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Short Communication

Comparison of Two Cysteine Endopeptidases from Latices of *Morrenia brachystephana* Griseb. and *Morrenia odorata* (Hook *et* Arn.) Lindley (Asclepiadaceae)

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The properties of morrenain b II, a proteinase isolated from the latex of *Morrenia brachystephana*, were compared with those of morrenain o II, a proteinase obtained from the latex of *Morrenia odorata*. Both peptidases were purified to homogeneity by acetone precipitation followed by cation exchange chromatography. The enzymes have pI values higher than 9.3 and similar molecular masses (close to 26 kDa) as determined by SDS-PAGE. They display maximum proteolytic activity within an alkaline pH range, and also exhibit esterolytic activity. The N-terminal sequences of morrenain o II and morrenain b II show a high degree of homology between each other and to other cysteine plant proteinases.

Key words: Latex peptidases / Morrenain / Protein purification / Thiol peptidases.

Proteolytic enzymes have important functions in plant physiology. They are involved in various processes like protein turnover, leaf senescence, breakdown of storage proteins, and regulatory mechanisms. Some plant species possess extremely high endopeptidase activity in certain tissues (fruits, germinating seeds, laticifers). These abundant proteinases might have no function in growth and development, but potential roles in the defense against pathogens and parasites (Feller, 1986) and in latex clotting (Moutim *et al.*, 1999). Knowledge of the biochemical properties of these enzymes could help to learn about their specific functions.

Papain is by far the best studied cysteine proteinase. Most plant cysteine peptidases belong to the papain family, including those of Asclepiadaceae. The present report describes the isolation and characterization of two proteases present in the latex of two species belonging to the genus *Morrenia* (Asclepiadaceae): *M. brachystephana* Griseb. and *M. odorata* (Hook *et* Arn.) Lindley.

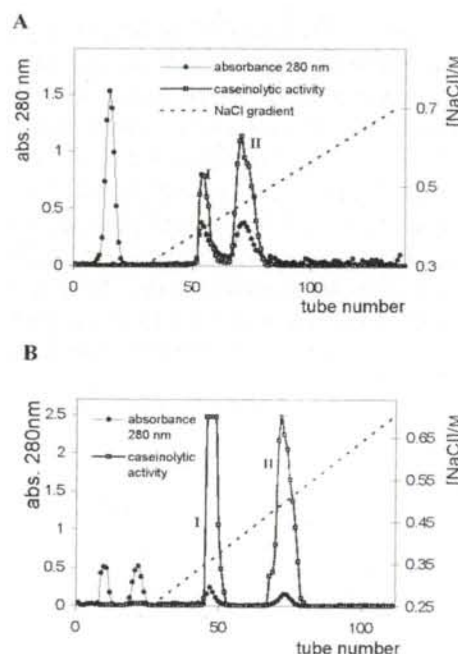

Fig. 1 Cation Exchange Chromatography.
(A) *Morrenia brachystephana*. Crude extract was precipitated with three volumes of could acetone and redissolved in the starting buffer. Enzyme preparation was loaded onto a CM Sepharose CL-6B Fast Flow column (Pharmacia K 15/30) equilibrated with 55 mM citrate-phosphate buffer (pH 6.4). The column was washed with the same buffer, then the reteined proteins were eluted with 200 ml of sodium chloride linear gradient (0.3–0.7 M) prepared in the same buffer.
(B) *Morrenia odorata*. A crude extract was precipitated with three volumes of cold acetone and redissolved in the starting buffer. The enzyme preparation was loaded onto a CM Sepharose CL-6B Fast Flow column (Pharmacia K 15/30) equilibrated with 50 mM phosphate buffer (pH 7.35) containing 5 mM EDTA. The column was washed with the same buffer, then the retained proteins were eluted with 200 ml of a linear sodium chloride gradient (0.25–0.7 M) prepared in the same buffer.

