Hieronymain I, a New Cysteine Peptidase Isolated from Unripe Fruits of *Bromelia hieronymi* Mez (Bromeliaceae)

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A new peptidase, named hieronymain I, was purified to homogeneity from unripe fruits of *Bromelia hieronymi* Mez (Bromeliaceae) by acetone fractionation followed by cation exchange chromatography (FPLC) on CM-Sepharose FF. Homogeneity of the enzyme was confirmed by mass spectroscopy (MALDI-TOF), isoelectric focusing, and SDS-PAGE. Hieronymain is a basic peptidase (pI > 9.3) and its molecular mass was 24,066 Da. Maximum proteolytic activity on casein (>90% of maximum activity) was achieved at pH 8.5–9.5. The enzyme was completely inhibited by E-64 and iodoacetic acid and activated by the addition of cysteine; these results strongly suggest that the isolated protease should be included within the cysteine group. The N-terminal sequence of hieronymain (ALPESIDWRAKGAVTEVKRQDG) was compared with 25 plant cysteine proteases that showed more than 50% of identity.

1. INTRODUCTION

Cysteine peptidases can be classified in more than 30 families, which can be grouped into five clans (CA, CD, CE, CX, and PA), according to a recent classification (Barret, 2001). The best known family of cysteine peptidases is the papain family (clan CA, family C1), which contains peptidases with a wide variety of activities, including endopeptidases with broad specifity (such as papain), or a narrow specifity (such as glycyl endopeptidase), aminopeptidases, and peptidases with both endo- and exopeptidase activities (like cathepsins B and H). Papain-like cysteine peptidases share similar amino acid sequences (Berti and Storer, 1995) and three-dimensional structures (Turk *et al.,* 1995).

Most plant cysteine peptidases belong to the papain family, including those of Bromeliaceae, the botanical family of pineapple. Bromeliaceae is a plant family whose members usually produce large amounts of proteinases with no apparent function in plant growth and development (Boller, 1986). A number of proteases from species belonging to Bromeliaceae have been isolated and characterized: stem and fruit bromelain, ananain, and comosain, obtained from *Ananas comosus* (Murachi, 1976; Ota *et al.,* 1985; Napper *et al.,* 1994; Rowan and Buttle, 1994; Lee *et al.,* 1997), as well as proteases from fruits of *Bromelia pinguin* (Toro-Goyco *et al.,* 1968, 1980), *B. hemispherica, B. palmeri*, and *B. sylvestris* (Cruz *et al.,* 1974; Hernández Arana *et al.,* 1983), *B. plumieri* (Montes *et al.,* 1990), *B. balansae* (Pardo *et al.,* 2000, Pardo *et al.,* 2001), and *Pseudananas macrodontes* (Natalucci *et al.,* 1996, López *et al.,* 2000, 2001).

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[3] Abbreviations: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid; BLAST, Basic Local Alignment Search Tool; CAPS, 3-(ciclohexylamino)-1-propanesulfonic acid; SP-Sepharose, sulfopropyl-Sepharose; E-64, *trans*-epoxysuccinyl-L-deleucylamido-(4-guanidino)butane; EDTA, ethylendiaminetetraacetic acid; IEF, isoelectric focusing; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MOPS, 3-(*N*-morpholino) propanesulfonic acid; TAPS, *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

The presence of endopeptidases in fruits of *Bromelia hieronymi* Mez (*Bromeliaceae*) had been previously reported (Priolo *et al.,* 1991). In this paper the isolation and partial characterization of the main cysteine proteinase of unripe fruits of *Bromelia hieronymi* Mez (*Bromeliaceae*) are reported by using an alternative purification strategy.

2. MATERIALS AND METHODS

2.1. Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, OH). AMPSO,[3] CAPS, cysteine, E-64, EDTA, IEF standards, glycine, iodoacetic acid, MOPS, sinapinic acid, TAPS, and Tris were purchased from Sigma Chemical Company (St. Louis, MO). Coomassie brilliant blue R-250, acrylamide, bisacrylamide, and low-range molecular weight standards were obtained from Bio-Rad (Hercules, CA). CM-Sepharose Fast Flow and Pharmalyte 3-10 were purchased from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were obtained from commercial sources and were of the highest purity available.

