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Research paper

Characterization of papain-like isoenzymes from latex of *Asclepias curassavica* by molecular biology validated by proteomic approach

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ABSTRACT

Latices from *Asclepias* spp are used in wound healing and the treatment of some digestive disorders. These pharmacological actions have been attributed to the presence of cysteine proteases in these milky latices. *Asclepias curassavica* (Asclepiadaceae), "*scarlet milkweed*" is a perennial subshrub native to South America. In the current paper we report a new approach directed at the selective biochemical and molecular characterization of asclepain cI (acI) and asclepain cII (acII), the enzymes responsible for the proteolytic activity of the scarlet milkweed latex. SDS-PAGE spots of both purified peptidases were digested with trypsin and Peptide Mass Fingerprints (PMFs) obtained showed no equivalent peptides. No identification was possible by MASCOT search due to the paucity of information concerning Asclepiadaceae latex cysteine proteinases available in databases. From total RNA extracted from latex samples, cDNA of both peptidases was obtained by RT-PCR using degenerate primers encoding Asclepiadaceae cysteine peptidase conserved domains. Theoretical PMFs of partial polypeptide sequences obtained by cloning (186 and 185 amino acids) were compared with empirical PMFs, confirming that the sequences of 186 and 185 amino acids correspond to acI and acII, respectively. N-terminal sequences of acI and acII, characterized by Edman sequencing, were overlapped with those coming from the cDNA to obtain the full-length sequence of both mature peptidases (212 and 211 residues respectively). Alignment and phylogenetic analysis confirmed that acI and acII belong to the subfamily C1A forming a new group of papain-like cysteine peptidases together with asclepain f from *Asclepias fruticosa*. We conclude that PMF could be adopted as an excellent tool to differentiate, in a fast and unequivocal way, peptidases with very similar physicochemical and functional properties, with advantages over other conventional methods (for instance enzyme kinetics) that are time consuming and afford less reliable results.

1. Introduction

Latex is a milky fluid composed of a liquid serum containing, either in suspension or solution, a complex mixture of molecules [1]. It is clear that the latex performs its functions through multiple modes of action, ranging from physical barriers to toxicity and defense. Some plants immediately secrete latex when the leaves, stems or fruits are injured. The latex bleeding proceeds for a few minutes until a clot form around the wounded area. The coagulation process is vital for plant defense against possible pathogen attack. Latex may act to shield the cambial meristem and the contents of the sieve tubes from predators, or ward off parasites or pathogens. Therefore, it seems reasonable to assume that the substances and enzymes needed for such purposes are present in latex [2,3]. It is well known that several hydrolytic enzymes – cellulases, polygalacturonases, chitinases, lipolytic and proteolytic enzymes – are highly expressed in laticifers [2,4–6]. The economic importance of some of these hydrolytic enzymes has prompted active investigations into its biochemical aspects.

Several names have been assigned to enzymes that cleave peptide bonds, including proteases, proteinases, peptidases, and proteolytic enzymes [7]. Here we will use the term peptidases (EC 3.4) as is suggested by the Nomenclature Committee of IUPAC-IUBMB (URL http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/). Cysteine peptidases (EC 3.4.22) are widely distributed in nature and are divided into clans that do not share sequence or structural identity and probably arose from different evolutionary lines. They have a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic

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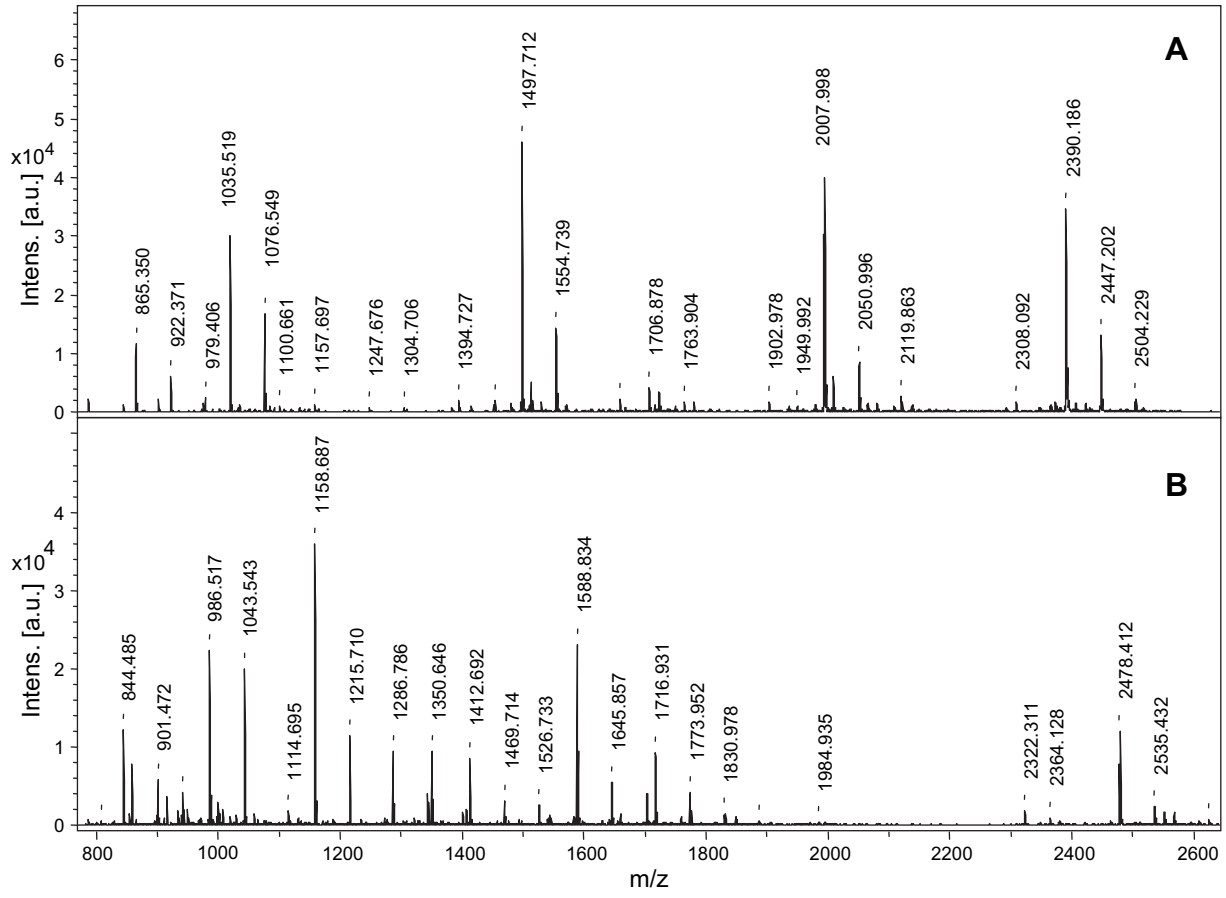

Fig. 1. MALDI-TOF mass spectra of tryptic digests from purified A) asclepain cI and B) asclepain cII, *m/z* values of prominent peaks are indicated in each graphic.

dyad. For these enzymes, the residue of cysteine is essential for their enzymatic activity [8,9]. The clan CA is the largest clan of cysteine peptidases with the papain-like family (C1) being the most studied. Family C1 has been subdivided into subfamily C1A, which comprises peptidases that contain disulfide bridges and accumulate in vesicles, the vacuole, or the apoplast, and family C1B, which comprises peptidases that lack disulfide bridges and are located in the cytoplasm [8]. Papain-like cysteine peptidases are synthesized as inactive proenzymes with N-terminal propeptide regions, which are removed upon activation. In addition to its inhibitory role, the propeptide is required for proper folding of the newly synthesized enzyme and its stabilization in denaturing pH conditions. Residues within the propeptide region also play a role in the transport of the proenzyme to lysosomes or acidified vesicles [10].

