20 ml of 0.5-0.8 M and 30 ml of 0.8-1.0 M; flow rate 0.5 ml/min) in the same buffer. Cation exchange chromatography was monitored spectrophotometrically by absorbance measurement at 280 nm.

Identification of elution fractions having proteolytic activity was performed by measuring caseinolytic activity [21]. Protein and peptide content (Bradford and Biuret tests) was assayed on these fractions [20, 22].

2.5. Characterization of Purified Proteases

2.5.1. Gradient SDS-PAGE Analysis

The active fractions (inhibited and non inhibited with 50 mM sodium iodoacetate) [23] were submitted to denaturing SDS polyacrylamide gradient (10-16%) electrophoresis with a sucrose gradient (0-9%) to amplify the resolution. Electrophoresis was performed according to Shägger and von Jagow [24]. After the electrophoretic run, gels were stained with Coomassie Brilliant Blue R- 250. Silver staining was also performed to improve the visualization of protein bands [25].

2.5.2. Isoelectric Focusing and Zymogram

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, USA) in a Mini IEF Cell (Model 111, Bio-Rad) as described by Obregón *et al*. [19]. The resulting gels were then fixed and stained with Coomassie Brilliant Blue R-250. Zymogram was performed to confirm the proteolytic activity of the protein bands according to Obregón *et al.* [19].

2.5.3. Mass Spectrometry Analyses

Matrix–Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI–TOF MS) was used for the determination of the molecular masses, as well as the degree of purity of active chromatographic fractions. MALDI-TOF mass spectra were acquired on a BRUKER Ultraflex 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 - sinapinic 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.

2.5.4. Determination of N-Terminal Sequence

The purified proteases were adsorbed onto a PVDF membrane (Millipore) and washed several times with deionized water. The N-terminal sequence was determined by Edman's automated degradation using an Applied Biosystems 492 Procise protein sequencer equipped with a PTH-amino acid analyzer Series 200 (Perkin Elmer). Protein homology searches were performed using the BLAST network service [26] (http://merops.sanger.ac.uk/ [27]), indicating the specific residues which are identical ("identities"), as well as those which are non-identical but having positive alignment scores ("positives").

2.5.5. Determination of Catalytic Type

The action of different inhibitors of cysteine proteases (50 mM sodium iodoacetate, 50 mM idoacetamide and 100 µM E-64) was evaluated by incubating the purified enzymes for 30 min at 25ºC [23]. The residual caseinolytic activity after each incubation assay was measured as indicated above [19].

2.5.6. Optimum pH

The effect of pH on enzyme activity of the two peptidases was measured with casein at 37º C in the pH range from 6.5 to 10.0 using 10 mM sodium salts of the "Good" buffers [28]: MES, MOPS, TAPS, AMPSO and CAPS.

2.5.7. Activity Toward Synthetic Substrates

2.5.7.1. Determination of Esterolytic Activity

These assays were carried out by the Silverstein´s method [29], modified according to optimal conditions of each enzyme. Preference for *N*-α-Cbz-*p*-nitrophenyl esters of some amino acids (Gln, Ala, Asp, Phe, Asn, Tyr, Gly, Leu, Val, and Pro) was determined. Assays were made at 40ºC in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 mM EDTA and 25 µM cysteine [17]. Determination of the µmoles of *p*-nitrophenolate produced during the reaction was achieved by performing a standard curve (15-70 µM *p*-nitrophenol).

2.5.7.2. Enzyme Kinetics Using N-Cbz aa p-Nitrophenyl Derivatives

Enzyme kinetics were followed with the most preferred *N*-Cbz-aa *p*-nitrophenyl derivative determined in the aforementioned assays for each enzyme. Substrate concentrations were in the range of 0.1-1 mM in the reaction mixture. Measures were made as described in ***2. 5. 6. 1.***

2.5.7.3. Determination of Kinetic Parameters Using PFLNA as Substrate

Amidasic activity was proved on PFLNA according to the method of Filippova *et al*. [30] with minor modifications [17]. The reaction mixture contained 1.5 ml of 0.1 M phosphate buffer pH 6.5 with 0.3 M KCl, 4×10^{-4} M EDTA, and 3×10^{-3} M DTT, 0.18 ml of substrate (from a 4 mM PFLNA stock solution in DMSO) and 0.12 ml of the enzyme (30 µg/ml). The *p*-nitroaniline released at 37ºC was detected spectrophotometrically at 410 nm. An arbitrary enzyme activity unit (U_{PFLNA}) was defined as the amount of protease that released one micromol of *p*-nitroaniline per minute in the assay conditions. Kinetic parameters using PFLNA as substrate were determined for each enzyme.

2.5.8. PMF by MALDI-TOF MS

Peptide mass fingerprint (PMF) is an analytical technique for protein identification. A key feature for PMF is that the genome of the organism has to be known and available. Briefly, the unknown protein of interest is cut into peptides by an enzyme (trypsin is one of the most used) which doesn't create too many peptides. The absolute masses (still unknown) of these peptides are determined by mass spectrometer (e.g. MALDI-TOF). The great advantage of this method for protein identification is that only the masses of the peptides have to be known. The main drawback is that the authentic protein sequence (or close homologue) must be present in the available databases.