2.2. Plant Material

Bromelia hieronymi Mez (folk name "chaguar") is a stoloniferous plant that has water-pounding rosettes, with spiny leaves. Fruits are fusiform and fibrous berries, approximately 2×5 cm long. Plant material was collected by Professor Lucas Roic from the University of Santiago del Estero, Argentina. Individual fruits were separated from the infrutescence, carefully cleaned with tap water, and stored at $-20°C$ until the beginning of the extraction procedure.

2.3. Crude Preparation

Crude extract was obtained by chopping and homogenizing frozen fruits (50 g) for 2 min in a domestic blender with 250 ml of cold 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA and 5 mM cysteine as protective agents. The homogenate was filtered through a twice-folded piece of gauze to remove plant debris and then centrifuged for 30 min at $16,000 \times g$. Supernatants were collected, filtered when needed, and immediately frozen at $-20°C$ until analysis. All operations were carried out at $0-4°C$ (López *et al.,* 2000).

2.4. Preliminary Purification of Crude Extract

Crude extract was treated with four volumes of cold $(-20°C)$ acetone with gentle agitation and left to settle for 20 min before centrifugation at $16,000 \times g$ for 30 min. The final acetone precipitate was redissolved with one volume of 0.1 M phosphate buffer, pH 6.0, and frozen until further use (Scopes, 1984).

2.5. Protein and Carbohydrate Content

Protein concentration was determined by the Coomassie Blue dye binding method (Bradford, 1976), using bovine serum albumin as standard. In chromatographic fractions, proteins were detected by measuring absorbance of eluates at 280 nm. Carbohydrate content was determined using the phenol-sulfuric method (Dubois *et al.,* 1956).

2.6. Proteolytic Activity Assays

The reaction mixture contained 1.1 ml of 1% (w/v) casein solution in 0.1 M glycine-NaOH buffer (pH 8.9) containing 15 mM cysteine and 0.1 ml of enzyme solution. The mixture was incubated at 37°C and the reaction stopped by the addition of 1.8 ml of 5% (w/v) trichloroacetic acid (TCA). Blanks were prepared by adding TCA to the enzyme, then adding the substrate. The test tubes were centrifuged at $7000 \times g$ for 20 min, and the absorbance of supernatants was measured at 280 nm. An arbitrary enzyme unit (Ucas) was used to express proteolytic activity (Natalucci *et al.,* 1996).

2.7. Effect of Inhibitors and Activity Enhancers

The effect of specific inhibitors (Salvesen and Nagase, 2001) on proteolytic activity was determined by measuring the residual activity on casein at pH 8.9 after preincubation at 37°C for 30 min in the presence of E-64 (100 μM) and iodoacetic acid (0.1 and 1.0 mM). Controls were prepared by preincubating the protease preparation with the appropriate solvent used to dissolve the inhibitors. The effect of activity enhancers was determined by adding different cysteine concentrations (0, 5, 10, 15, 20, 30, and 50 mM) to the reaction mixture, and then caseinolytic activity was measured as mentioned.

2.8. Effect of pH on Enzyme Activity

Proteolytic activity vs. pH was measured on 1% casein solution containing 15 mM cysteine within the pH

range 6.0–12.5 using 50 mM sodium salts of the following "Good" buffers (Good and Izawa, 1972): MES, MOPS, TAPS, AMPSO, and CAPS.