Plant peptidases in recent years have been the subject of intensive research and have been proposed as part of several important processes [11] such as plant development [12], root symbiosis [13], plant defense against pathogens [14] and insects [3,15], programmed cell death [16], and seed germination [17]. Papain-like peptidases are implicated in pathogen perception, disease resistance signaling, defense against insects, and senescence [11]. Despite the large amount of information gathered in recent years on plant peptidase function, little is known about the functions of latex peptidases. Agrawal and coworkers [15] in phylogenetically independent analyses have demonstrated a positive correlation between *Asclepias* spp. latex exudation and cysteine peptidase activity, thus sharing a correlated evolutionary history. It is possible that latex cysteine peptidases act in the degradation of proteins during laticifer development or promotion of coagulation or as responsible for strong toxicity.

In the current paper we report a new approach directed at the selective characterization of papain-like peptidase isoenzymes. The methodology used included techniques of protein purification, molecular biology, and proteomics. For this purpose latex of *Asclepias curassavica*, which is known to present two cysteine endopeptidases that share some physical and biochemical features– asclepain cI (acI) and asclepain cII (acII) [2,18], was taken as starting material.

2. Materials and methods

2.1. Plant material

A. curassavica L., "*scarlet milkweed*", (Asclepiadaceae) is an erect, evergreen perennial subshrub [19]. Native to South America, *A. curassavica* has become a naturalized weed in tropical and subtropical areas distributed throughout the world. This species presents non-articulated laticifers in all organs. Latex was obtained from plants grown in La Plata, Province of Buenos Aires, Argentina. A voucher specimen (UNR 1130) has been deposited at the UNR herbarium (Faculty of Agricultural Sciences, University of Rosario, Argentina).

2.2. Peptidases isolation and purification

Crude extract (CE) was prepared by ultracentrifugation of latex obtained by superficial incisions of petiols, according to Liggieri and coworkers [2]. AcI and acII peptidases were purified from the CE by cation exchange chromatography following the method of Liggieri and coworkers [2].

Table 1
Primer design for Asclepiadaceae latex endopeptidase molecular cloning. A) and B) show primers design with N-terminal sequences. C) and D) show primers design with catalytic site sequences.

A)
Primer **NTapo1** (5′-GTTGAATTGCCAGATTCTGTAGATTGG- 3′)

Enzyme	N-Terminal sequence	References
Araujiain h II	V**PDS**I**DWR**EKDAVLPIRNQGQ	[25]
Araujiain h III	**LP**E**SVDWR**KKNLVFPVRNQGQ	[25]
Asclepain c I	**LP**N**SVDWR**QKGVVFPIRDQGK	[2]
Asclepain c II	**LP**SF**VDWR**QKGVVFPIRNQGQ	[18]
Asclepain f	**VELPDSVDWR**EKGVVFPIRNQGK	[23]
Funastrain c II	**LP**N**SVDWR**QKGVVSAIRNQGK	[26]
Morrenain o II	**LPDSVDWR**KKNLVFPVRNQGK	[24]

B)
Primer **NTapo2** (5′-CCAGATTCTGTAGATTGGCGG- 3′)

Enzyme	N-Terminal sequence	References
Araujiain h II	V**PDS**I**DWR**EKDAVLPIRNQGQ	[25]
Araujiain h III	L**P**E**SVDWR**KKNLVFPVRNQGQ	[25]
Asclepain c II	L**P**SF**VDWR**QKGVVFPIRNQGQ	[18]
Asclepain f	L**PDSVDWR**EKGVVFPIRNQGK	[22]
Asclepan c I	L**P**N**SVDWR**QKGVVFPIRDQGK	[2]
Funastran c II	L**P**N**SVDWR**QKGVVSAIRNQGK	[26]
Morrenain o II	L**PDSVDWR**KKNLVFPVRNQGK	[24]

C)
Primer **CAapo1** 5′-CCTATCAGAAATCAAGGAAAATGTGGGAGTTGCTGG-3′

Enzyme	Protein sequence	References
Asclepain f	LPDSVDWREKGVVF**PIRNQGKCGSCW**TFSA	[23]
Asclepain c I	LPNSVDWRQKGVVF**PIRDQGKCGSCW**TFSA	[2]
Asclepain c II	LPSFVDWRQKGVVF**PIRNQGQCGSCW**TFSA	[18]

D)
Primer **CAapo2** 5′-ATCAAGGAAAATGTGGGAGTTGCTGG-3′

Enzyme	Protein sequence	References
Asclepain f	LPDSVDWREKGVVFPIR**NQGKCGSCW**TFSA	[23]
Asclepain c I	LPNSVDWRQKGVVFPIR**DQGKCGSCW**TFSA	[2]
Asclepain c II	LPSFVDWRQKGVVFPIR**NQGQCGSCW**TFSA	[18]

Degenerated primers were designed encoding conserved N-terminal and active site sequences from Asclepiadaceae latex endopeptidases (bold sequences) obtained by automated Edman's degradation. CAapo1 and CAapo2 were used as nested primers to improve the efficiency of amplification of specific cDNAs.

2.3. SDS-PAGE analysis

Purified samples of acI and acII were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with tricine cathodic buffer in 10% polyacrylamide gels [20]. Gels were stained with Coomassie brilliant blue R-250.

2.4. Peptide mass fingerprint (PMF) analysis by MALDI-TOF MS

Bands corresponding to purified peptidases were cut out, washed with milli Q water and acetonitrile several times to remove dye and dried under vacuum. The gel fragments were treated with 0.1 M NH_4HCO_3 containing 10 mM DTT for 30 min at 37 °C, centrifuged, washed with acetonitrile for 5 min, and then incubated in darkness in 0.1 M NH_4HCO_3 with 50 mM iodoacetamide for 20 min at 25 °C for Cys sulfhydryl alkylation. Digestions were carried out with 4 ng/μl trypsin during 12 h at 37 °C. The peptides obtained were recovered by extraction with 0.5 ml/ml of acetonitrile (ACN), dried in a SpeedVac vacuum centrifuge and redissolved in 1 ml/l of trifluoroacetic acid (TFA). Each sample was spotted on a Ground Steel plate and mixed with freshly prepared matrix solution (10 mg/ml of α-cyano-4-hydroxycinnamic acid in aqueous solution containing 30% ACN and 0.1% TFA). PMFs were obtained by MALDI-TOF MS with UltraFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptide masses were acquired with Flex Control Software in a range of *ca.* 1000–3500 *m*/*z*. External calibration was performed using peptide calibrants.

