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Cloning, Sequencing, and Identification Using Proteomic Tools of a Protease from *Bromelia hieronymi* Mez

Mariela Anahí Bruno · Sebastián Alejandro Trejo · Francesc Xavier Avilés · Néstor Oscar Caffini · Laura Maria Isabel López

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Abstract Fruits of *Bromelia hieronymi*, a tropical South American plant, possess a high content of peptidases with potential biotechnological uses. Total RNA was extracted from unripe fruits and peptidase cDNA was obtained by 3′RACE-PCR. The consensus sequence of the cysteine peptidase cDNA contained 875 bp, the 690 first ones codifying for a hypothetical polypeptide chain of the mature peptidase, named Bh-CP1 (molecular mass 24.773 kDa, pI 8.6, extinction molar coefficient 58,705 M^{-1} cm^{-1}). Bh-CP1 sequence shows a high percentage of identity with those of other cysteine plant proteases. The presence of highly preserved residues is observed, like those forming the catalytic site (Gln19, Cys25, His159, and Asn175, papain numbering), as well as other six Cys residues, involved in the formation of disulfide bounds. Molecular modeling results suggest the enzyme belongs to the $\alpha+\beta$ class of proteins, with two disulfide bridges (Cys23–Cys63 and Cys57–Cys96) in the α domain, while the β domain is stabilized by another disulfide bridge (Cys153–Cys203). Additionally, peptide mass fingerprints (PMFs) of the three peptidases previously isolated from *B. hieronymi* fruits (namely hieronymain I, II, and III) were performed and compared with the theoretical fingerprint of PMF of Bh-CP1, showing a partial matching between the in silico-translated protein and hieronymain II.

Keywords *Bromelia hieronymi* · Bromeliaceae · Cloning · Sequencing · Cysteine endopeptidase · Hieronymain II · Proteomic tools

M. A. Bruno · N. O. Caffini · L. M. I. López (✉)
Laboratorio de Investigación de Proteínas Vegetales, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115, C.C. 711, B1900AVW La Plata, Argentina
e-mail: lmilopez@biol.unlp.edu.ar

S. A. Trejo · F. X. Avilés
Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Campus Universitari 08193, Bellaterra, Cerdanyola del Vallès, Barcelona, Spain

Introduction

Proteolytic enzymes constitute one of the most important group of enzymes currently used in food technology as meat tenderizers, in preparation of bread and beer, as well as cheesemaking. Proteolytic enzymes are obtained from a wide range of sources, and the most recent innovations in this regard relate to their recombinant production [1].

In addition, many commercial proteolytic enzymes from animal, vegetable, fungal, or bacterial origin are used to obtain protein hydrolysates to increase food digestibility, decrease their allergenicity, or to obtain small peptides with biological activities. Some examples are the use of alcalase, trypsin, chymotrypsin, and pepsin on soy protein [2]; pancreatin, alcalase, and bromelain on caseins [3]; alcalase on egg yolk proteins; [4] and cardosins on goat and sheep milk [5].

According to a recent review, information on most plant proteases is restricted to a few number of families: Asclepiadaceae, Asteraceae, Bromeliaceae, Caricaceae, Moraceae, Poaceae, Cucurbitaceae, Euphorbiaceae, and Fabaceae [6].

One of the best known plant proteases, bromelain, is obtained from pineapple (*Ananas comosus*, Bromeliaceae), containing at least four peptidases (stem bromelain, fruit bromelain, ananain, and comosain) that have already been purified and characterized [7, 8].

Bromelia hieronymi (Bromeliaceae) is an indigenous plant from the North of Argentina that has been previously studied by us. From the unripe fruits of this species, three cysteine peptidases have been isolated and characterized [9–11]. Recently, a partially purified enzyme preparation from *B. hieronymi* fruits was able to clot milk and to hydrolyze bovine casein and milk whey proteins. This enzyme preparation is appropriate for cheese making, as an alternative or in addition to calf rennet. On the other hand, this new enzyme preparation showed to be suitable for the production of milk protein hydrolysates that can be used in the designing of new dietary products, as well as to obtain potentially bioactive peptides [12, 13]. In the present work, a protease named Bh-CP1 from *B. hieronymi* fruits has been cloned, sequenced, and compared with other plant proteases. The use of proteomics tools allowed us to detect their correspondence with one of the three endopeptidases from *B. hieronymi* previously isolated and biochemically characterized.