2.9. Isoelectric Focusing and Zymogram

Isoelectric focusing (IEF) was developed on 5% polyacrylamide gels containing broad pH range ampholytes (Biolyte 3-10, Bio-Rad) in a Mini IEF Cell (Model 111, Bio-Rad). Samples were precipitated with 3 volumes of cold ($-20°C$) acetone and centrifuged, with the protein sediments redissolved and precipitated once again with acetone and finally redissolved in half a volume of deionized water. About 1–10 µg of protein was loaded in each case. Focusing was carried out under constant voltage conditions in a stepped procedure: 100 V for 15 min, 200 V for 15 min, and 450 V for 60 min. Gels were fixed and then stained with Coomassie Brilliant Blue R-250. The pI markers used (Sigma) were: amyloglucosidase (pI 3.50), trypsin inhibitor (pI 4.55), β-lactoglobulin a (pI 5.20), carbonic anhydrase II (pI 5.85), carbonic anhydrase I (pI 6.55), myoglobin (pI 6.85 and 7.35), lectins from *Lens culinaris* (pI 8.15, 8.45, and 8.65), and trypsinogen (pI 9.30).

To visualize proteolytic activity, zymograms were performed. An agarose gel was imbibed during 20 min at room temperature with substrate solution (1% casein in Tris-HCl buffer, pH 8.0) and then washed twice with distilled water. Unstained IEF gels were contacted for 15 min at 55°C with the agarose gel. Proteolytic activities became visible as clear bands on the stained agarose gels (Westergaar *et al.,* 1980).

2.10. FPLC Cation-Exchange Chromatography

Cation-exchange chromatography was performed onto a column (Pharmacia XK 16/40, with AK16 adaptors) packed with CM-Sepharose Fast Flow (Pharmacia) and equilibrated with 50 mM Tris-HCl buffer (pH 8.5) at 20°C. After washing the column with the same buffer, the retained proteins were eluted with 75 ml of a linear sodium chloride gradient (0.0–0.15 M) in the starting buffer. The main fraction was rechromatographed in the same way but pH of buffer was slightly lower (7.5) and a little broad sodium chloride gradient (0.0–0.3 M) was used.

2.11. SDS-PAGE

SDS-PAGE was performed in a Miniprotean III Cell (Bio-Rad) according to Laemmli (1970). 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 by Coomassie Brilliant Blue R-250. The molecular weight markers used (Bio-Rad) were: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase bovine (31.0 kDa), ovalbumin (45.0 kDa), serum albumin bovine (66.2 kDa), and phosphorylase B (97.4 kDa).

2.12. Titration of Active Sites with E-64

Titration of the active site was performed as described by Barrett and Kirschke (1981) with some modifications. The enzyme (0.6 µM) was preincubated with the activation buffer (50 mM Tris-HCl, pH 7.5, containing 15 mM cysteine). Fractions (75 µl) were incubated with 25 µl of different concentrations (0–2 µM) of E-64 for 30 min at 37°C and the residual activity was then measured on casein as described previously. The enzyme concentration was established by determining both protein content (Bradford, 1976) and molecular mass value (mass spectrometry).

2.13. Mass Spectrometry

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was used for the determination of purity degree as well as molecular weight of chromatographic fractions. MALDI-TOF mass spectra were acquired on a BRUKER BIFLEX 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) in 0.1% TFA in water/acetonitrile 2:1, and a 1–10 µM protein solution. From this mixture, 1 µl was spotted onto the sample slide and allowed to evaporate to dryness. A protein of known molecular mass (trypsinogen) was used as standard for mass calibration.

2.14. N-Terminal Sequence

The N-terminal sequence was determined by Edman's automated degradation using an Applied Biosystems (model 476) peptide sequencer. Protein homology searches were performed using the BLAST network service (Altschul *et al.,* 1997).

3. RESULTS AND DISCUSSION

Crude extracts obtained from unripe fruits of *Bromelia hieronymi* showed high caseinolytic activity (7.5 Ucas/ml of crude extract; 37.5 Ucas/g of fruit).

As crude extracts contained phenolic compounds, which could oxidize and irreversibly react with proteins, a previous treatment with 4 volumes of acetone was carried out: the redissolved acetone precipitate (RAP) contained 74% of proteins, 80% of total caseinolytic activity, and only 9.9% of soluble sugars with respect to crude extracts. This preparation exhibited maximum activity (higher than 80%) between pH 7.3 and 10.7 (Fig. 1). As can be seen in Fig. 2, no activity loss was observed when RAP was incubated at 37°C during 120 min or at 55°C during 40 min, whereas at 60°C, 80% of activity remained during a period of 30 min. The enzyme was almost completely inactivated by heating for 20 min at 75°C. Enzyme activity increases with temperature, but at 75°C the reaction rate notably diminishes after 2 min because of enzyme denaturation (Fig. 3). Low sodium chloride concentrations (0.2 M) do not affect caseinolytic activity, but diminishes with the increase of salt concentration (52% of residual activity at 2.5 M NaCl).