MASCOT search tool (URL http://www.matrixscience.com) was used for identification of tryptic maps. 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) Peptide Mass Tolerance: ±1000 ppm; (8) 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 were considered significant ($p < 0.05$).

2.5. cDNA cloning

Total RNA was isolated from two latex drops, obtained by superficial incisions of petioles, using the RNAeasy Plant Mini Kit (Qiagen, Barcelona, Spain) following the manufacturer's protocol. Double-stranded cDNA was synthesized by 3′ rapid amplification of cDNA ends – polymerase chain reaction – 3′RACE-PCR [21]. cDNA template was generated with First Strand cDNA Synthesis Kit for RT-PCR, AMV (avian myeloblastosis virus) reverse transcriptase (Roche Applied Science, Mannheim, Germany) and R_0R_1polidT primer (5′-CCGGAATTCACTGCAGGGTACCCAATACGACTCACTATAG GGCTTTTTTTTTTTTTTTT-3′). Reaction was carried out in a thermal cycler XPCycler (Bioer Technology Co., Ltd, Hangzhou, China) following the program: 10 min at 25 °C, 120 min at 42 °C, 5 min at 99 °C, and 5 min at 10 °C. cDNA was PCR-amplified with the following specific oligonucleotides: NtApo1 (5′-GTTGAATTGCCAGA TTCTGTAGATTGG-3′), NtApo2 (5′-CCAGATTCTGTAGATTGGCGG-3′) or degenerated primers Nd_{1-8} (5′-yTkCCdGATTCCGATGTTTGGmG-3′, y = C or T, m = A or C and k = G or T), and the adaptor primer R_0 (5′-CCGGAATTCACTGCAG-3′). The PCR mixture containing 5 μl of the cDNA previously obtained, 25 pmol of adaptor primer and 25 pmol of one of the specific primers, 20 nmol of deoxynucleotide triphosphates, 5 U EcoTaq/*Thermus aquaticus* polimerase, 5 μl buffer EcoTaq 10×, 2 μl $MgCl_2$ 50 mM (all of above reagents purchased from Ecogen, Barcelona, Spain) was subjected to reaction in the same thermal cycler following the program: 1× (5 min at 95 °C; 15 min at 72 °C); 30× (1 min at 94 °C; 1 min at 46 °C; 2 min at 72 °C); 1× (15 min at 72 °C); 1× (16 h at 10 °C).

Design of specific primers was performed from most conserved amino acid sequences of peptidases (N-terminal and active site catalytic Cys containing regions) from the Asclepiadaceae family [2,18,22–26]. Nucleotide sequences corresponding to such amino acid sequences were obtained with Backtranslation Tool, available on the WEB (http://www.entelechon.com/bioinformatics/backtranslation.php?lang=eng).

To improve the efficiency of amplification of specific cDNAs a second set of PCR cycles was carried out using two combinations of nested primers: asclepiadaceae-specific primer CAapo1 (5′-CCTATCAGAAATCAAGGAAAATGTGGGAGTTGCTGG-3′) and adaptor primer R1 (5′-GGTACCCAATACGACTCACTATAGGGC-3′), and asclepiadaceae-specific primer CAapo2 (5′-ATCAAGGAAAATGTGGGA GTTGCTGG-3′) with the same adaptor primer (R1).

PCR products were visualized on 20 g/l agarose gel as bands of about 760 bp, the expected size based on known molecular masses of papain-like peptidases, and extracted by QIAEX II Agarose Gel Extraction (Qiagen). The isolated cDNA was inserted into pGEM-T Easy vector (Promega Biotech Iberica, Alcobendas, Spain). *Escherichia coli* XL1-Blue competent cells were transformed and cultured on LB plates with 100 μg/ml ampicillin, 112 μg/ml IPTG and 80 μg/ml X-Gal overnight at 37 °C. White colonies were selected for transfer to LB medium with 50 μg/ml ampicillin and cultured overnight at 37 °C; afterwards plasmids with inserts were extracted using commercial purification kit (GFX Micro Plasmid Prep Kit, GE HealthCare Biosciences, Uppsala, Sweden). Purified plasmids were

A

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1                    AT CAA GGA AAA TGT GGG AGT TGC TGG ACA TTC TCG GCA GTT GCT TCA     47
1   LPNSVDWRQKGVVFPIRD  Q   G   K   C   G   S   C   W   T   F   S   A   V   A   S     33
48   ATA GAA ACC CTA ATT GGA ATT AAA GAA GGC CGT ATG ATT GCA TTA TCC GAG CAA GAG CTA   107
34    I   E   T   L   I   G   I   K   E   G   R   M   I   A   L   S   E   Q   E   L    53
108  TTG GAC TGT GAG AGA ACA AGT TTT GGG TGT AAA GGA GGT TAC TAT GCT AAC GCA TTC GCA   167
54    L   D   C   E   R   T   S   F   G   C   K   G   G   Y   Y   A   N   A   F   A    73
168  TAT GTA GCA AAA AAT GGT ATT ACC TCT CGG GAT AGA TAT CCT TAT ATT TTT CAA CAA GGA   227
74    Y   V   A   K   N   G   I   T   S   R   D   R   Y   P   Y   I   F   Q   Q   G    93
228  CAA TGT TAT CAA AAG GAA AAA GTG GTC AAA ATT AGT GGT TAT AGG AAC GTA CGT AGA AAT   287
94    Q   C   Y   Q   K   E   K   V   V   K   I   S   G   Y   R   N   V   R   R   N   113
288  GAC GAG AAA GAA CTT CAA CTT GTT GTA GCA CAA CAA GTT GTG AGT ATT GGC ATC AAA TCT   347
114   D   E   K   E   L   Q   L   V   V   A   Q   Q   V   V   S   I   G   I   K   S   133
348  AGC AGC AGA GAT TTT CAG CAT TAT CGT CAG GGT ATA TTT AAT GGA GCT TGC GGT CCA AAG   407
134   S   S   R   D   F   Q   H   Y   R   Q   G   I   F   N   G   A   C   G   P   K   153
408  TTG GAT CAT GCA GTG AAT ATT GTT GGA TAC GGT TCT GAA GGT GGA GCT AAT TAT TGG ATC   467
154   L   D   H   A   V   N   I   V   G   Y   G   S   E   G   G   A   N   Y   W   I   173
468  GTG AGA AAC TCT TGG GGC ACA GGT TGG GGA GAG GGT GGA TAT GCA AGG TTA CCA ATG TAT   527
174   V   R   N   S   W   G   T   G   W   G   E   G   G   Y   A   R   L   P   M   Y   193
528  TCA GGA CAA GTT GGA GGT TAT TGT GGA ATT GTC AGT CAG GCG TCT TAT CCT GTT TAT TAG   587
194   S   G   Q   V   G   G   Y   C   G   I   V   S   Q   A   S   Y   P   V   Y     212
588  CACTTATCAAAAAATTATGTTATTTATCAAGAAAACATATATATGTAAGTATGTCCGATCCGTATGAAGTAATTTTTAT  666
667  ATGAATTAAATAAAAACTTTTTTATATAAAAAAAAAAAAAAAA                                    710
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B