Materials and Methods

Plant Material

B. hieronymi Mez (folk name “chaguar”) is a stoloniferous plant having water pounding rosettes, with spiny leaves. Flowers are located in the axil of little bracts, giving place to terminal, purple glabrous panicles. Fruits are fusiform and fibrous berries, about 2×5 cm long [10]. Plant material was collected in Santiago del Estero, Argentina. A voucher specimen (Leg. Venturi, LP 7050) is deposited at the herbarium of the Vascular Plant Division, Faculty of Natural Sciences and Museum, La Plata University, Argentina.

Chemicals

Ampicillin, dithiotreitol (DTT), and iodoacetamide were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Coomassie Brilliant Blue R-250, acrylamide, and bisacrylamide were obtained from Bio-Rad (Hercules, CA, U.S.A.). EcoTaq DNA polymerase was obtained from ECOGEN Barcelona (Barcelona, Spain). Trypsin Gold and pGEM-T Easy

vector were products from Promega Corporation (Madison, WI, USA). DNA Molecular weight markers X and III, IPTG, X-Gal, and SalI restriction endonuclease were obtained from Roche (Roche Diagnostics-Roche Applied Science, Indianapolis, IN, U.S.A.). Peptide calibrants and α-cyano-4-hydroxycinnamic acid were purchased from Bruker Daltonics (Bremen, Germany). *Escherichia coli* XL1-Blue was purchased from Stratagene (La Jolla, CA, USA). Other reagents used were of the highest grade available.

Peptidases Isolation and Purification

Crude extract was obtained by chopping and homogenizing frozen fruits with 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA and 5 mM cysteine as protective agents. The homogenate was filtered and centrifuged and supernatants were collected and treated with acetone, as described in a previous work [9]. The partially purified extract was used to obtain three purified peptidases by ionic exchange chromatography: hieronymain I [9], hieronymain II [10], and hieronymain III [11].

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Miniprotean III Cell (Bio-Rad) according to Laemmli [14]. Current was kept constant at 40 mA during stacking and then increased to 60 mA and kept constant for 40 min. Gels (12.5% polyacrylamide) were stained with Coomassie Brilliant Blue R-250.

RNA Isolation

Fruits were carefully cleaned with tap water. About 2 g of unripe fruits devoided of seeds were crushed in a mortar with liquid nitrogen. Fractions of 0.2 g were employed for total RNA extraction, using the comercial kit Invisorb Spin Plant RNA Mini Kit (Invitek, Berlin). Total RNA was extracted as recommended by the manufacturers employing spin columns that traps nucleic acids and then eluted with 50 μl RNase-free elution buffer provided by the kit.

Specific Primers

Forward primers were designed taking into account the N-terminal amino acid sequences of the three peptidases (hieronymain I, II, and III) previously isolated from *B. hieronymi* as well as the nucleotidic sequence of fastuosain [15], a peptidase isolated from *Bromelia fastuosa*, on the basis of the high percentage of identity of the N-terminal sequences (nine amino acids): 100% of identitiy with hieronymain II and III and 78% with hieronynmain I. On the other hand, two reverse primers were used: an oligo(dT)R_1R_0 primer, that was employed in RT-reaction, and the R_0 primer used in PCR (Table 1). Primers were synthesized at TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany).