Proteolytic activity of RAP was irreversible inhibited by E-64 and iodoacetic acid. The addition of cysteine (0–50 mM) increased proteolytic activity up to a maximum value when cysteine concentration was raised to 15 mM. These results strongly suggest that the enzyme preparation contains cysteine-type proteases, as

Fig. 2. Thermal stability of RAP. Activity on casein at pH 8.9 after 2 min at 37°C was taken as 100%. Data points represent the mean value of five determinations and each experiment was repeated twice.

all the other studied proteases belonging to the family Bromeliaceae (Barrett *et al.,* 1998).

Isoelectric focusing of RAP followed by zymogram analysis showed six protein bands (pI = 5.9, 6.4, 7.6, 8.3, and two bands >9.3), most of them proteolytically actives, but only three of which (pI = 6.4, 8.3, and >9.3) proved to be important (Fig. 4). On the basis of these results, cation exchange chromatography was selected for the next purification step.

Fig. 1. Effect of pH on proteolytic activity. Proteolytic activity was measured on 1% casein solution containing 15 mM cysteine. Data points represent the mean value of four determinations and each experiment was repeated twice.
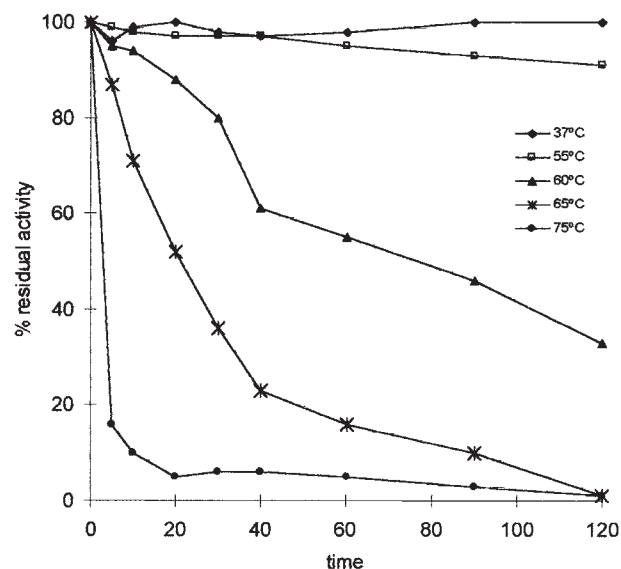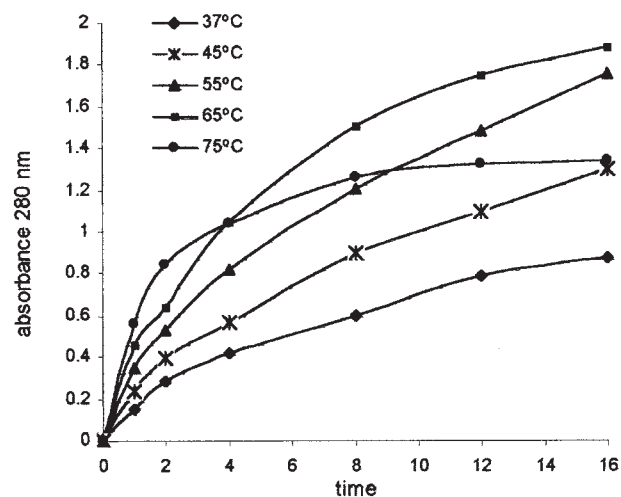

Fig. 3. Enzyme activity of crude extract as a function of temperature. Enzyme activity was determined on casein at pH 8.9.
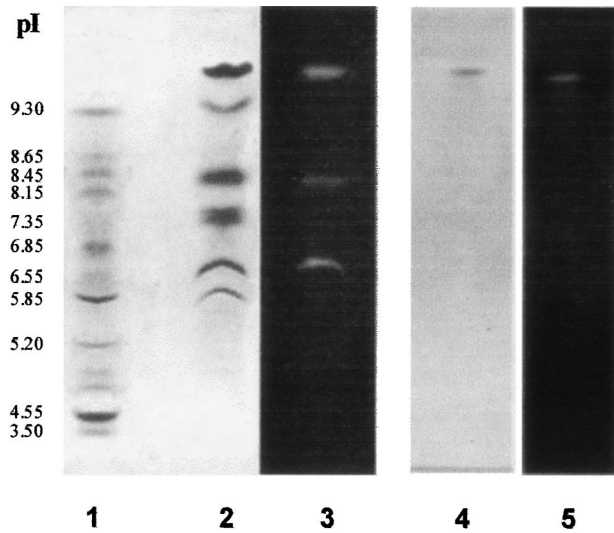
Fig. 4. Isoelectric focusing and zymogram of the redissolved acetone precipitate (RAP). Lane 1, pI markers; lane 2, RAP; lane 3: RAP zymogram; lane 4, hieronymain I; lane 5, hieronymain zymogram.

FPLC cation-exchange chromatography of RAP (Fig. 5) allowed the separation of two basic active fractions, the main of which was rechromatographed (Fig. 6), affording a unique fraction (pI > 9.3, MW = 24,066 Da) that was named hieronymain I, which appeared ho-
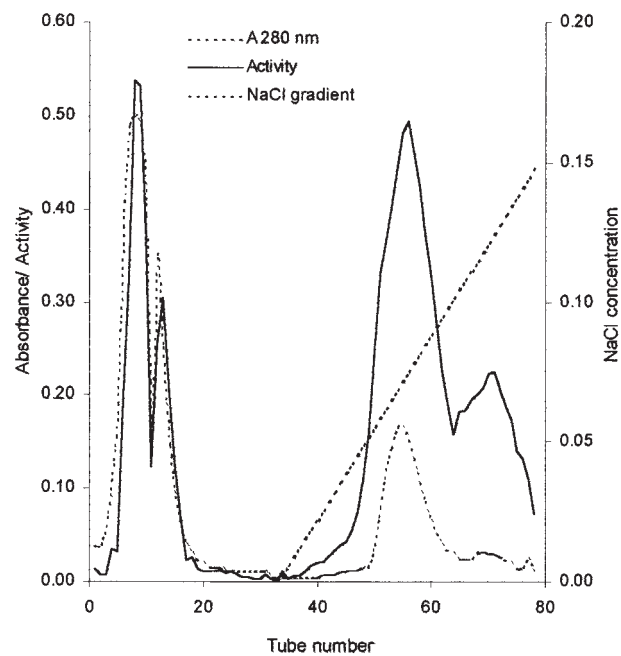

Fig. 6. Rechromatography of the main proteolytic fraction of RAP (CM-Sepharose Fast Flow). Flow rate, 1.0 ml/min. Fractions of 1.8 ml were collected.

mogeneous by IEF (Fig. 4), SDS-PAGE (Fig. 7), and mass spectrometry (Fig. 8). Molecular mass of hieronymain I determined by SDS-PAGE (25 kDa) is higher

Fig. 5. Cation exchange chromatography (CM-Sepharose Fast Flow) of RAP. Flow rate, 1.0 ml/min. Fractions of 1.8 ml were collected.
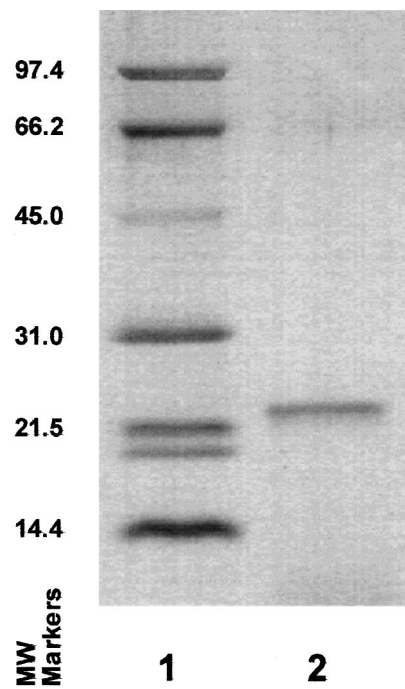
Fig. 7. SDS-PAGE of hieronymain I. Lane 1, low-molecular-weight markers; lane 2, hieronymain I.
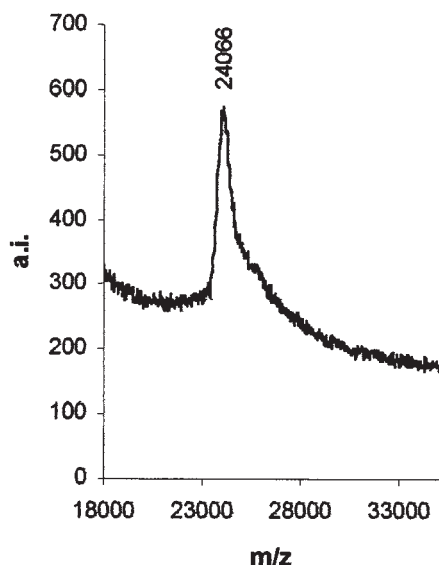
Fig. 8. Mass spectrometry of hieronymain I.

Fig. 9. Titration of active site with E-64.

than those obtained by other methods, as reported for other plant proteases (Harrach *et al.,* 1998).

The purification scheme is presented in Table 1. Yielding of hieronymain I was good enough (21.4%), taking into account that the enzyme is one of several active fractions. The purification factor is very low (1.9-fold), but this is a common fact in plant organs with high proteolytic activity, where proteases represent the bulk of protein content of crude extracts (López *et al.,* 2000).

Maximum proteolytic activity of hieronymain I on casein (>90% of maximum activity) was achieved at pH 8.5–9.5 (Fig. 1). The enzyme was completely inhibited by E-64 and iodoacetic and activated by the addition of cysteine (11 times in relation to the same preparation without the addition of cysteine), suggesting that the isolated protease should be included within the cysteine group.

As far as is known, E-64 and cysteine endopeptidases have been found to react to each other on an equimolecular basis, so this inhibitor can be used for titration of the enzyme active site. In the titration assay
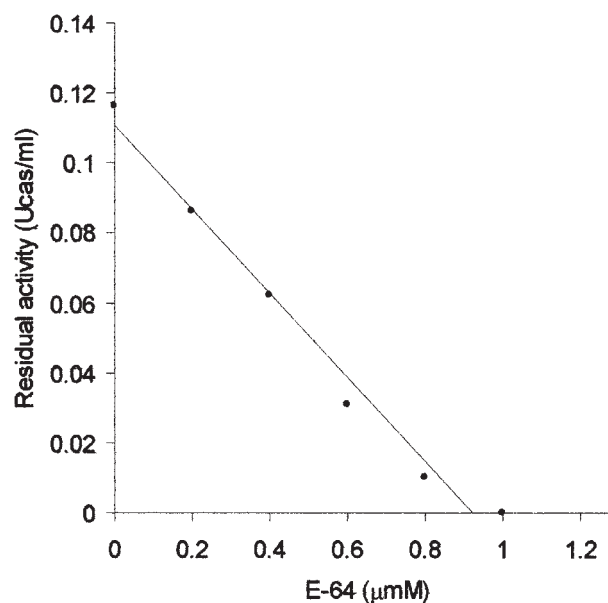
the residual enzyme activity gave a straight line (residual activity vs. E-64 concentration) that intersected the abscissa at 0.92 μM E-64, corresponding to 57% of active enzyme (Fig. 9).

The N-terminus sequence of hieronymain I (ALPE SIDWRAKGAVTEVKRQDG), compared with those of other 25 plant endopeptidases possessing more than 50% identities, is shown in Table 2. A high conservation degree can be observed: Pro2 and Gln19 (papain numbering) and the motifs DWR and GAV are present in the all sequences. The presence of Pro2 would be related with the prevention of unwanted proteolysis by aminopeptidases and Gln19 would help the formation of the "oxyanionic hole" (Rawlings and Barret, 1994).

ACKNOWLEDGMENTS

Table 1. Purification Scheme of Hieronymain I

Step	Activity (U$_{cas}$)	Protein (mg)	Specific activity (U$_{cas}$/mg)	Purification (n-fold)	Yield (%)
Crude	42.9	3.67	11.7	1.0	100.0
RAP	34.3	2.73	12.5	1.1	80.0
First chromatography	15.3	1.07	14.3	1.2	35.7
Hieronymain I	9.2	0.42	21.9	1.9	21.4

Table 2. N-Terminal Amino Acid Sequences of Cysteine Plant Endopeptidases

Plant protease/Source	N-terminal sequence	Reference	% homology
Hieronymain I	ALPESIDWRAKGAVTEVKRQDG		100.0
Arabidopsis thaliana	LPESIDWRKKGAVAEVKDQGG	Koizumi *et al.* (1993)	77
Papaya proteinase IV	LPESVDWRAKGAVTPVKHQGY	Buttle *et al.* (1990)	73
Oryzain α	ALPESVDWRTKGAVAEIKDQGG	Watanabe *et al.* (1991)	73
Pseudotsuga mentziesii	LPESIDWREKGAVTAVKNQGS	Tranbarger and Misra (1996)	73
Zingiber officinale	LPDSIDWREKGAVVPVKNQGG	Choi and Laursen (2000)	73
Zea mays L.	LPESVDWRAKGAVAEVKDQ	Pechan *et al.* (1999)	73
Pisum sativum	VPSSIDWRKKGAVTDVKDQ	Cercos *et al.* (1999)	68
Balansain I	AVPESIDWRDYGAVTSVKNQG	Pardo *et al.,* (2000)	68
Carica candamarcensis	YPESIDWRKKGAVTPVKNQGS	Jaziri *et al.* (1994)	68
Chymopapain (EC 3.4.22.6)	YPQSIDWRAKGAVTPVKNQGA	Taylor *et al.* (1999)	68
Macrodontain I	AVPQSIDWRDYGAVNEVKNQGP	López *et al.* (2000)	64
Bromelain (EC 3.4.22.32)	AVPQSIDWRDYGAVTSVKNQNP	Ritonja *et al.* (1989)	64
Dianthus caryophyllus	LPESVDWRKKGAVSHVKDQGQ	Jones *et al.* (1995)	64
Pea endopeptidase	LPEDFDWREKGAVTPVKDQGS	Guerrero *et al.* (1990)	64
Oryzain β	LPESVDWREKGAVAPVKNQGQ	Watanabe *et al.* (1991)	64
Vicia sativa	VPSSIDWRNKGAVTGVKDQGQ	Shutov and Vaintraub (1987)	62
Comosain	VPQSIDWRNYGAVTSVKNQG	Napper *et al.* (1994)	59
Zinnia elegans	DLPKSVDWRKKGAVSPVKNQGQ	Ye and Varner (1996)	59
Papain (EC 3.4.22.2)	IPEYVDWRQKGAVTPVKNQGS	Kamphuis *et al.* (1984)	59
Ananain (EC 3.4.22.31)	VPQSIDWRDSGAVTSVKNQGR	Lee *et al.* (1997)	59
Papaya peptidase III (EC 3.4.22.30)	LPENVDWRAKGAVTPVRHQGS	Revell *et al.* (1993)	59
Ricinus communis	VPASVDWRKKGAVTSVKDQGQ	Schmid *et al.* (1998)	59
Mung bean endopeptidase	VPASVDWRKKGAVTDVKDQGQ	Yamuchi *et al.* (1992)	59
Macrodontain II	AVPQSIDWRDYGAVNEVKNQNP	López *et al.* (2001)	55
CYP-7 (*Nicotiana tabacum*)	DLPADFDWRDHGAVTGVKNQGS	Linthorst *et al.* (1993)	55

a Shared amino acids are underlined.

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