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1                    AT CAA GGA AAA TGT GGG AGT TGC TGG ACA TTC TCG GCA GTT GCT       44
1   LPSFVDWRQKGVVFPIRN  Q   G   K   C   G   S   C   W   T   F   S   A   V   A       32
45   TCA ATA GAA ACC TTA ATT GGA ATT AAA GAA GGT CGT ATG ATT GCA TTA TCC GAG CAA GAG   104
33    S   I   E   T   L   I   G   I   K   E   G   R   M   I   A   L   S   E   Q   E    52
105  CTC TTG GAC TGC GAA ACA ACA AGT TAT GGG TGT AAA GGA GGT CAC TAT GAT AAT GCA TTC   164
53    L   L   D   C   E   T   T   S   Y   G   C   K   G   G   H   Y   D   N   A   F    72
165  GCA TAT GTA GCC AAG AAG GGT ATT ACC TCT GAG GAA AAA TAT CCA TAT GTT TTT CGA CAA   224
73    A   Y   V   A   K   K   G   I   T   S   E   E   K   Y   P   Y   V   F   R   Q    92
225  GGA CAA TGT TCT CAA AAG AAA AAA GTG GCC AAA ATT TCT GGT TAC AGG AGA GTA CCT GGA   284
93    G   Q   C   S   Q   K   K   K   V   A   K   I   S   G   Y   R   R   V   P   G   112
285  AAT AAC GAA GGA CAA CTT CAA AGT GCT GTA GCA CAA CAA GTA GTG AGT GTT GCC GTA AAA   344
113   N   N   E   G   Q   L   Q   S   A   V   A   Q   Q   V   V   S   V   A   V   K   132
345  TCT AAA AGC GAC GAT TTC CGA TTT TAT AGT GGG GGC ATA TTT AGT GGA GCT TGC GGA AAA   404
133   S   K   S   D   D   F   R   F   Y   S   G   G   I   F   S   G   A   C   G   K   152
405  GTA TTA GAT CAT GCA GTA AAT ATT GTT GGA TAT GGT TCT GAA AGT GGA GCT AAT TAT TGG   464
153   V   L   D   H   A   V   N   I   V   G   Y   G   S   E   S   G   A   N   Y   W   172
465  ATC ATG AGA AAC TCT TGG GGT ACA AAT TGG GGA GGG AAT GGA TAT ATG AAG GTT CCA AAG   524
173   I   M   R   N   S   W   G   T   N   W   G   G   N   G   Y   M   K   V   P   K   192
525  AAT TCA AAA CAG CTT GGA GGT TAT TGT GGA ATT GCC TTC TTG CCC TCT TAT CCT GTT TGA   584
193   N   S   K   Q   L   G   G   Y   C   G   I   A   F   L   P   S   Y   P   V     211
585  GAGTCACCAAAACTTATGTAATTTGTTAAGACAACGCACATAATATATATATATATGTGTGTGTGTGTGTGTACGTAC  664
665  GTATGAAATGATTTCTATATGAATTAAATAAATCTTTTTTATATAAAAGAAAAAAAAAAAAAA                727
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Fig. 2. cDNA sequence and deduced protein sequence of A) acI and B) acII. AcI and acII amino acid sequences determined at the protein level are boxed. Cys25 and His156 – the catalytic dyad – and other residues that help to stabilize the dyad are circled. Stop codon, polyadenylation signal and polyA tail are underlined.

digested with *Eco*RI restriction enzyme (2 U), identified by PCR (program and reaction mixtures same as above, using CAapo1 and R1 primers) and analyzed by electrophoresis on 10 g/l agarose gel. Positive plasmids were sequenced by the Sequencing Services of Veterinary Faculty, Autonomous University of Barcelona (Barcelona, Spain). Chromatograms were analyzed with the software Chromas v2.13 (Technelysium Pty. Ltd).

2.6. Obtaining the full-length sequence of mature acI and acII

cDNA sequences obtained from the clones were analyzed with Translating Blast Service (Translated query – Translated db [tblastx] tool) that converts a nucleotide query sequence into protein sequences in all 6 reading frames and then compares this to an NCBI nucleotide database which has been translated in all six reading frames (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Translations&PROGRAM=blastx&BLAST_PROGRAMS=blastx&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn). In translated Blast NCBInr database was restricted to the *Viridiplanteae* kingdom in order to identify the presence of plant cysteine endopeptidase amino acid sequence conserved elements.

The translated sequences obtained were subjected to theoretical tryptic digestion by means of SequenceEditor 3.1 software (Bruker Daltonics, Biotools 3.1). Comparison of this theoretical mass maps with empirical PMF of purified peptidases (Section 2.4.) helped to identify each peptidase. Then N-terminal sequences previously obtained by Edman [2,18] were overlapped to the partial sequence obtained by cloning and thus the complete sequences of the mature enzymes were obtained.

2.7. Amino acid sequence alignment and phylogenetic trees construction

Multiple sequence alignment was performed with CLUSTAL W2 [27] and Basic Local Alignment from the National Center for

Biotechnology Information (URL http://blast.ncbi.nlm.nih.gov/Blast.cgi), conserved domains (CD) were identified with NCBI's CD-Search service (URL http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, search performed against database: cdd-27036 PSSMs) [28] and evolutionary distance model was constructed with PSI-BLAST tool, that allow construction of guide trees between sequences with more than 75% mismatched amino acids (Grishin), tree method: neighbor joining, distance: Grishin (protein).

2.8. Theoretical properties and modeling

Theoretical physicochemical properties of the deduced amino acid sequences (pI, Mw and molar extinction coefficient) were predicted by the GPMAW v6.0 software algorithms (Lighthouse data, DK-5230 Odense M, Denmark). Data obtained were compared with those previously experimentally determined [2,18]. Putative phosphorylation motifs were searched with NetPhos 2.0 server (URL http://www.cbs.dtu.dk/services/NetPhos/).

3. Results and discussion

3.1. PMF analysis

Asclepain cI and cII, two peptidases isolated from latex of *A. curassavica,* were purified by cation exchange chromatography. Purified proteins separated by SDS-PAGE were cleaved *in situ* with the residue-specific peptidase trypsin and hydrolyzed derived peptides were eluted. Trypsin produces a series of peptides of different molecular mass characteristic of each particular protein. PMF spectra of acI and acII are shown in Fig. 1 A and B, respectively. The peptide map analysis showed the enzymes have no equivalent peptides, despite their high degree of sequence identity. The peptide profiles of the query proteins were compared with theoretical peptide libraries generated from sequences in the MASCOT database in order to identify the studied proteins. Usually as few as three to four masses that match closely are often enough to obtain a significant match [29] but no identification was possible due to the limited amount of information concerning Asclepiadaceae latex cysteine proteinases deposited in the available databases.