Proteins were separated by SDS-PAGE and visualized by staining the gels according to the colloidal Coomassie method [31]. The selected bands were cut, washed with milli Q water and acetonitrile several times to remove the dye, and dried under vacuum. The gel fragments were covered with a solution of 100 mM NH_4HCO_3 containing 10 mM DTT for 30 min at 37ºC, then centrifuged and washed with acetonitrile for 5 min. The fragments were incubated in a solution of 100 mM NH_4HCO_3 containing 50 mM iodoacetamide for 20 min at room temperature in darkness, and washed with milli Q water and acetonitrile alternatively. The tryptic digestion was achieved by using a NH_4HCO_3 100 mM pH 8.5 buffer containing trypsin (4 ng/µl) for 12 h at 37ºC. The resulting peptides were recovered by extraction with 50% (v/v) acetonitrile and then dried in a SpeedVac, redissolved in 0.1% (v/v) TFA and analyzed by MALDI-TOF MS using 4-HCCA as matrix.

Identification of the tryptic map was tried by searching with the Mascot tool (http://www.matrixscience.com/. Accessed online: 04/29/2008) according to the following search parameters: (1) Type of search, Peptide Mass Fingerprint; (2) Enzyme, trypsin; (3) Database, SwissProt 55.2; (4) Taxonomy, Viridiplantae; (5) Variable modifications, Carbamidomethyl (C), Oxidation (M); (6) Mass values, Monoisotopic; (7) Protein Mass, 24 kDa; (8) Peptide Mass Tolerance: ± 1000 ppm; (9) Peptide Charge State, 1+. Probability Based Mowse Score: Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 56 are significant ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1. Purification of Araujiains aI and aIII

Purification of peptidases from CE by afforded seven fractions Fig. (**1**).

Fractions I, II and III displayed high proteolytic activity, which they were named araujiain aI, aII and aIII, respectively. The purification scheme consisted of a simple chromatographic procedure. Table **1** shows the obtained results.

Homogeneity of araujiains aI and aIII was confirmed by means of SDS-PAGE gradient gel with sucrose. This was revealed with silver staining (Fig. **2**), where it can also be seen that the purified fractions are not susceptible to autodigestion.

Molecular masses of the purified proteases, estimated by gradient SDS-PAGE, are about 23.5 kDa, which are of the same order of those obtained for other proteases from *Asclepiadaceae* [12-14]. The results obtained by SDS-PAGE were confirmed by mass spectrometry (MALDI-TOF): molecular masses for araujiain aI and aIII were 23464.7 Da and 23488.8 Da, respectively Figs. (**3A** and **B**). The marked difference in mobility observed by gradient SDS-PAGE between araujiains aI and aIII (Fig. **2**) could be due to some inaccuracy of this technique. Sizing accuracy may vary for both proteins, owning to their characteristics, such as differences in amino acid composition, isoelectric point, etc. The sizing precision that can be achieved by SDS-PAGE is approximately 2-7% [32].

IEF and zymogram analysis of both purified enzymes showed they had pI values of 8.5 (araujiain aI) and higher than 9.3 (araujiain aIII) (data not shown). This basic nature was also observed in the proteases isolated from the latex of other *Asclepiadaceae* [15, 33].

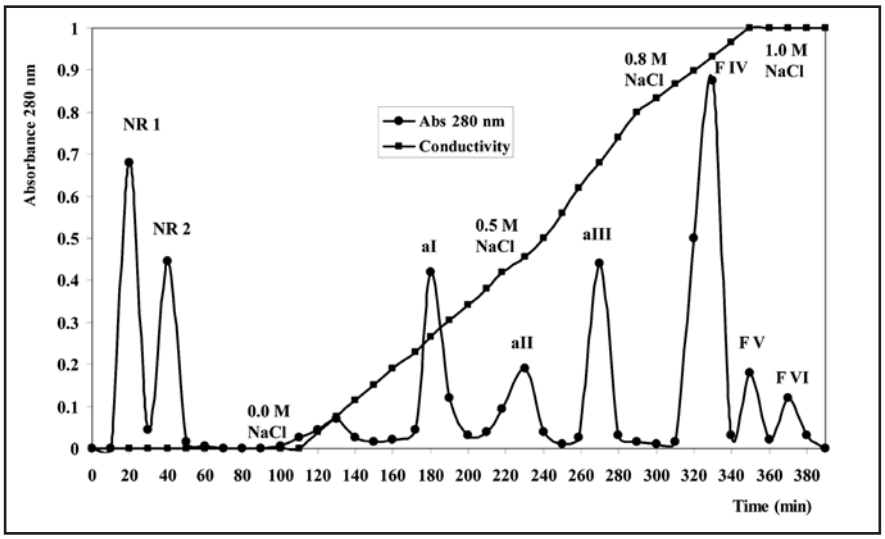


Figure 1. Chromatographic profile (cation exchange) of *Araujia angustifolia* latex CE.