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The latex, collected from cut stems, was centrifugated (16 000 *g*) to obtain a crude extract (Arribére *et al.*, 1998, 1999). These preparations showed high proteolytic activity when assayed on casein, and required a reducing agent, such as cysteine, for maximal activity. The aqueous supernatant layers containing the soluble proteins were purified by acetone precipitation followed by cation exchange chromatography (Figure 1). Two basic proteolytically active fractions were obtained from each species; in both cases the second fraction was homogeneous by SDS polyacrylamide gel electrophoresis (Figure 2), and therefore these fractions were selected for further characterization. The fraction II obtained from *M. brachystephana* latex was called morrenain b II, and the fraction II corresponding to *M. odorata* was called morrenain o II. These designations were chosen following the traditional nomenclature for proteinases obtained from latex of the Asclepiadaceae family members (Rodríguez-Romero and Hernández-Arana, 1998).

Isoelectric focusing of morrenain b II and morrenain o II showed a unique band with a pI higher than 9.3 (Figure 3); the enzymes also showed similar molecular masses as determined by SDS-PAGE: M_r 26 000 and 25 800, respectively (Figure 2). These results are in good agreement with those obtained for other proteinases from Asclepiadaceae: the M_rs of *Asclepias syriaca* proteinases are 21 000 and 23 000 (Brockbank and Lynn, 1979), proteinases from *Asclepias glaucescens* have a M_r of 23 000 (Barragán *et al.*, 1985), the four calotropins isolated from *Calotropis gigantea* have molecular masses ranging from 23.0 to 27.0 kDa (Abraham and Joshi, 1979a,b; Pal and Sinha, 1980), and araujiain h I isolated from the latex of *Araujia hortorum* (Priolo *et al.*, 2000) has a M_r of 24 000.

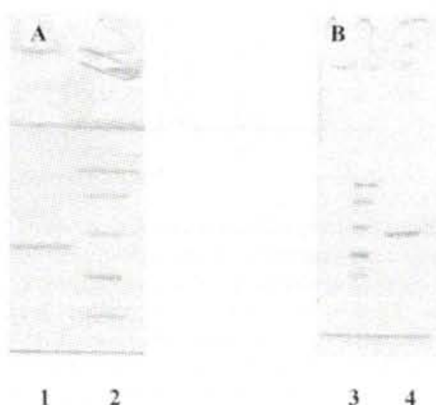

Fig. 2 SDS-PAGE Analysis.
(A) Lane 1, morrenain b II; lane 2, low range molecular weight standars (Bio-Rad): phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovoalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soy trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (B) Lane 3, low range molecular weight standars (Bio-Rad); Lane 4, morrenain o II. Electrophoresis was carried out according to Laemmli (1970). The gel (14% T) was stained with Coomassie Brillant Blue R250 (Sigma Chemical Co.).

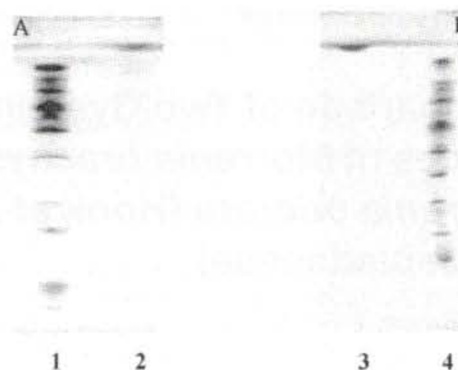

Fig. 3 Isoelectric Focusing.
(A) *Morrenia brachystephana*. Lane 1, pI markers; lane 2; morrenain b II. (B) *Morrenia odorata*. Lane 3, morrenain o II; lane 4, pI markers. Isoelectric focusing was performed in 5% polyacrylamide gels containing broad pH range ampholytes (Biolyte 3–10, Bio-Rad). Samples were precipitated with 3 volumes of cold acetone, centrifuged and the precipitates redissolved in deionized water; this procedure was repeated twice. Focusing was carried out under constant voltage conditions in a stepwise procedure: 100 V for 15 min, 200 V for 15 min, and 450 V for 60 min. Gels were fixed and then stained by Coomassie Brillant Blue R250.