3.2. Molecular cloning of acI and acII cDNAs and characterization of deduced amino acid sequence

Degenerated primers were designed encoding conserved N-terminal and active site sequences obtained by automated Edman's degradation of asclepain c I [2] and asclepain c II [18] from *A. curassavica* L.; asclepain f [23] from *Asclepias fruticosa* L. [syn: *Gomphocarpus fruticosus*] (accession number: FM201283); araujiain h II and h III [25] from *Araujia hortorum*; morrenain o II [24] from *Morrenia odorata* and funastrain c II [26] from *Funastrum clausum* (Table 1); all species belonging to the Asclepiadaceae family. These peptidase sequences were selected to ensure that the primers designed could be useful in a general way for cloning any cysteine peptidase from latex of the Asclepiadaceae family. Furthermore, if one considers the high degree of conservation of the amino terminal sequences, as well as, the conserved surroundings of the catalytic Cys25 of papain-like endopeptidases, it is very likely that the primers Ntapo1, Ntapo2, Capo1 and Capo2 could be useful for the molecular cloning of other members of the C1A subfamily.

Total RNA was isolated from latex of *A. curassavica* and cDNAs encoding internal segments of two different putative cysteine endopeptidases were obtained by RT and 3′RACE-PCR methods (GenBank accession number: FM877966 and FM877967). Twelve cDNA sequences obtained from different clones were analyzed, and alignment results revealed two consensus sequences of 710 and 727 nucleotides with 82% identities [score (S): 590, expected value (E): $1 \times e^{-172}$] among them, both containing one stop codon, a polyA-signal and a poly(A) sequence (Fig. 2A and B). The nucleotide sequences translated in all six reading frames produced significant alignments with mRNA of cysteine endopeptidases isolated from latex. The shorter sequence (710 nucleotides) showed high degree of identity with asclepain f, of *A. fruticosa* (74% identities, $S = 360$, $E = 9 \times e^{-98}$), papaya proteinase omega and proteinase IV from *Carica papaya* (46% identities, $S = 134$, $E = 1 \times e^{-60}$ and 47% identities, $S = 141$, $E = 1 \times e^{-56}$, respectively) while the larger nucleotide sequence (727 nucleotides) exhibits such high identity only with asclepain f (86% identities, $S = 315$, $E = 1.9 \times e^{-89}$).

To further confirm that the cDNA from latex codes for acI and acII and not for other cysteine peptidases present in latex, the theoretical mass maps of the translated sequences were compared with those of the purified acI and acII. The experimentally determined masses of peptides derived from both peptidases match well (0.6 Da of mass tolerance) with those in silico fragments generated by the simulated digestion of the putative sequences (10 and 8 coincident peptides for acI and acII, respectively; Table 2). The resultant peptide fragments that matched covered a 50% and 43% of the acI and acII entire sequences, respectively. Thus, confirming the sequence of 710 and 727 nucleotides correspond to acI and acII, respectively. The proposed sequences for the mature enzymes are shown in Fig. 2A and B, containing 212 (acI) and 211 (acII) amino acids and including those residues coming from the N-terminal sequence determined at the protein level.

When acI and acII sequences were analyzed by GPMAW v6.0 the calculated molecular masses were 23 513 and 23 057 Da, which are of the same order of those obtained for other peptidases from Asclepiadaceae [18,30]; the molar extinction coefficients were 48 010 and 44 170 M^{-1} cm^{-1}, respectively. The calculated pI values were 9.78 for acI and 9.82 for acII, in agreement with the experimental values, higher than 9.3 reported by Liggieri and coworkers [2,18]. Then, acI and acII are more basic than papain with a pI of 8.75

Table 2
Theoretical and experimental masses of trysin-digested peptides of **A**) asclepain cI and **B**) asclepain cII. A.

Experimental PMF	Theoretical PMF	Fragment position	Peptide fragment sequence
A)			
m/z	*m/z*		
1120.55	1120.65	104–112	ISGYRNVRR
1148.58	1148.55	143–153	QGIFNGACGPK
1394.73	1394.67	65–77	GGYYANAFAYVAK
1497.71	1497.65	176–189	NSWGTGWGEGGYAR
1706.87	1706.81	45–58	MIALSEQELLDCER
1722.88	1722.79	86–98	YPYIFQQGQCYQK
1936.97	1936.90	84–98	DRYPYIFQQGQCYQK
1979.00	1979. 93	86–100	YPYIFQQGQCYQKEK
2065.03	2065.93	59–77	TSFGCKGGYYANAFAYVAK
2390.19	2390.19	154–175	LDHAVNIVGYGSEGGANYWIVR
B)			
m/z	*m/z*		
1019.51	1019.53	1–8	LPSFVDWR
1043.52	1043.63	9–17	QKGVVFPIR
1412.67	1412.66	65–77	GGHYDNAFAYVAK
1540.76	1540.75	65–78	GGHYDNAFAYVAKK
1588.80	1588.80	79–91	GITSEEKKYPYVFR
2322.21	2322.24	110–132	VPGNNEGQLQSAVAQQVVSVAVK
2478.29	2478.34	109–132	RVPGNNEGQLQSAVAQQVVSVAVK
2551.18	2551.24	153–175	VLDHAVNIVGYGSESGANYWIMR

Cysteines have been treated with iodoacetamide to form carbamidomethyl-cysteine (Cys-CAM). For theoretical PMF Cys-CAM, oxidized Cys and monoisotopic masses of the occurring amino acid residues were considered. The experimental and theoretical mass values matches were selected with a mass tolerance of 0.6 Da.

and less than philibertain g I from *Philibertia gilliesii* Hook. *et* Arn. (Asclepiadaceae) with an isoelectric point >10.25 [30]. According to the information provided by the NetPhos 2.0 server both sequences include putative phosphorylation motifs. Ser105, Ser134 and Tyr187 showed the highest scores (>0.95) of the predicted phosphorylation sites for acI; whereas Ser82, Ser96, Ser133 and Tyr86 displayed the highest scores (>0.95) for acII.

3.3. Molecular phylogeny and classification

In homology studies BLAST program database searches reveal that the deduced amino acid sequences have a 75% of identity (159/211) among them, with 84% of positives amino acids (178/211), expected value: $6 \times e^{-89}$. Fig. 3 showed the high degree of conservation between acI, acII and other plant cysteine peptidases that belong to subfamily C1A (papain family). The highest score (75% and 82% identity with acI and acII respectively) was detected with the sequence of asclepain f from *A. fruticosa L.* (accession number: FM201283). CD database searches allowed the identification of one conserved domain in both peptidases, the cd02248 (peptidase C1A subfamily according to MEROPS database nomenclature [8], expected values: $2 \times e^{-61}$ and $4 \times e^{-60}$ for acI and acII, respectively. The conserved features of this CD are the active site [31,32] and the sub site S2 which is the dominant specificity sub site in papain-like cysteine peptidases [33]. In fact, all papain-like peptidases share their general architecture, consisting of two lobes with a V-shaped active site cleft at the interface of the lobes, with a catalytic dyad comprising residues Cys 25 and His 159 (according to the papain numbering) situated at the opening of the cleft, one from each lobe. The activity of cysteine endopeptidases is governed by the catalytic dyad that exist as a Cys^{-}…His^{+} zwitterion – a prerequisite for enzyme catalysis [6]. Both acI and acII presented the catalytic Cys at position 25, while His was located at position 156 (Fig. 2); this arrangement is also observed in asclepain f [23], araujiain aII [34], philibertain gA and philibertain gB [30], all enzymes from latex of Asclepiadaceae family. Besides the catalytic dyad, other amino acids: Asp158, Asn175, Glu50, and Gln19 (papain numbering), are known to be important in catalysis and stabilization of the zwitterionic form of the dyad [6], *cfr* Fig. 2.