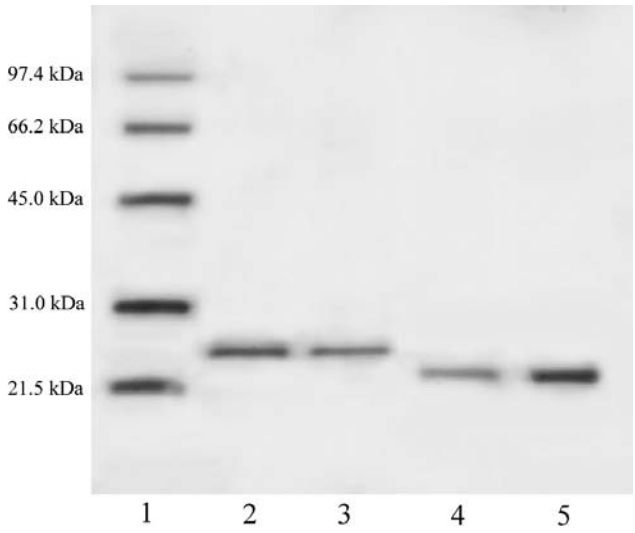


Figure 2. Gradient SDS-PAGE (10-16%), silver staining. **Lane 1**: Bio Rad Molecular weight markers: Phosphorylase b, 97.4 kDa; Serum albumin, 66.2 kDa; Ovalbumin, 45.0 kDa; Carbonic anhidrase, 31.0 kDa; Trypsin inhibitor, 21.5 kDa; and Lysozyme, 14.4 kDa. **Lanes 2** and **4**: araujiain aI and aIII, respectively; **Lanes 3** and **5**: araujiain aI and aIII inhibited with sodium iodoacetate, respectively.

3.2. Characterization of the Two Endopeptidases

3.2.1. Effect of pH and Specific Inhibitors on Proteolytic Activity

The effect of pH on the proteolytic activity is shown in Figs. (**4A** and **B**). Optimum pH (higher than 80% of maximum activity) for araujiain aI and aIII was comprised in the range of 9.2-9.7, and 6.8-9.6, respectively. The restricted profile of caseinolytic activity for araujiain aI showed a strong contrast with that of araujiain aIII, which was wider.

Different specific inhibitors of cysteine proteases (50 mM sodium iodoacetate, 50 mM iodoacetamide and 100 μM E-64) inhibited totally and irreversibly the enzyme activity in all cases.

3.2.2. Determination of Esterolytic Activity Toward N-α-Cbz-aa p-Nitropheyl Ester Derivatives

Araujiain aI showed a great preference for the Ala derivative, followed by those of Phe, Gln and Asp, which were hydrolyzed in a much lesser extent Figs. (**5A** and **B**). As araujiain aI, araujiain aIII demonstrated higher affinity for *N*-α-Cbz-Ala *p*-nitrophenyl ester; however, the enzyme showed only 45% of preference for the following derivatives, Gln and Asp. This behavior was also observed for other cysteine peptidases purified from the latex of plants belonging to the *Asclepiadaceae* family. When kinetic studies were performed with the most preferred substrate for each peptidase, they did not show a Michaelian behavior.

3.2.3. Determination of Kinetic Parameters Using PFLNA as Substrate

As a Michaelian behavior was observed for araujiain aIII using this substrate, their kinetic parameters were calculated by a linear regression analysis by means of Hanes and Hofstee equations. In the conditions of the test, araujiain aI showed no activity on PFLNA. The Km obtained for araujiain aIII was 5.14 mM, much higher than those obtained for papain (0.34 mM), bromelain (0.30 mM) and ficin (0.43 mM) [31]. The relation kcat/Km calculated was 2.38 seg^{-1} mM^{-1}. These results showed a low affinity for this substrate.

3.2.4. N-Terminal Sequence Analysis

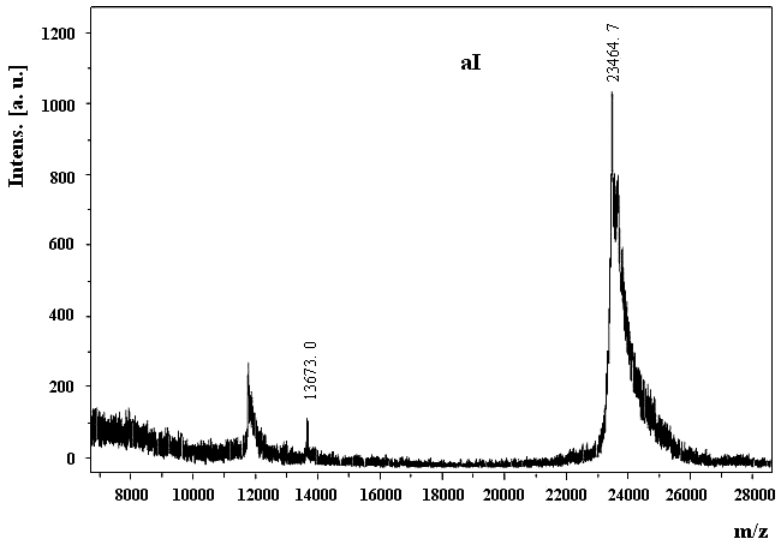
The comparison of the N-terminus of the two araujiains with similar sequences of other proteases is shown in Tables **2** and **3**. The two N-terminus sequences were analyzed with the BLAST network [26, 27]: according to this analysis, the N-terminus of the both enzymes showed high similarity with other plant cysteine proteases, and especially with those obtained from species belonging to the *Asclepias* genus. Considerable homology with *Caricaceae* cysteine peptidases was also noted: caricain and cysteine proteinase CC-III showed 86% and 85% of homology for araujiain aI, whereas caricain and papain showed 83% and 79% of homology with araujiain aIII. Papain, considered as the archetype of cysteine peptidases, showed a remarkably degree of homology with araujiain aI and aIII (81% and 79% respectively). The conserved motifs DWR, QG, as well as the residues K, V, P are clearly distinguished for the two proteinases isolated from *A. angustifolia* latex: these motifs are shared with papain, as can be seen in their N- terminal sequences, and many other papain-like endopeptidases. Interestingly, Pro2 is often conserved in the mature peptidases of family C1, and it is suggested that this prevents attack by aminopeptidases, since the Xaa-Pro bonds are inaccessible for this type of enzymes [34]. The N-terminal aminoacid of araujiain aI was valine, whereas aIII had leucine. The homology between araujiain aI and araujiain aIII was 79% [35] (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). A 73% of homology was found among the two proteases N-terminal sequences.

All the studied features (pI values, optimum pH, endoesterolytic patterns as well as kinetic values) indicate that both proteases belong to the cysteine peptidases (CPs)

Table 1. Purification of the Proteolytic Components Present in the Latex of *Araujia angustifolia*

Sample	Vol/ml	Protein mg/ml	Total Proteins (mg)	U_{CAS}/ml	Total U_{CAS}	Specific Activity (U_{CAS}/mg)	Purification (Fold)	Yield (%)
CE	2	2.233	4.466	8.52	17.04	3.8	-	-
Araujian aI	6	0.098	0.586	0.19	1.14	1.9	0.50	13.0
Araujiain aIII	8	0.176	1.405	0.49	3.92	2.7	0.71	31.2

A)



B)

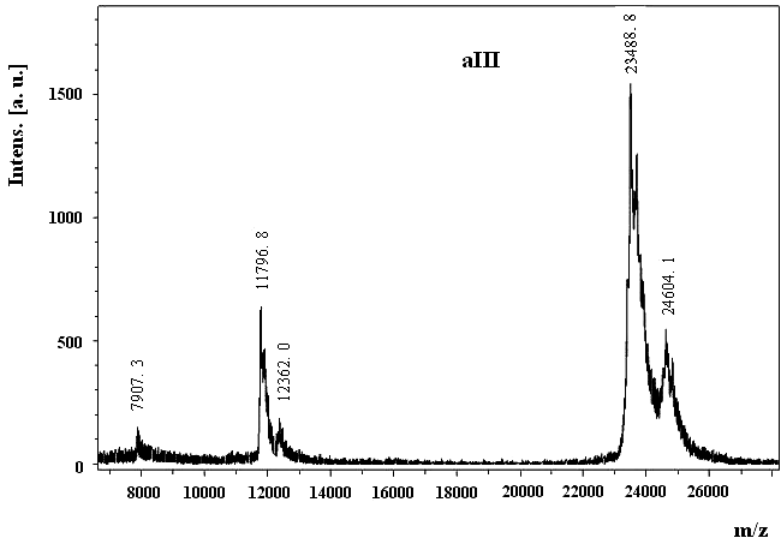


Figure 3. Mass spectra (MALDI-TOF) of araujiain aI (**A**), and araujiain aIII (**B**).