cDNA Synthesis and Amplification

To prepare the cDNA, a commercial kit (First-Strand cDNA Synthesis Kit for RT-PCR, AMV, Roche), and oligo(dT)R_1R_0 was employed. The reaction was carried out using the thermal cycler EZ Cycler (Ericomp Inc., San Diego, CA, USA). The complete retrotranscription was performed in four successive steps: 10 min at 25 °C; 120 min at 42 °C; 5 min at 99 °C; and 5 min at 10 °C. cDNA amplification was achieved using the same thermal cycler

Table 1 Primers used for cDNA cloning of BH-CP1 protease

Primer	Orientation	Sequence (5′→3′)
Nt-H_1	Forward	GCGCTGCCTGAAAGTATTGATTGGAG
Nt-H_{2-3}	Forward	GCGGTGCCTCAAAGTATTGATTGGAG
oligo(dT)R_1R_0	Reverse	CCGGAATTCACTGCAGGGTACCCAATACGACTCACTAT AGGGCTTTTTTTTTTTTTTTT
R_0	Reverse	CCGGAATTCACTGCAG

employing the specifics primers Nt-H_1, Nt-H_{2-3}, and R_0 and EcoTaq DNA polymerase (Ecogen, from *Thermus aquaticus*) according to the following protocol: a first cycle of 95 °C for 5 min and 72 °C for 15 min; then 40 cycles composed by three steps: 1 min at 94 °C, 1 min at 48 °C, and 2 min at 72 °C, followed by a final extension step at 15 min at 72 °C, then the reaction mix was kept at 10 °C for 16 h. Amplification products were analyzed by horizontal electrophoresis (Blue Marine 200, Serva; power supply, Biotech EPS 200, Pharmacia) on 2% agarose gel containing ethidium bromide to visualize DNA bands using an UV trans-illuminator. The molecular mass of the DNA fragments was estimated by comparison with molecular weight markers (Roche, DNA Marker X and III).

Cloning and Sequencing of cDNA

Amplified products of about 900 bp, representing the expected size for molecular masses of plant cysteine peptidases, were gel-purified employing a commercial DNA extraction kit (QIAEX II Agarose Gel Extraction, QIAGEN GmbH, Hilden, Germany). Isolated cDNA was ligated into the vector pGEM-T Easy (Promega Corporation, Madison, WI, USA) and transformed into competent *E. coli* XL1-Blue cells [16] and cultivated on Petri dish with LB medium containing ampicillin (100 μg/ml), IPTG (50 μg/ml), and X-Gal (50 μg/ml) overnight at 37 °C. White colonies were selected and transferred to liquid LB medium containing ampicillin (50 μg/ml) and cultured overnight at 37 °C. Plasmidial DNA containing inserts were extracted employing a commercial DNA preparation (Fast Plasmid Mini Kit, Eppendorf). Purified plasmids were digested with *Sal* I restriction endonuclease (Roche) and analized on 1% agarose gel electrophoresis. Plasmids containing inserts were sequenced in both directions (Servei de Seqüenciació i Síntesi d'Àcids Nucleics, Facultat de Veterinària de la UAB, Barcelona, Spain). Chromathograms were analyzed with the software Chromas v.2.0 (Technelysium Pty Ltd.).

Sequence Analysis

cDNAs sequences from selected clones were analyzed with the Clustal-W multiple alignment program [17] to obtain a consensus sequence, which was traslated in the six possible reading frames by using the Translate Tool program (http://www.expasy.ch/tools/dna.html) to identify the presence of conserved motifs in the amino acid sequences of cysteine endopeptidases. Deduced amino acid sequences were used as inputs for database searching using the Basic Local Alignment Search Tool (BLAST) algorithm [18].

Peptide Mass Fingerprinting

In-gel protein digestion was performed using the In-Gel DigestZP Kit (Millipore, Billerica, MA, USA). Protein bands corresponding to hieronymain I, II, and III were excised from

SDS-PAGE with a scalpel, diced into 1-mm^3 pieces and introduced into the Zip-Plate wells, treated with 25 mM NH_4HCO_3/5% acetonitrile (ACN) and then with 25 mM NH_4HCO_3/50% ACN (washing/destaining solutions) and finally dehydrated with pure ACN. Proteins were then reduced with 10 mM DTT in 25 mM NH_4HCO_3/5% ACN buffer (pH 8.0) for 30 min at room temperature. Cys sulphydrils were alkylated with 50 mM iodoacetamide in 25 mM NH_4HCO_3/5% ACN for 30 min at 37 °C. Washing/destaining solutions were used for additional washing (twice) and finally the gel pieces were dehydrated with pure ACN, and then dried. Trypsin (0.02 μg) in 25 mM NH_4HCO_3/5% ACN was added to rehydrate the gel pieces in each well and the digestion was carried out for 3 h at 37 °C. The tryptic peptides obtained were extracted from the gel with 0.2% trifluoroacetic acid (TFA) and captured on the C18 resin applying vacuum. Finally, the tryptic peptides were eluted with 50% ACN/0.1% TFA. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) was used for protein identification by peptide mass fingerprint (PMF). Analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The sample was spotted on an MTP GroundSteel 600/384 target 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). External calibration was performed using a peptide calibration standard mixture (Bruker Daltonics, 1,047.19–3,149.57 Da). Peptide masses were acquired with a range of 800–3,000 m/z. PMF spectra of samples analyzed were acquired and compared. Comprehensive peak assignments were accomplished using the Bruker Daltonics BioTools 3.1 software [19].

Results and Discussion

Cloning of Bh-CP1

Total RNA was extracted from *B. hieronymi* fruits grinded in liquid nitrogen and used to obtain the cDNA encoding a putative cysteine peptidase by 3′RACE-PCR. The first cDNA strand was prepared by retrotranscription using the oligo(dT)R_1R_0 primer. This product was used as a template for PCR amplification, using the specific forward primers Nt-H_1 and Nt-$H_{2–3}$ and the reverse primer R_o. The amplified products were checked by agarose gel electrophoresis. Best amplification results were obtained with Nt-$H_{2–3}$ primer and 48 °C annealing temperature (Fig. 1). Fragments of 900 bp were observed, the expected size (24 kDa) for cysteine proteinase found in plant fruits. Selected bands in agarose gel electrophoresis were cut out and the cDNA was gel-purified, ligated into the pGEM-T Easy vector, and transformed into competent *E. coli* (XL1-Blue) cells. cDNA sequences obtained from different clones were analyzed, and after alignment a consensus sequence was obtained and named Bh-CP1. The full sequence of the enzyme was deposited in the EMBL nucleotide sequence database (GenBank accession number FN645748.1).

Sequence Analysis of Bh-CP1

The consensus sequence of Bh-CP1 (Fig. 2) was composed by 875 nucleotide residues, the initial 690 residues coding for the mature peptidase sequence, followed by the stop codon (tga), the polyadenylation signal (aataaa), and the polyadenine tail consisting of 24 residues.

The deduced protein was composed by 230 amino acids and contained seven cysteine residues, like most plant cysteine endopeptidases [7].

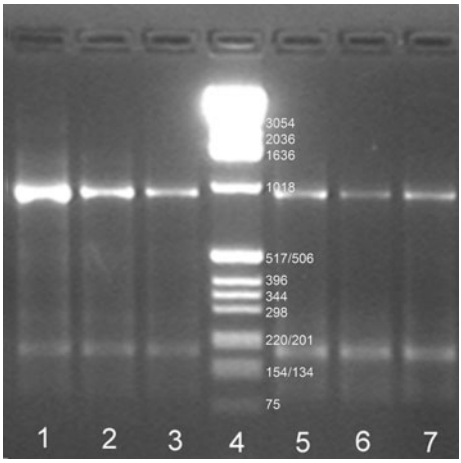

Fig. 1 Agarose gel electrophoresis of RT-PCR products using Nt-H_{2-3} primer. *Lanes 1–3*: undiluted samples, at 48, 50.8, and 54 °C, respectively. *Lane 4*: DNA standards (DNA MW Marker X, Roche). *Lanes 5–7*: samples diluted (1/10), at 48, 50.8, and 54 °C, respectively

The calculated molecular mass and the molar extinction coefficient at 280 nm of Bh-CP1 were 24.773 kDa and 58,705 M^{-1} cm^{-1}, respectively, obtained by analysis with ProtParam tool from the ExPASy Proteomics server (http://www.expasy.org). The calculated pI was 8.6, close to that determined for hieronymain II [10]. Data values were calculated on the basis of the existence of three disulfide bridges in the active form the enzyme.

```
1    A  V  P  Q  S  I  D  W  R  D  Y  G  A  V  T  S  V  K  N  Q
1    gcggtgcctcaaagtattgattggagagactatggtgccgtcacaagtgtcaagaatcaa
21   G  R  C  G  S  C  W  S  F  S  A  I  A  T  V  E  G  I  Y  K
61   ggcagatgcggttcttgctggtcattctctgcaattgcgacggtcgagggaatttacaaa
41   I  K  T  G  N  L  V  S  L  S  E  Q  E  V  L  D  C  A  V  S
121  atcaaaacagggaacttagtatctctatcggagcaagaagttctcgattgtgctgttagc
61   H  G  C  K  G  G  W  V  D  K  A  Y  N  F  I  I  S  N  N  G
181  cacgggtgcaaaggcgggtgggtggacaaggcctacaacttcatcatatctaacaacggt
81   V  T  S  A  A  Y  Y  P  Y  K  G  Y  Q  G  T  C  G  A  N  S
241  gtgacctccgcagcttactatccttataaaggataccaaggcacctgcggtgccaatagc
101  V  P  N  A  A  Y  I  T  G  Y  K  Y  V  Q  R  N  N  E  R  S
301  gtgcccaatgcagcctacattactggttataaatatgtgcagaggaacaacgaacgcagc
121  M  M  Y  A  L  S  N  Q  P  I  A  A  L  I  D  A  S  G  K  N
361  atgatgtacgctttgtcgaatcaacctatagctgctctcattgacgctagcgggaaaaac
141  F  Q  Y  Y  K  G  G  V  Y  S  G  P  C  G  T  S  L  N  H  A
421  tttcaatattacaaaggcggtgtgtatagcggaccttgtggaactagtctcaatcatgcc
161  I  T  V  I  G  Y  G  Q  D  S  S  G  I  K  Y  W  I  V  K  N
481  atcaccgttataggttacgggcaagatagcagcggaataaaatattggatagtaaagaac
181  S  W  G  T  S  W  G  E  R  G  Y  I  R  M  A  R  D  V  S  S
541  tcatggggtacttcatggggtgagcgtggatatattcgtatggcgagagatgtgtcatct
201  S  G  I  C  G  I  A  M  A  P  L  F  P  T  L  D  S  G  A  N
601  tcaggaatatgtggaattgccatggctcctcttttcccactctagattcaggagccaat
221  V  E  V  L  K  M  V  S  E  T  stop
661  gtcgaagttcttaagatggtttctgaaacttgaagctccgtatgagtcggaagcgatgat
721  ctagcaatgcactacaatgtgctataaataaaggatgttcgcatccaattgtacttccct
781  tgcttcgacgttgtggttttctgttatatttcgttgtatccataccagtgagtatgtacg
821  tttgaatatctacatttgaattaatgtgctccttaattaaaaaaaaaaaaaaaaa
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Fig. 2 Nucleotide and deduced amino acid sequences of Bh-CP1.The stop signal (tga) and the polyadenylation signal sequence (aataaa) are *underlined*. *White on a black background* sequence corresponds to Nt-H_{2-3} primer. *Black on a gray background* correspond to cysteine residues

Amino acid sequence of Bh-CP1 was analyzed using the BLAST algorithm [18], revealing that Bh-CP1 belongs to the C1A subfamily, whose archetype protease is papain: identities=100/216 (46%), positives=146/216 (67%), gaps=9/216 (4%). The highest percentage identity was observed with those of ananain [8], followed by fastuosain [15], fruit bromelain [20], macrodontain [21], and stem bromelain [22] (78%, 82%, 83%, 77%, and 68%, respectively), all of them endopeptidases belonging to the Bromeliaceae family.

The alignment of primary structures is shown in Fig. 3. As expected, a strong conservation was observed, particularly for those amino acid residues which are essential for catalytic activity: Cys25 and His159 (papain numbering), which constitute the catalytic dyad in all cysteine peptidases, as well as active-site residues Gln19 and Asn175 (papain numbering), characteristic of Clan CA. Also, Phe141, Trp177, and Trp181 (papain numbering), involved in the hydrophobic pocket of the catalytic site, are conserved in Bh-CP1 [23]. In addition the Cys26 residue of the active site, six Cys residues are found: Cys23, Cys57, Cys63, Cys96, Cys153, and Cys204, probably involved in disulfide bridges, as it happens in papain [24].

A phylogenetic tree of plant cysteine peptidases including BhCP1, as inferred by the neighbor joining method [25], is shown in Fig. 4. It is seen that the endopeptidases from Bromeliaceae clearly constitute a separate group that would indicate a common ancestor, thus providing a contribution to chemosystematics of the Bromeliaceae family.

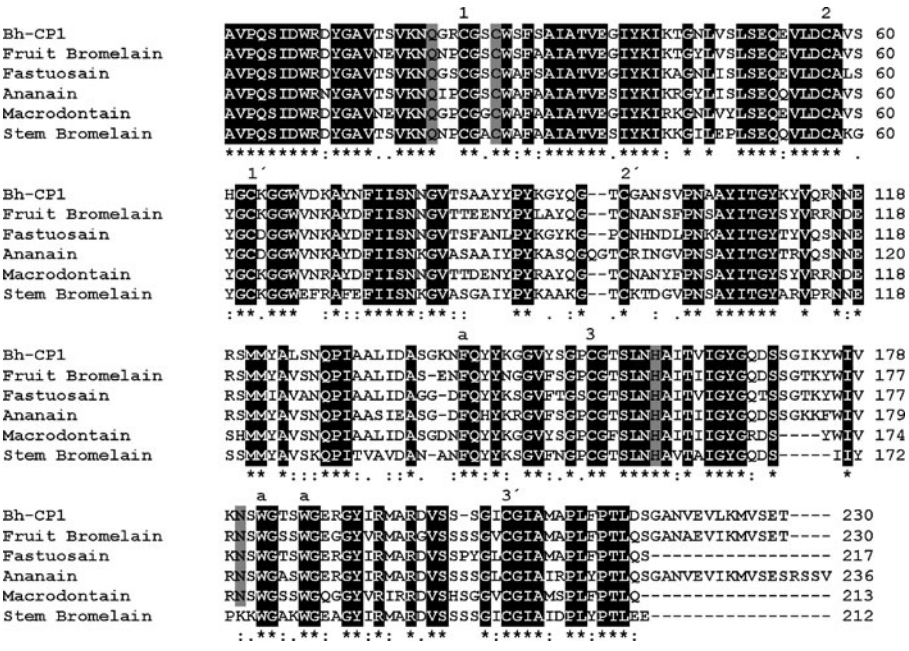

Fig. 3 Sequence alignment of Bh-CP1 with proteases from the Bromeliaceae family. Bh-CP1 sequence was aligned with the sequences of fruit bromelain, fastuosain, ananain, macrodontain, and stem bromelain. Active-site residues are *black on a gray* background. Conserved motifs and conserved amino acid residues are *white on a black* background. Cysteines involved in disulfide bridges are *underlined* (1–1′, 2–2′, and 3–3′). *Dashes* represent gaps introduced to maximize alignment. *a* indicate the amino acids involved in the hydrophobic pocket. *Asterisks* mean that residues in the column are identical in all sequences in the alignment, *colons* indicate conserved substitutions, and *dots* mean that residues in the column are semi-conserved substitutions. *No symbol* means variant residue

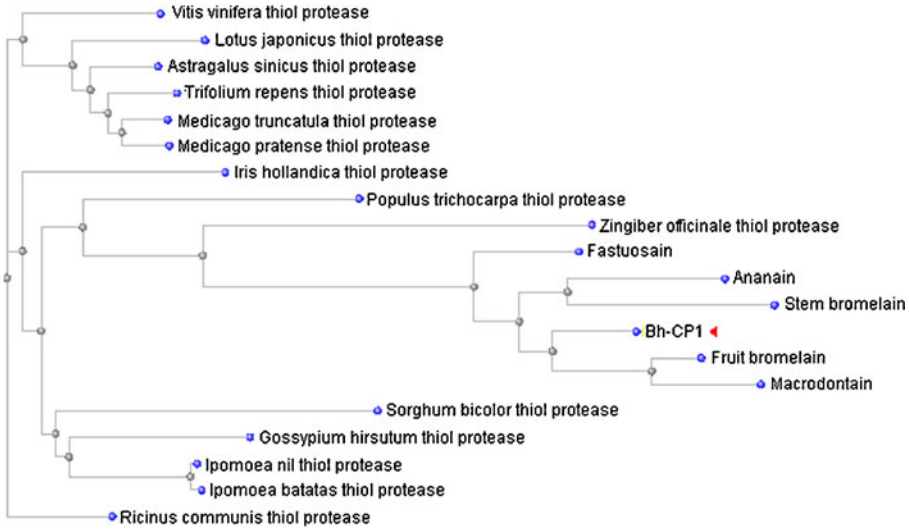

Fig. 4 Phylogenetic tree of plant cysteine peptidases that includes Bh-CP1. Evolutionary distance model was constructed with the PSI-BLAST tool. Tree method used: neighbor joining, distance according to Grishin, restricted to 0.5 (maximum sequence difference)

Homology Modeling of the Mature Protein Sequence

The structural homology model (Fig. 5) was predicted based on the of Bh-CP1 sequence and employing the data processed at the ESyPred3D Web Server 1.0 [26], that determines the three-dimensional model for proteins based on the homology with a known structure (papain, in this case). The model was interpreted with PyMOL 1.2r2 (Shrödinger LLC, Portland, OR 97204, USA), suggesting that the enzyme belongs to the α+β class of proteins, with two disulfide bridges (Cys23–Cys63 and Cys57–Cys96) in the α domain, while the β domain is stabilized by another disulfide bridge (Cys153–Cys203). The model shows the presence of six alpha-helixes (Trp8–Tyr11, Cys26–Thr43, Glu51–Leu55, Val68–Asn78, Glu118–Asn127, and Lys139–Tyr143) and six beta-sheets (Thr108–Tyr112, Ile130–Ile134, Asn158–Asp169, Gly172–Lys179, Tyr191–Met194, and Leu210–Tyr213). A central groove divides the peptidase into two domains, with the active site located towards the bottom of this groove.

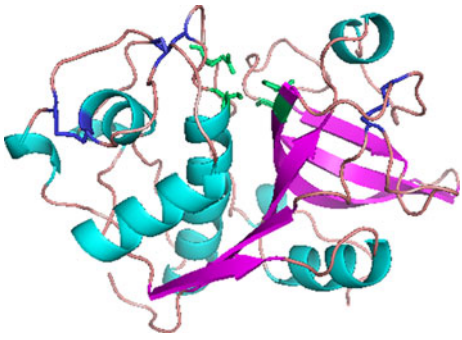

Fig. 5 3D-JIGSAW model of Bh-CP 1. alpha-helixes, *cyan*; beta-sheets, *magenta*; loops, *salmon*; active site, *lime green*; disulphide bridges, *tv-blue*

Table 2 Molecular mass values of tryptic digests from hieroymain I, hieroymain II, hieroymain III

Hieronymain I	Hieronymain II	Hieronymain III
1,086.548	978.565	1,000.749
1,285.694	*1,000.541*	*1,071.680*
1,296.686	*1,071.515*	*1,179.605*
1,347.736	*1,179.475*	1,195.598
1,363.738	1,195.475	1,760.892
1,457.774	1,211.469	1,948.914
1,609.761	*1,992.008*	2,019.994
2,203.094	*2,081.053*	2,211.072
2,229.154	*2,097.044*	2,283.157
2,376.244	*2,113.056*	2,434.228
2,447.274	*2,221.084*	2,526.247
2,553.312	2,316.114	2,844.376
2,584.454		
2,635.423		

Matching peptides with theoretical tryptic digestion of Bh-CP1 are shown in italics

Peptide Mass Fingerprinting Analysis

PMFs of the three peptidases previously isolated from *B. hieronymi* fruits (hieronymain I, II, and III) were performed (Table 2) and compared with the deduced PMF of Bh-CP1. The tolerance limit for positive matches was set at 0.045 Da. No mass peptide coincidences were found when comparing hieronymain I and Bh-CP1 and only two mass peptides of hieronymain III matched the mass of Bh-CP1; however, when hieronymain II and Bh-CP1 peptide masses were confronted, eight matches afforded (84.9% intensity coverage and 29.6% sequence coverage). For PMF of Bh-CP1 it was considered that Cys residues had

Table 3 Experimental and theoretical masses of trypsin-digested peptides of Hieronymain II and Bh-CP1, respectively

Experimental PMF of Hieronymain II	Theoretical PMF of Bh-CP1	Δ Mass	# MC	Peptide	Position	Known modifications
1,000.541	1,000.521	−0.020	0	VPQSIDWR	2–9	
1,071.515	1,071.558	0.043	0	AVPQSIDWR	1–9	
1,179.475	1,179.158	0.043	0	NSWGTSWGER	180–189	
1,992.008	1,992.019	0.011	1	AVPQSIDWRDYGAVTSVK	1–18	
2,081.053	2,081.041	−0.012	0	SMMYALSNQPIAALIDASGK	120–139	
2,097.044	2,097.035	−0.009	0	SMMYALSNQPIAALIDASGK	120–139	1×MSO
2,113.056	2,113.030	−0.026	0	SMMYALSNQPIAALIDASGK	120–139	2×MSO
2,221.084	2,221.067	−0.017	1	CGSCWSFSAIATVEGIYKIK	23–42	DEAM

Cysteines have been treated with iodoacetamide to form carbamidomethyl-cysteine (Cys-CAM). For theoretical PMF Cys-CAM, oxidized Cys, oxidized Met (MSO), deamidation (DEAM), 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 0.045 Da

#*MC* number of missed cleavages

been modified with iodoacetamide to form carbamidomethyl-cysteine, as well as some frequent post-translational modifications for this type of proteases, as Met oxidation and Gln and Asn deamidation (Table 3). Taking into account that during the proenzyme processing more than one molecular forms of the mature enzyme can be generated, differing only in their N-terminal residue [27]; in this case the proteolytic cleavage of the proenzyme could be happened either to the right or to the left side of the Ala position and therefore the peptide VPQSIDWR was included.

This proteomic approach provides additional information on structural characterization of hieronymain I, II, and III and affords strong evidence that Bh-CP1 would correspond to hieronymain II.

Bromeliaceae is a family of monocotyledonean plants of around 3,170 species and about 60 genera, including species characterized by possesing an unusual content of endopeptidases. Nevertheless, the sequence of only five Bromeliaceae peptidases has been up to date reported. In the present article the full deduced sequence of a mature proteolytic enzyme of *B. hieronymi* is notified, corresponding to an isoform of hieronymain II, according to results provided by peptide mass fingerprint, a proteomic tool used for the first time in the characterization of Bromeliaceae endopeptidases.

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