A phylogenetic tree inferred by Neighbor joining (Fig. 4) showed that the enzymes from Asclepiadaceae latex form a separate group;

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papain                          IPEYVDWRQKGAVTPVKNQGSCGSCWAFSAVVTIEGIIKIRTGNLNEYSE 50
caricain                        LPENVDWRKKGAVTPVRHQGSCGSCWAFSAVATVEGINKIRTGKLVELSE 50
cysteineprotease-4H.annuus      LPKSVDWRKKGAVAPVKNQGQCGSCWAFSTVAAVEGINQIVTGNLTMLSE 50
asclepain-cII                   LPSFVDWRQKGVVFPIRNQGKCGSCWTFSAVASIETLIGIKEGRMIALSE 50
asclepain-f                     LPDSVDWREKGVVFPIRNQGKCGSCWTFSAVASIETLNGIKKGHMIALSE 50
asclepain-cI                    LPNSVDWRQKGVVFPIRDQGKCGSCWTFSAVASIETLIGIKEGRMIALSE 50
ervatamin-B                     LPSFVDWRSKGAVNSIKNQKQCGSCWAFSAVAAVESINKIRTGQLISLSE 50
                                :*. ****.**.* .::.* .*****:**:*.::* :  *  *.:   **

papain                          QELLDCDRRS-YGCNGGYPWSALQLVAQYG-IHYRNTYPYEGVQRYCRSR 98
caricain                        QELVDCERRS-HGCKGGYPPYALEYVAKNG-IHLRSKYPYKAKQGTCRAK 98
cysteineprotease-4H.annuus      QELIDCDTTFNNGCNGGLMDYAFAYVMRSG-LHKEEEYPYIMSEGTCDEK 99
asclepain-cII                   QELLDCETTS-YGCKGGHYDNAFAYVAKKG-ITSEEKYPYVFRQGQCS-  96
asclepain-f                     QELLDCETIS-QGCKGGHYNNAFAYVAKNG-ITSEEKYPYIFRQGQCY-  96
asclepain-cI                    QELLDCERTS-FGCKGGYYANAFAYVAKNG-ITSRDRYPYIFQQGQCY-  96
ervatamin-B                     QELVDCDTAS-HGCNGGWMNNAFQYIITNGGIDTQQNYPYSAVQGSCKP- 98
                                ***:**:     **:**    *:  :   * :  .. ***   :   *

papain                          EKGPYAAKTDGVRQVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYRGGI 148
caricain                        QVGGPIVKTSGVGRVQPNNEGNLLNAIAKQPVSVVVESKGRPFQLYKGGI 148
cysteineprotease-4H.annuus      KDVSETVTISGYHDVPRNNEDSFLKALANQPISVAIEASGRDFQFYSGGV 149
asclepain-cII                   -QKKKVAKISGYRRVPGNNEGQLQSAVAQQVVSVAVKSKSDDFRFYSGGI 145
asclepain-f                     -QKEKVVKISGYKRVPRNNGGQLQSAVAQQVVSVAVKCESKDFQFYDRGI 145
asclepain-cI                    -QKEKVVKISGYRNVRRNDEKELQLVVAQQVVSIGIKSSSRDFQHYRQGI 145
ervatamin-B                     -YRLRVVSINGFQRVTRNNESALQSAVASQPVSVTVEAAGAPFQHYSSGI 147
                                      .. .*   *   :   :   :*.* :*: ::. .  *: *  *:

papain                          FVGPCGNKVDHAVAAVGYGPN----YILIKNSWGTGWGENGYIRIKRGTG 194
caricain                        FEGPCGTKVDHAVTAVGYGKSGGKGYILIKNSWGTAWGEKGYIRIKRAPG 198
cysteineprotease-4H.annuus      FDGHCGTELDHGVAAVGYGTTKGLDYVIVRNSWGPKWGEKGYIRMKRKTG 199
asclepain-cII                   FSGACGKVLDHAVNIVGYGSESGANYWIMRNSWGTNWGGNGYMKVPKNSK 195
asclepain-f                     FSGACGPILDHAVNIVGYGSKGGANYWIMRNSWGTNWGENGYMRIQKNSK 195
asclepain-cI                    FNGACGPKLDHAVNIVGYGSEGGANYWIVRNSWGTGWGEGGYARLPMYSG 195
ervatamin-B                     FTGPCGTAQNHGVVIVGYGTQSGKNYWIVRNSWGQNWGNQGYIWMERNVA 197
                                * * **   :*.*   ****     * :::****  **  **  :

papain                          NSYGVCGLYTSSFYPVKN 212
caricain                        NSPGVCGLYKSSYYPTKN 216
cysteineprotease-4H.annuus      KPHGMCGLYMMASYPT-  215
asclepain-cII                   QLGGYCGIAFLPSYPV-  211
asclepain-f                     HYEGHCGIAMQPSYPV-  211
asclepain-cI                    QVGGYCGIVSQASYPVY- 212
ervatamin-B                     SSAGLCGIAQLPSYPT-- 213
                                   * **:   . **.
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Fig. 3. Multiple Sequence Alignment by CLUSTAL-W of putative mature protein sequences of asclepain cI and asclepain cII against papain (P00784), caricain (P10056), cysteineprotease-4H.annuus (Q84M26), asclepain f (B5BLP0) and ervatamin B (P60994). "*" means that residues in the column are identical in all sequences in the alignment. ":" indicates conserved substitutions. "." means that residues in the column are semi-conserved substitutions.

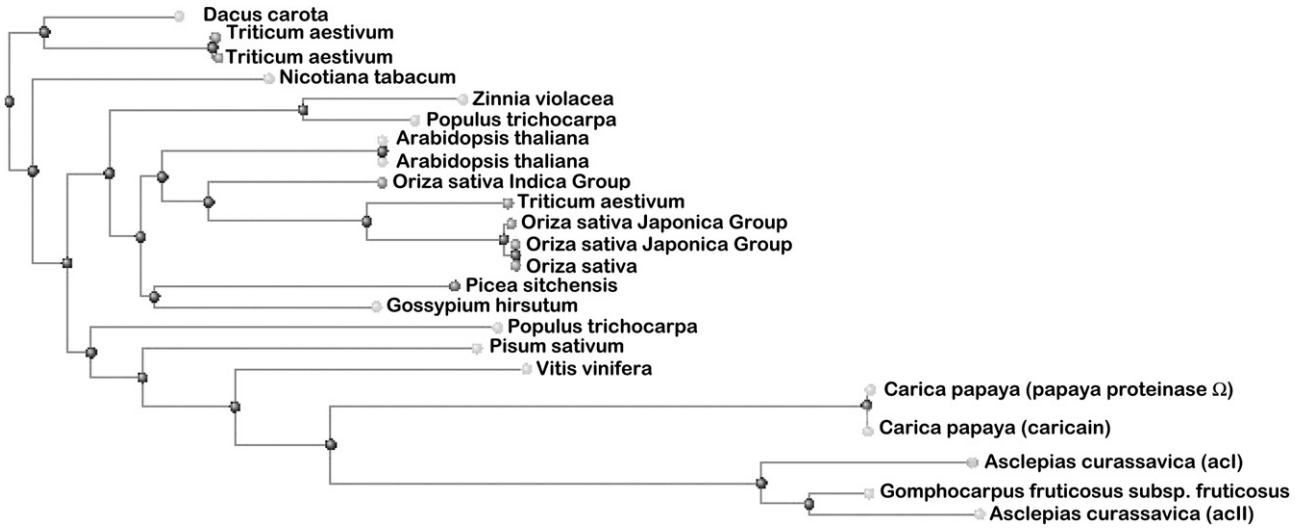

Fig. 4. Phylogenetic tree of plant cysteine peptidases that includes asclepain cI and cII. Evolutionary distance model was constructed with the PSI-BLAST tool, with the database restricted to the *Viridiplanteae* kingdom. Tree method used: neighbor joining, distance according to Grishin (protein) retricted to 0.5.