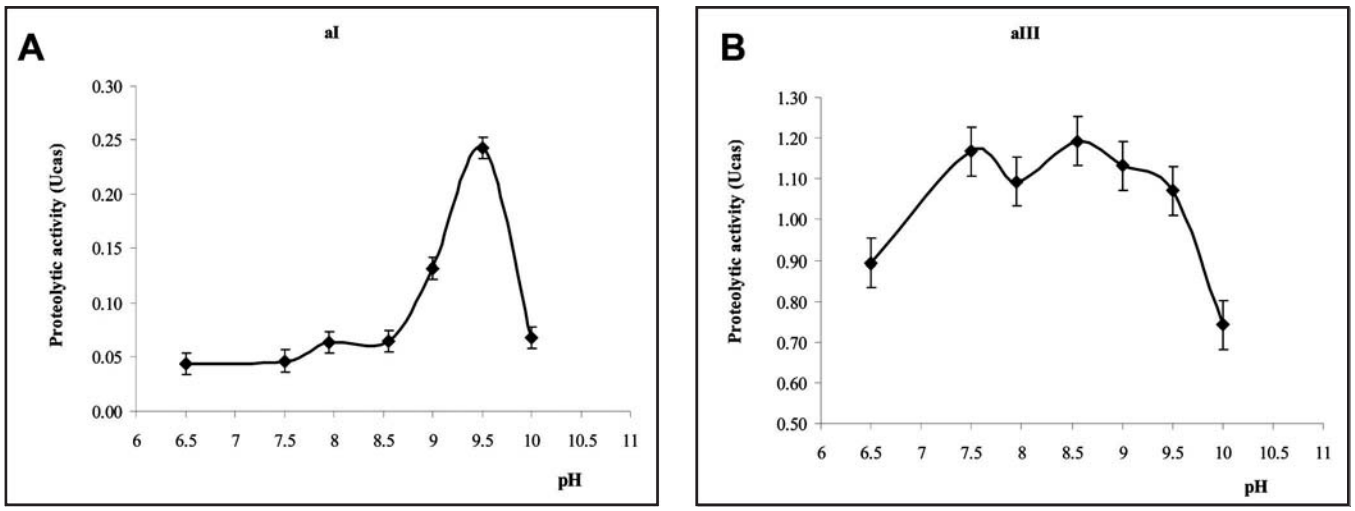


Figure 4. Effect of pH on proteolytic activity of the three endopeptidases: **A**, araujiain aI; **B**, araujiain aIII.

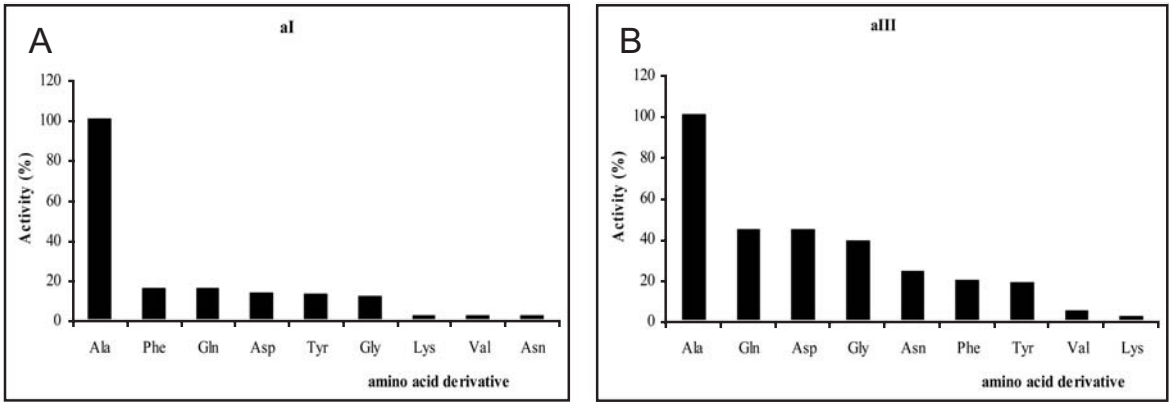


Figure 5. Comparative endoesterolytic activity (U_{cbz}) of the araujiains aI (**A**) and aIII (**B**) with different *N*-α-Cbz-amino acid *p*-nitrophenyl esters derivatives.

Table 2. Sequence Analysis of Araujiain aI

Protease / *Plant Source*	N-terminal Sequence	Identities	Positives
Araujiain aI/*Araujia angustifolia*	1 VPDSIDWREKDAVLPIRNQGQC 22		
Araujiain hII/*Araujia hortorum*	1 VPDSIDWREKDA-LPIRNQGQC 21 VPDSIDWREKDA LPIRNQGQC	21/22 (95%)	21/22 (95%)
Asclepain f/*Asclepias fruticosa*	2 PDSVDWREKGVVFPIRNQGKC 22 PDS+DWREK V PIRNQG+C	16/21 (76%)	18/21 (85%)
RD21A-like cysteine protease/*Triticum aestivum*	2 LPESIDWREKGAVAPVKNQGQC 23 +P+SIDWREK AV P++NQGQC	16/22 (72%)	20/22 (90%)
Asclepain cII/*Asclepias curassavica*	5 VDWRQKGVVFPIRNQGQC 22 +DWR+K V PIRNQGQC	13/18 (72%)	15/18 (83%)
Cysteine proteinase GP-I/*Zingiber officinale*	3 LPDSIDWREKGAVVPVKNQGGC 24 +PDSIDWREK AV+P++NQG C	16/22 (72%)	20/22 (90%)
Araujian aIII/*Araujia angustifolia*	2 PESVDWRKKNLVFPIRNQGQC 22 P+S+DWR+K+ V PIRNQGQC	15/21 (71%)	19/21 (90%)
Morrenain oII/*Morrenia odorata*	2 PESVDWRKKNLVFPIRNQGQC 22 P+S+DWR+K+ V PIRNQGQC	15/21 (71%)	19/21 (90%)
Morrenain bII/*Morrenia brachystephana*	2 PDSVDWRKKNLVFPVRNQG 20 PDS+DWR+K+ V P+RNQG	13/19 (68%)	17/19 (89%)
Philibertain g 1/*Philibertia giliesii*	1 LPASVDWRKEGAVLPIRHQGQC 22 +P S+DWR++ AVLPIR+QGQC	15/22 (68%)	20/22 (90%)
Cysteine proteinase GP-II/*Zingiber officinale*	3 LPDSIDWRENGAVVPVKNQGGC 24 +PDSIDWRE AV+P++NQG C	15/22 (68%)	19/22 (86%)
Araujian hIII/*Araujia hortorum*	2 PESVDWRKKNLVFPVRNQGQC 22 P+S+DWR+K+ V P+RNQGQC	14/21 (66%)	19/21 (90%)
Cysteine proteinase CC-III/*Vasconcellea cundinamarcensis*	2 PESIDWRKKGAVTPVKNQGSC 22 P+SIDWR+K AV P++NQG C	14/21 (66%)	18/21 (85%)
Chymopapain/*Carica papaya*	2 PQSIDWRAKGAVTPVKNQGAC 22 P SIDWR K AV P++NQG C	14/21 (66%)	16/21 (76%)
Mexicain/*Jacaratia mexicana*	2 PESIDWREKGAVTPVKNQNPC 22 P+SIDWREK AV P++NQ C	14/21 (66%)	17/21 (80%)

(Table 2) contd….

Protease / *Plant Source*	N-terminal Sequence	Identities	Positives
Asclepain cI/*Asclepias curassavica*	2 PNSVDWRQKGVVFPIRDQGKC 22 P+S+DWR+K V PIR+QG+C	13/21 (61%)	18/21 (85%)
Chymomexicain/*Jacaratia mexicana*	2 PESIDWRDKGAVTPVKNQNPC 22 P+SIDWR+K AV P++NQ C	13/21 (61%)	17/21 (80%)
Funastrain cII/*Funastrum clausum*	2 PNSVDWRQKGVVSAIRNQGKC 22 P+S+DWR+K V IRNQG+C	13/21 (61%)	17/21 (80%)
Macrodontain-1/*Pseudonanas macrodontes*	2 VPQSIDWRDYGAVNEVKNQGPC 23 VP SIDWR+ AV ++NQG C	13/22 (59%)	16/22 (72%)
Ervatamin-C (ERV-C)/*Tabernaemontana divaricata*	1 LPEQIDWRKKGAVTPVKNQGSC 22 +P+ IDWR+K AV P++NQG C	13/22 (59%)	18/22 (81%)
Cysteine proteinase/*Vasconcellea cundinamarcensis*	2 PQRMDWRKKGAVTPVKNQGGC 22 P +DWR+K AV P++NQG C	12/21 (57%)	16/21 (76%)
Papain/*Carica papaya*	1 IPEYVDWRQKGAVTPVKNQGSC 22 +P+ +DWR+K AV P++NQG C	12/22 (54%)	18/22 (81%)
Caricain/*Carica papaya*	1 LPENVDWRKKGAVTPVRHQGSC 22 +P+++DWR+K AV P+R+QG C	12/22 (54%)	19/22 (86%)

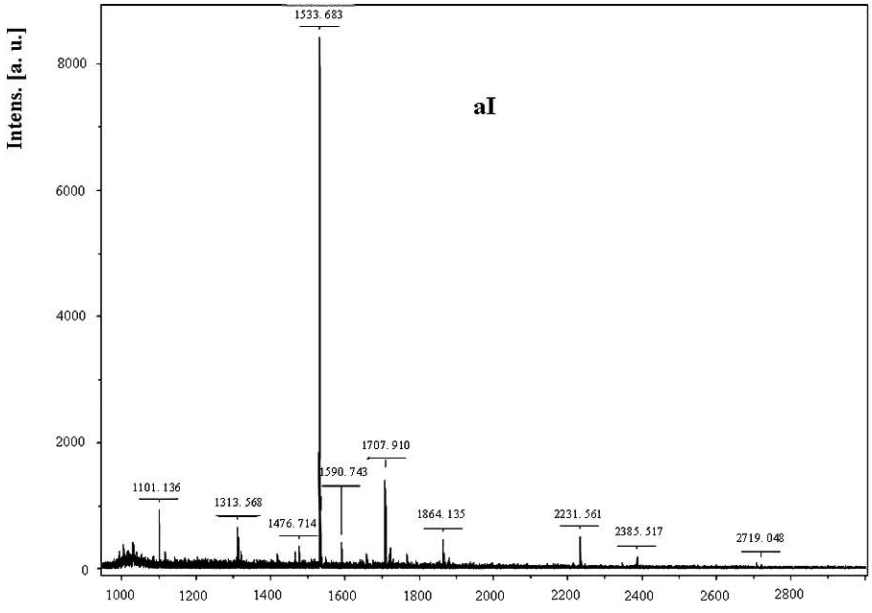
Table 3. Sequence Analysis of Araujiain aIII

Protease / *Plant Source*	N-Terminal Sequence	Identities	Positives
Araujiain aIII/*Araujia angustifolia*	1 LPESVDWRKKNLVFPIRNQGQCGS 24		
Asclepain f/*Asclepias fruticosa*	2 PDSVDWREKGVVFPIRNQGKCGS 24 PDS+DWREK V PIRNQG+CGS	18/23 (78%)	20/23 (86%)
Asclepain cII/*Asclepias curassavica*	5 VDWRQKGVVFPIRNQGQCGS 24 +DWR+K V PIRNQGQCGS	15/20 (75%)	17/20 (85%)
Araujian hII/*Araujia hortorum*	2 PDSIDWREKDAL--PIRNQGQCGS 23 P+S+DWR+K+ L PIRNQGQCGS	17/24 (70%)	21/24 (87%)
RD21A-like cysteine protease/*Triticum aestivum*	2 LPESIDWREKGAVAPVKNQGQCGS 25 LPES+DWR+K V P++NQGQCGS	17/24 (70%)	21/24 (87%)
Araujian hIII/*Araujia hortorum*	2 PESVDWRKKNLVFPVRNQGQC 22 P+S+DWR+K+ V P+RNQGQC	14/21 (66%)	19/21 (90%)
Cysteine proteinase CC III/*Vasconcellea cundinamarcensis*	2 PESIDWRKKGAVTPVKNQGSCGS 24 PES+DWRKK V P++NQG CGS	16/23 (69%)	19/23 (82%)
Ervatamin-C (ERV-C)/*Tabernaemontana divaricata*	1 LPEQIDWRKKGAVTPVKNQGSCGS 24 LPE +DWRKK V P++NQG CGS	16/24 (66%)	19/24 (79%)
Morrenain bII/*Morrenia brachystephana*	2 PDSVDWRKKNLVFPVRNQG 20 PDS+DWR+K+ V P+RNQG	13/19 (68%)	17/19 (89%)
Protease Omega/*Carica papaya*	1 LPENVDWRKKGAVTPVRHQGSCGS 24 LPE+VDWRKK V P+R+QG CGS	17/24 (70%)	20/24 (83%)
Caricain/*Carica papaya*	1 LPENVDWRKKGAVTPVRHQGSCGS 24 LPE+VDWRKK V P+R+QG CGS	17/24 (70%)	20/24 (83%)
Asclepain cI/*Asclepias curassavica*	2 PNSVDWRQKGVVFPIRDQGKCGS 24 P+S+DWR+K V PIR+QG+CGS	15/23 (65%)	20/23 (86%)

(Table 3) contd….

Protease / *Plant Source*	N-Terminal Sequence	Identities	Positives
Funastrain cII/*Funastrum clausum*	2 PNSVDWRQKGVVSAIRNQGKCGS 24 P+S+DWR+K V IRNQG+CGS	15/23 (65%)	19/23 (82%)
Morrenain oII/*Morrenia odorata*	2 PDSVDWRKKNLVFPVRNQGKXGS 24 PDS+DWR+K+ V P+RNQG+ GS	15/23 (65%)	20/23 (86%)
Cysteine proteinase GP-I/*Zingiber officinale*	3 LPDSIDWREKGAVVPVKNQGGCGS 26 LP+S+DWR+K V P++NQG CGS	15/24 (62%)	20/24 (83%)
Papain/*Carica papaya*	1 IPEYVDWRQKGAVTPVKNQGSCGS 24 +PE VDWR+K V P++NQG CGS	15/24 (62%)	19/24 (79%)

A)



B)

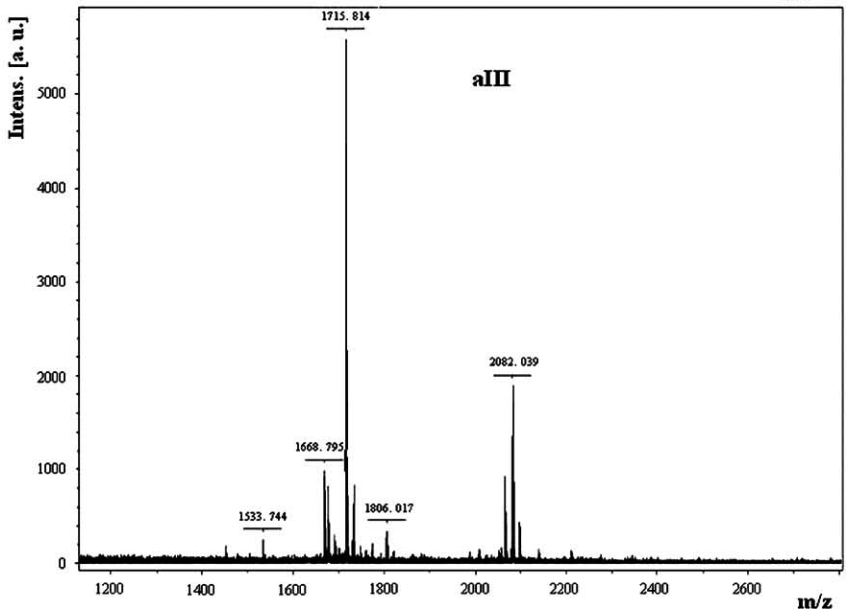


Figure 6. Peptide mass fingerprint of araujiain aI (**A**), and araujiain aIII (**B**).

group, subfamily C1A (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 proteinase. As the two enzymes are located in the same histological compartment, specific functions could not be expected for each of them.

3.2.5. Peptide Mass Fingerprint

It is well known that plant cysteine proteinases are present in multiple enzyme forms, as those found in the latexes of *Carica papaya*, *Vasconcellea* spp., *Ficus* spp. and in the stem of *Ananas comosus* [3, 6]. Chymopapain is present in at least five isoforms, whereas there are two forms of caricain [3]. *Vasconcellea cundinamarcensis* latex has six or seven proteinases, some of which could be isoforms [36]. All these multiple enzyme forms were first detected by their heterogeneous chromatographic profiles [6, 37]; some of these isoforms were confirmed by cDNA analyses [38, 39]. Although all the biochemical findings would indicate that araujiains aI and aIII are not multiple enzyme forms of the same enzyme, PMF MALDI-TOF analysis could give stronger evidence to substain this idea. Protein identification by PMF has shown to be an excellent tool to differentiate in a fast and unequivocal way proteases with very similar physicochemical and functional properties. In this sense PMF takes advantage on other conventional methods (for instance, enzyme kinetics) that are time-consuming and afford less reliable results.

The peptide mass fingerprint analyses of araujiains aI and aIII (Figs. **6A** and **B**) showed they have equivalent but also different peptides (due to their different amino acid sequence as well as potential posttranslational modifications) (Tables **4** and **5**) which demonstrated that they are different enzymes but sharing a high degree of homology. The presence of coincident peptide masses between studied enzymes would reflect the presence of conserved domains, which are unique for the species, and perhaps many other domains of other cysteine proteases belonging to the *Asclepiadaceae*, *Caricaceae* and *Bromeliaceae* families. On the other hand, the existence of non coincident peptide masses would demonstrate that they constitute different enzymes, with the same biological function and sharing similar biochemical features. Searches using the Mascot tool [40] were made in order to

Table 4. PMF Numeric Results for Araujiain aI Calculated by the Bruker Daltonics Flex Analysis 2.2 Software

m/z	SN	Quality Fac.	Res.	Intens.	Area
1101.282	17.1	2563	2508	755.47	700
1313.676	17.7	1711	11577	637.37	168
1476.768	10.1	1145	10980	336.65	114
1533.712	259.5	88126	9818	8784.17	3950
1590.745	12.6	1449	10190	406.64	176
1707.849	45.9	23504	9717	1498.74	787
1721.882	11.4	10118	8454	306.00	79
1863.974	15.2	2326	9375	417.24	255
2231.092	22.7	2919	9290	408.85	320

Table 5. PMF Numeric Results for Araujiain aIII Calculated by the Bruker Daltonics Flex Analysis 2.2 Software

m/z	SN	Quality Fac.	Res.	Intens.	Area
1533.747	9.7	1183	11786	232.36	72
1667.809	17.1	14399	12416	375.00	67
1677.905	26.3	7224	13811	796.88	300
1715.816	149.0	1606	11536	5011.67	2258
1731.822	20.5	255	15288	661.92	251
1806.018	11.4	1565	15122	283.35	113
2065.014	34.5	13570	12943	768.48	395
2082.038	73.1	1926	12336	1616.65	930
2096.049	15.4	482	13579	339.86	193

identify studied proteins with these tryptic maps (Figs. **7A** and **B**), but no identification was possible due to the absence of enough plant cysteine proteinases in the available databases.

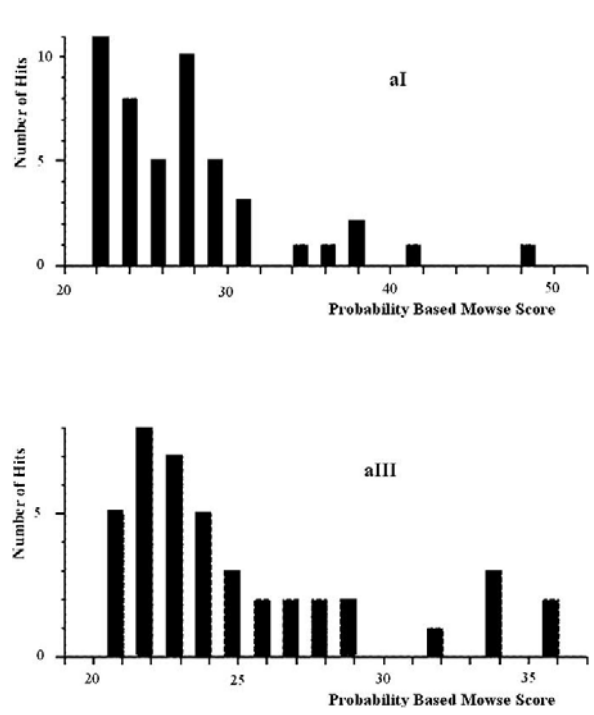


Figure 7. Mascot tool search results for (**A**) araujiain aI and (**B**) araujiain aIII.

A close homology between araujiains aI and aIII was found by PMF, which supported the results obtained by other conventional methods. The robustness of the PMF method, together with the aforementioned results strongly suggests the presumption that the studied peptidases could be isoenzymes. Nevertheless, the complete amino acid sequence of the two isolated endopeptidases, as well as deeper studies of the genes codifying for them are needed to confirm this idea [41].

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