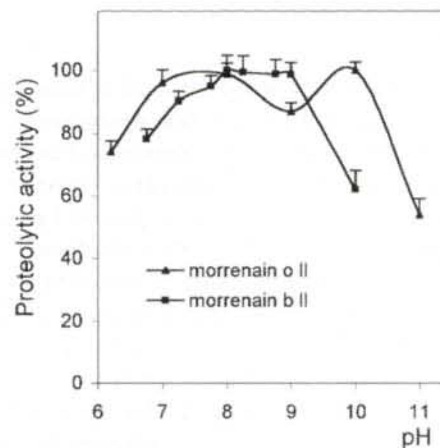

Fig. 4 Effect of pH on Proteolytic Activity of Morrenain b II and Morrenain o II.
The effect of pH on the proteolytic activity of both enzymes was measured with casein (pH range 6.0 to 11.0) using 10 mM sodium salts of the following 'Good' buffers (Good and Izawa, 1972): MES, MOPS, TAPS, AMPSO and CAPS (Sigma Chemical Co.).

Morrenain b II showed maximal proteolytic activity with casein within the pH range 7.5 to 9.0, while morrenain o II exhibited a similar but somewhat broader pH profile, with highest activity in the pH range 7.0–10.0 (Figure 4). Other cysteine endopeptidases like ananain (Rowan and Buttle, 1994), caricain (Taylor *et al.*, 1994) and asclepain (Brockbank and Lynn, 1979) also have a broad pH optimum, which is centered around neutral pH. Inhibition assays with E-64 and iodoacetic acid suggested that –SH

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Table 1 Kinetic Parameters.

	N-α-CBZ-*p*-nitrophenyl Ala		N-α-CBZ-*p*-nitrophenyl Asp		N-α-CBZ-*p*-nitrophenyl Gly	
	Morrenain b II	Morrenain o II	Morrenain b II	Morrenain o II	Morrenain b II	Morrenain o II
K_m (mM)	4.1×10^{-2}	4.3×10^{-5}	2.3×10^{-3}	4.9×10^{-6}	4.1×10^{3}	2.0×10^{-5}
k_{cat} (s^{-1})	6.6	7.3	9.6×10^{-1}	1.7	3.5×10^{6}	2.0
k_{cat}/K_m (s^{-1} M^{-1})	1.6×10^{5}	1.7×10^{8}	4.1×10^{2}	3.5×10^{8}	$8{,}5 \times 10^{5}$	9.9×10^{7}

The assays for determination of K_m and k_{cat} of the isolated enzymes were performed using N-α-CBZ-amino acids-*p*-nitrophenyl esters of alanine, aspartic acid, and glycine (Sigma Chemical Co.). The substrate stock solution (5 mM) was prepared in acetonitrile. The reaction mixture consisted of 50 µl of the sample, 2.0 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 2 mM EDTA and 50 µl of the substrate. The initial reaction rate was determined by the continuous liberation of *p*-nitrophenol followed spectrophotometrically at 405 nm every 8 s during 3 min in a Beckmann DU 640 device equipped with a chamber held at 37 °C. Final concentration of substrates varied from 2.4 to 119 µM. Before the assay the enzymes were activated with 1 mM of cysteine. K_m and k_{cat} were calculated by regression analysis using the nonlinearized form of the Michaelis-Menten equation.

Table 2 Comparison of the N-Terminal Amino Acid Sequence of Morrenain b II with Related Enzymes.

Plant source/enzyme	Sequence	Identity	Positives	References
Morrenia brachystephana morrenain b II	LPDSVDWRKKNLVFPVRNQGKKGG			
Morrenia odorata	LPDSVDWRKKNLVFPVRNQGK G	95%	95%	
morrenain o II	LPDSVDWRKKNLVFPVRNQGKXG	(22/23)	(22/23)	
Asclepias syriaca	LP+S+DWR+KN+VFP++NQG	70%	100%	Lynn *et al.* (1980)
asclepain a	LPNSIDWRQKNVVFPIKNQG	(14/20)	(20/20)	
Zinnia elegans	LP SVDWRKK V PV+NQG+ G	69%	77%	Ye and Varner (1996)
	LPKSVDWRKKGAVSPVKNQGQCG	(16/23)	(18/23)	
Asclepias syriaca	LP+ VDWRKK +VFP+RNQG+	66%	90%	Lynn *et al.* (1980)
asclepain b	LPNFVDWRKNGVVFPIRNQGQ	(14/21)	(19/21)	
Oryza sativa	LP+SVDWR+K V PV+NQG+ G	65%	82%	Watanabe *et al.* (1991)
	LPESVDWREKGAVAPVKNQGQCG	(15/23)	(19/23)	
Carica papaya	LP++VDWRKK V PVR+QG G	65%	78%	Dubois *et al.* (1988)
papaya proteinase Ω	LPENVDWRKKGAVTPVRHQGSCG	(15/23)	(18/23)	
Arabidopsis thaliana	LP SVDWRKK V P++NQG G	65%	73%	Gan and Amasino (1995)
	LPVSVDWRKKGAVTPIKNQGSCG	(15/23)	(17/23)	
Carica candamarcensis	P+S+DWRKK V PV+NQG G	63%	76%	Walreavens *et al.* (1993)
(syn. *Carica pubescens*)	PESIDWRKKGAVTPVKNQGSCG	(14/22)	(17/22)	
cysteine proteinase III (CC-III)				
cysteine proteinase IV (CC-IV)				
Brassica napus	LP +VDWRKK V P++NQG G	60%	73%	Noh and Amasino (1999)
	LPIAVDWRKKGAVTPIKNQGSCG	(14/23)	(17/23)	

The N-terminal sequences of both proteases were determined by automated Edman degradation using a Beckman LF3000 protein sequencer. Homology between each other was compared using the BLAST2 sequences (Tatusova and Madden, 1999). Sequence homology searches were performed using the BLAST network services (Altschul *et al.*, 1997). Percentages of identical residues are indicated (column 'identity'), as well as the proprotion of nonidentical residues with positive alignment scores ('positives'). Conserved sequence motifs are underlined.

groups are involved in the catalytic mechanism of these proteinases.

Most of the specifity requirements for interaction during catalysis of cysteine proteinases have been defined by the use of small synthetic substrates instead of large protein substrates because of complex interactions between the enzymes and a polypeptide chain, leading to diverse cleavage patterns (Ménard and Storer, 1998). In addition to their proteolytic activity, morrenains exhibit esterolytic activity like other cysteine peptidases such as papain (Glazer and Smith, 1971) and bromelain (Silverstein, 1974). Measurements of endoesterolytic activity were performed according to Silverstein (1974). N-α-CBZ-*p*-nitrophenyl esters of alanine, glycine and aspar-

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Table 3 Comparison of the N-Terminal Amino Acid Sequence of Morrenain o II with Related Enzymes.

Plant source/enzyme	Sequence	Identity	Positives	References
Morrenia odorata	LPDSVDWRKKNLVFPVRNQGKXGSXWTFSAVASI			
(morrenain o II)				
Morrenia brachystephana	LPDSVDWRKKNLVFPVRNQGK G	95%	95%	
(morrenain b II)	LPDSVDWRKKNLVFPVRNQGKKG	(22/23)	(22/23)	
Asclepias syriaca	LP+S+DWR+KN+VFPV+NQG	70%	100%	Lynn *et al.* (1980)
asclepain a	LPNSIDWRQKNVVFPIKNQG	(14/20)	(20/20)	
Carica candamarcensis	S+DWR+K V PVRNQG GS WTFS+VA++	70%	86%	Walreavens *et al.* (1993)
cysteine proteinase I	S I DWRQKGAVTPVRNQGSXGSXWTFSSVAAV	(22/31)	(27/31)	
(EC 3.4.22.)				
Asclepias syriaca	LP+ VDWRKK +VFP+RNQG+	66%	90%	Lynn *et al.* (1980)
asclepain b	LPNFVDWRKNGVVFP I RNQGQ	(14/21)	(19/21)	
Carica papaya	LP++VDWRKK V PVR+QG GS W FSAVA++	64%	78%	Dubois *et al.* (1988)
caricain II (EC 3.4.22.30)	LPENVDWRKKGAVTPVRHQGSCGSCWAFSAVATV	(22/34)	(27/34)	
precursor papaya				
proteinase Ω				
Brassica napus	LP SVDWRKK V P+++QG GS W FSAVA+I	64%	75%	Noh and Amasino (1999)
	LPVSVDWRKKGAVTP I KDQGLCGSCWAFSAVAAI	(22/34)	(26/34)	
Zinnia elegans	LP SVDWRKK V PV+NQG+ GS W FS VA++	64%	75%	Ye and Varner (1996)
	LPKSVDWRKKGAVSPVKNQGQCGSCWAFSTVAAV	(22/34)	(26/34)	
Arabidopsis thaliana	LP SVDWRKK V P++NQG G W FSAVA+I	64%	72%	Asamizu *et al.* (1998)
senescence-specific	LPVSVDWRKKGAVTP I KNQGSCGCCWAFSAVAAI	(22/34)	(25/34)	
cysteine protease				
Oryza sativa	LP+SVDWR+K V PV+NQG+ GS W FSAV+++	61%	81%	Watanabe *et al.* (1991)
oryzain β chain precursor	LPESVDWREKGAVAPVKNQGQCGSCWAFSAVSTV	(21/34)	(28/34)	
Carica candamarcensis	P+S+DWRKK V PV+NQG GS W FS + ++	60%	78%	Walreavens *et al.* (1993)
cysteine proteinase IV	PES I DWRKKGAVTPVKNQGSXGSXWAFST I VTV	(20/33)	(26/33)	
(EC 3.4.22.)				
Carica papaya	+P+ VDWR+K V PV+NQG GS W FSAV +I	58%	72%	Mitchel *et al.* (1970)
papaya proteinase I	I PEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVT I	(20/34)	(25/34)	
Zingiber officinale	LPDS+DWR+ V PV+NQG GS W FS VA++	58%	72%	Choi *et al.* (1999)
	LPDS I DWRENGAVVPVKNQGGCGSCWAFSTVAAV	(20/34)	(25/34)	
Arabidopsis thaliana	LP SVDWRK V PV+NQG+ GS W FS V ++	58%	69%	Sato *et al.* (2000)
cysteine endopeptidase	LPTSVDWRKNGAVTPVKNQGQCGSCWAFSTVVAV	(20/34)	(24/34)	

See the legend of Table 2 for explanations.

tic acid were chosen for the determination of the kinetic parameters of both enzymes, because crude extracts showed a preference for them. Morrenain b II showed the highest catalytic efficiency (k_{cat}/K_m) with the glycine derivative and morrenain o II with the aspartic acid derivative (Table 1); the alanine and glycine derivatives may be considered as very good substrates for morrenain b II (Table 1).

The N-terminal sequences of both proteinases were determined by automated Edman degradation. For morrenain b II the sequence LPDSVDWRKK NLVFPVRNQG KKGG was identified, and the N-terminal amino acids of morrenain o II are LPDSVDWRKK NLVFPVRNQG KXGSXWTFSA VASIXT. These sequences show 95% identity and are also highly homologous to other plant cysteine proteinases (Tables 2 and 3), especially to those belonging to the clan CA, family C1 (Barrett *et al.*, 1998). The N-terminus of morrenain b II shows a degree of identity between 60% and 69% with other proteinases such as those isolated from *Zinnia elegans*, *Oryza sativa*, cysteine proteinase III and IV from *Carica candamarcensis*, papaya proteinase Ω from *Carica papaya*, and the peptidases from *Arabidopsis thaliana* and *Brassica napus*. An endopeptidase with even higher homology to morrenain o II is the cysteine proteinase I isolated from *Carica candamarcensis* (70% identity).

The evaluation of the data in Tables 2 and 3 revealed that the positions for some amino acids (Pro2, Pro15, Lys10, Val13, Gly23, and Trp26 observed in the larger sequences) as well as some motifs (SVDWRKK and NQG, and FS in larger sequences) are notably conserved, suggesting that morrenain b II and morrenain o II probably share a common ancestral gene, with cysteine proteases obtained from taxonomically unrelated plant species.

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