moreover this cluster contained two distinct subgroups: acI on one side and acII and asclepain f (*A. fruticosa*) on the other. It is noteworthy that the peptidases from latex (Asclepiadaceae and Caricaceae family) formed a group apart, that would indicate a common ancestor.

3.4. Future perspectives

Latices from Asclepiadaceae have been used in folk medicines as antiparasitic agents against gastrointestinal nematodes and in wound healing in addition to other ailments. The pharmacological actions of these milky latices have been attributed to the presence of cysteine peptidases [35–37]. Recently it was established by Shivaprasad and co-workers [37] that the cysteine proteases from *A. curassavica* latex affect haemostasis by exhibiting thrombin like activity mediated by specific cleavage of fibrinogen. This is considered as the basis for the use of plant latex traditionally to stop bleeding on fresh cuts. Examples of other potential biomedical uses are: gastric phytobezoar [38], gastric antiulcerogenic [39], antitumoral agent [40] among a wide variety of uses of papain-like peptidases that have not yet been investigated for acI and acII. Whereas, some potential biotechnological applications comprise the use of these enzymes as meat tenderizers (such as Panol® Purified Papain, Liquipanol® T100 available in the market), component of detergent formulations [41], surfactants synthesis [42] among others.

4. Conclusions

Peptidases asclepain cI and asclepain cII from *A. curassavica* are two isoenzymes belonging to the C1A subfamily (MEROPS database nomenclature). This subfamily is composed of papain-like cysteine peptidases, including some peptidases of bacteria, protozoa, arthropod, mammalian and plant origin; most members of the subfamily are endopeptidases. In addition to its peptidase activity acI and acII hydrolyze a wide variety of synthetic esters and acI also hydrolyzes amide bonds [2,18]. In this paper we have designed a set of primers based on highly conserved sequences of Asclepiadaceae latex cysteine endopeptidases potentially useful for cloning other latex peptidases of the family C1A. We have also presented the partial nucleotide sequence of two cysteine peptidases cDNA cloned from RNA of *A. curassavica* latex. By PMF analysis of acI and acII we have confirmed which cloned cDNA correspond to each isoenzyme. Additionally PMF allowed us to complete the sequences of the mature peptidases by adding the N-terminus determined by Edman's method. PMF could be adopted as an excellent tool to differentiate, in a fast and unequivocal way, peptidases with very similar physicochemical and functional properties. In this sense PMF has advantages on other conventional methods (for instance enzyme kinetics) that are time consuming and afford less reliable results. The robustness of the method even allowed the differentiation of the homologue isoenzymes of the latex of *A. curassavica*.

Based on previous substrate specificity studies and due to the kinship of acI and acII with papain, these enzymes are promising for other biomedical and biotechnological applications.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biochi.2009.07.017.

References

[1] C.D.T. Freitas, J.S. Oliveira, M.R.A. Miranda, N.M.R. Macedo, M. Pereira Sales, L.A. Villas-Boas, M.V. Ramos, Enzymatic activities and protein profile of latex from *Calotropis procera*, Plant Physiol. Biochem. 45 (2007) 781–789.
[2] C. Liggieri, M.C. Arribére, S.A. Trejo, F. Canals, F.X. Avilés, N. Priolo, Purification and biochemical characterization of asclepain c I from the latex *Asclepias curassavica L.* Protein J. 23 (2004) 403–411.
[3] K. Konno, C. Hirayama, M. Nakamura, K. Tateish, Y. Tamura, M. Hattori, K. Kdino, Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex, Plant J. 37 (2004) 370–378.
[4] A. Kush, E. Goyvaerts, M.L. Chye, N.H. Chua, Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree), Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 1787–1790.
[5] F. Fiorillo, C. Palocci, S. Soro, G. Pasqua, Latex lipase of *Euphorbia characias* L.: an aspecific acylhydrolase with several isoforms, Plant Sci. 172 (2007) 722–727.
[6] R. Ghosh, S. Chakraborty, C. Chakrabarti, J.K. Dattagupta, S. Biswas, Structural insights into the substrate specificity and activity of ervatamins, the

papain-like cysteine proteases from a tropical plant, *Ervatamia coronaria*, FEBS J. 275 (2008) 421–434.
[7] A.J. Barrett, N.D. Rawlings, J.F. Woessner, Handbook of Proteolytic Enzymes, Academic Press, London, 1998, pp. XXV.
[8] N.D. Rawlings, F.R. Morton, C.Y. Kok, J. Kong, A.J. Barrett, *MEROPS*: the peptidase database, Nucleic Acids Res. 36 (2008) D320–D325.
[9] L. Polgár, in: A.J. Barret, N.D. Rawlings, J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, Elsevier, Amsterdam, 2004, pp. 1072–1079.
[10] N. Rawlings, A. Barrett, Introduction the clans and families of cysteine endopeptidases, in: A. Barrett, N. Rawlings, J. Woessner (Eds.), Handbook of Proteolytic Enzymes, second ed. Elsevier Academic Press, Amsterdam, 2004, pp. 1051–1071.
[11] R.A.L. van der Hoorn, Plant proteases: from phenotypes to molecular mechanisms, Annu. Rev. Plant Biol. 59 (2008) 191–223.
[12] B. Bölter, A. Nada, H. Fulgosi, J. Soll, A chloroplastic inner envelope membrane protease is essential for plant development, FEBS Lett. 580 (2006) 789–794.
[13] N. Takeda, C. Kistner, S. Kosuta, et al., Proteases in plant root symbiosis, Phytochem 68 (2007) 111–121 T.
[14] J.R. Pagano, F.F. Mendieta, G. Muñoz, R. Daleo, M.G. Guevara, Roles of glycosylation on the antifungal activity and apoplast accumulation of StAPs (*Solanum tuberosum* aspartic proteases), Int. J. Biol. Macromol 41 (2007) 512–520.
[15] A.A. Agrawal, M.J. Lajeunesse, M. Fishbein, Evolution of latex and its constituent defensive chemistry in milkweeds (*Asclepias*): a phylogenetic test of plant defense escalation, Entomol. Exp. Appl. 128 (2008) 126–138.
[16] H.J. Chen, D.J. Huang, W.C. Hou, J.S. Liu, Y.H. Lin, Molecular cloning and characterization of a granulin-containing cysteine protease SPCP3 from sweet potato (*Ipomoea batatas*) senescent leaves, J. Plant Physiol. 163 (2006) 863–876.
[17] S. Sheokand, P. Dahiya, J.L. Vincent, N.J. Brewin, Modified expression of cysteine protease affects seed germination, vegetative growth and nodule development in transgenic lines of *Medicago truncatula*, Plant Sci. 169 (2005) 966–975.
[18] C. Liggieri, W.D. Obregón, S.A. Trejo, N. Priolo, Biochemical analysis of a papain-like protease isolated from the latex of *Asclepias curassavica L.* Acta Biochim. Biophys. Sin. 41 (2) (2009) 154–162.
[19] M.J. Dimitri, Enciclopedia Argentina de Agricultura y Jardinería Editorial, Vol. 1, Acme SACI, Buenos Aires, 1988, pp. 895.
[20] H. Shägger, G. Von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.
[21] M.A. Frohman, On beyond classic RACE (rapid amplification of cDNA ends), PCR Methods Appl. 4 (1) (1994) 40–58.
[22] S.A. Trejo, L.M.I. López, C.V. Cimino, N.O. Caffini, C.L. Natalucci, Purification and characterization of a new plant endopeptidase isolated from latex of *Asclepias fruticosa L.* (Asclepiadaceae), J. Protein Chem. 20 (4) (2001) 445–453.
[23] S.A. Trejo, L.M. López, N.O. Caffini, C.L. Natalucci, F. Canals, F.X. Avilés, Sequencing and characterization of asclepain f: the first cysteine peptidase cDNA cloned and expressed from *Asclepias fruticosa* latex, Planta 230 (2) (2009) 319–328.
[24] S.E. Vairo Cavalli, A. Cortadi, M.C. Arribére, P. Conforti, N.O. Caffini, N.S. Priolo, Comparison of two cysteine endopeptidases from latices of *Morrenia brachystephana* Griseb. and *Morrenia odorata* (Hook et Arn.) Lindley (*Asclepiadaceae*), Biol. Chem. Hoppe-Seyler 382 (2001) 879–883.
[25] W.D. Obregón, M.C. Arribére, S. Morcelle del Valle, C. Liggieri, N.O. Caffini, N.S. Priolo, Two new cysteine endopeptidases obtained from the latex of *Araujia hortorum* fruits, J. Protein Chem. 20 (2001) 17–25.
[26] S. Morcelle del Valle, N. Caffini, N. Priolo, Proteolytic properties of *Funastrum clausum* latex, Fitoterapia 75 (2004) 480–490.
[27] R. Chenna, H. Sugawara, T. Koike, et al., Multiple sequence alignment with the Clustal series of programs, Nucleic Acids Res. 31 (2003) 3497–3500.
[28] A. Marchler-Bauer, J.B. Anderson, P.F. Cherukuri, CDD: a conserved domain database for protein classification, Nucleic Acids Res. 33 (D) (2005) 192–196.
[29] J. Barrett, P.M. Brophy, J.V. Hamilton, Analysing proteomic data, Int. J. Parasitol. 35 (2005) 543–553.
[30] C. Sequeiros, M.J. Torres, S.A. Trejo, J.L. Esteves, C.L. Natalucci, L.M.I. López, Philibertain g I, the most basic cysteine endopeptidase purified from the latex of *Philibertia gilliesii* Hook. *et* Arn. (*Apocynaceae*, Protein J. 24 (2005) 445–453.
[31] S.D. Lewis, F.A. Johnson, J.A. Shafer, Effect of cysteine-25 on the ionization of histidine-159 in papain as determined by proton nuclear magnetic resonance spectroscopy. Evidence for a His-159–Cys-25 ion pair and its possible role in catalysis, Biochemistry 20 (1) (1981) 48–51.
[32] N.D. Rawlings, A.J. Barret, Evolutionary families of peptidases, Biochem. J. 15 (1993) 205–218.
[33] F. Lecaille, E. Authié, T. Moreau, C. Serveau, F. Gauthier, G. Lalmanach, Subsite specificity of trypanosomal cathepsin L-like cysteine proteases. Probing the S2 pocket with phenylalanine-derived amino acids, Eur. J. Biochem. 268 (9) (2001) 2733–2741.
[34] Obregón, W.D., Liggieri, C., Morcelle, S., Trejo, S., Aviles, F.X., Priolo, N., Biochemical and Pmf MALDI-TOF analyses of three novel papain-like plant proteinases. Protein Pept. Lett. 16 (10), in press.
[35] G. Stepek, J.M. Behnke, D.J. Buttle, I.R. Duce, Natural plant cysteine proteinases as anthelmintics? Trends Parasitol. 20 (2004) 322–327.
[36] C.E. Salas, M.T.R. Gomes, M. Hernandez, M.T.P. Lopes, Plant cysteine proteinases: evaluation of the pharmacological activity, Phytochem. 69 (12) (2008) 2263–2269.
[37] H.V. Shivaprasad, R. Rajesh, B.L. Nanda, K.K. Dharmappa, B.S. Vishwanath, Thrombin like activity of *Asclepias curassavica L.* latex: action of cysteine proteases, J. Ethnopharmacol. 123 (2009) 106–109.
[38] A.J. Dwivedi, F. Chahin, S. Agrawal, J. Patel, M. Khalid, Y. Lakra, Gastric phytobezoar: treatment using meat tenderizer, Dig. Dis. Sci. 46 (2001) 1013–1015.
[39] C.F. Chen, S.M. Chen, S.Y. Chow, P.W. Han, Protective effects of *Carica papaya* Linn on the exogenous gastric ulcer in rats, Am. J. Chin. Med. 9 (1981) 205–212.
[40] A. Bellelli, M. Mattioni, V. Rusconi, M.L. Sezzi, L. Bellelli, Inhibition of tumor growth, invasion and metastasis in papain-immunized mice, Invasion Metastasis 10 (1990) 142–169.
[41] S.S. Khaparde, R.S. Singhal, Chemically modified papain for applications in detergent formulations, Bioresour. Technol. 78 (1) (2001) 1–4.
[42] S.R. Morcelle, C.S. Liggieri, M.A. Bruno, N. Priolo, P. Clapés, Screening of phytoproteases for the synthesis of arginine-based surfactants, J. Mol. Catal., B Enzym. 57 (2009) 177–182.

Glossary

acI: asclepain cI
acII: asclepain cII
AMV: avian myeloblastosis virus
ACN: acetonitrile
blast: Basic Local Alignment Search Tool
CD: conserved domains
CE: crude extract
DTT: dithiothreitol
cDNA: complementary desoxyribonucleic acid
E: expected value
IPTG: isopropyl-beta-D-thiogalactopyranoside
LB: Luria-Bertani Broth
MALDI TOF-MS: matrix assisted laser desortion ionization time of flight mass spectrometry
PCR: polymerase chain reaction
PMF: peptide mass fingerprint
PSI-blast: Position-Specific Iterated-blast
RACE: rapid amplification of cDNA ends
RNA: ribonucleic acid
RT: retrotranscription
S: score
TFA: trifluoroacetic acid
X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside