Isolation and Characterization of Hieronymain II, Another Peptidase Isolated from Fruits of *Bromelia hieronymi* Mez (Bromeliaceae)

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From unripe fruits of *Bromelia hieronymi* Mez (Bromeliaceae), a partially purified protease preparation was obtained by acetone fractionation of the crude extract. Purification was achieved by anionic exchange chromatography (FPLC) on Q-Sepharose HP followed by cationic exchange chromatography (SP-Sepharose HP). Homogeneity of the new enzyme, named hieronymain II, was confirmed by SDS-PAGE and mass spectroscopy (MALDI-TOF-TOF). The molecular mass of was 23,411 Da, and maximum proteolytic activity (more than 90% of maximum activity) was achieved at pH 7.5–9.0 on casein and at pH 7.3–8.3 on Z-Phe-Arg-*p*-nitroanilide. The enzyme was completely inhibited by E-64 and iodoacetic acid and activated by the addition of cysteine. The N-terminal sequence of hieronymain II (AVPQSIDWRVYGAV) was compared with those of 12 plant cysteine proteases which showed more than 70% of identity. Kinetic enzymatic assays were made on Z-Phe-Arg-*p*-nitroanilide ($K_m = 0.72\,mM$, $k_{cat} = 1.82\,seg^{-1}$, $k_{cat}/K_m = 2.54\,seg^{-1}\,mM^{-1}$). No detectable activity could be found on PFLNA or Z-Arg-Arg-*p*-nitroanilide.

KEY WORDS: *Bromelia hieronymi*; Bromeliaceae; cysteine proteinase; plant peptidases.

1. INTRODUCTION

With hundreds of genes encoding proteases, plants are equipped with a large proteolytic machinery that irreversibly regulates the fate of proteins. This machinery has generally been viewed in a housekeeping role, serving to remove non-functional proteins and to release of amino acids for recycling. However, proteases also appear to play key roles in the regulation of biological processes in plants, such as the recognition of pathogens and pests and the induction of effective defense responses (van der Hoorn and Jones, 2004).

Five catalytic types of peptidases can now be recognized, in which serine, threonine, cysteine, aspartic or metallo groups play primary roles in catalysis. The serine, threonine and cysteine peptidases are catalytically

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very different from the aspartic and metallopeptidases in that the nucleophile of the catalytic site is part of an amino acid, whereas it is an activated water molecule in the other two groups. In cysteine peptidases the nucleophile is the sulfhydryl group of a Cys residue and the catalytic mechanism is similar to that of serine-type peptidases in that the proton donor is a His residue. Although there is evidence in some cysteine peptidases that a third residue is required to orientate the imidazolium ring of the His, there are a number of families in which only a catalytic dyad is necessary. Up to date forty one families of cysteine peptidases are recognized, grouped in six clans. Most plant cysteine peptidases belong to the papain family (Clan CA, familiy Cl), including those of Bromeliaceae, the botanical family of pineapple (Rawlings and Barrett, 2004).

Up to date a number of proteases from species belonging to Bromeliaceae have been isolated and characterized (Bruno *et al*., 2003). In the present paper a new proteolytic enzyme isolated from unripe fruits of *Bromelia hieronymi* Mez (Bromeliaceae) is reported.

2. MATERIALS AND METHODS

2.1. Chemicals

Agarose, 3-[(1,1-dimethyl-2-hydroxyethyl) amino]-2-hydroxy-propanesulfonic acid (AMPSO), casein, 3-(ciclohexylamino)-L-propanesulfonic acid (CAPS), cysteine, dithiothreitol, E-64, isoelectrofocusing (IEF) standards, glycine, iodoacetic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *p*-GIu-Phe-Leu-*p*-nitroanilide (PFLNA), sinapinic acid, *N*-tris (hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) and Tris were purchased from Sigma Chemical Company (St. Louis, MO). Coomassie brillant blue R-250, acrylamide, bisacrylamide and low-range molecular weight standards were obtained from Bio-Rad (Hercules, CA). Z-Phe-Arg-*p*-nitroanilide and Z-Arg-Arg-*p*-nitroanilide was provided from Bachem (Torrance, CA). Q-Sepharose High Performance, sulphopropyl-sepharose (SP-Sepharose) High Performance and Pharmalyte 3-10 were purchased from General Electric Amersham Biosciences (Buckinghamshire, UK). 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 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 $2 \times 5 \, cm$ long. Plant material was collected by Ing. 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. Preparation of Plant Extracts

Crude extract was obtained by homogenizing 50 g of unripe fruits on 0.1 *M* sodium phosphate buffer (pH 6.0) containing 5 m*M* EDTA and 5 m*M* cysteine as protective agents (López *et al*., 2000). The crude extract was partially purified by precipitation with 4 volumes of cold acetone for 20 min and further centrifugation at 16,000 *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). This redissolved acetone precipitate (RAP) was characterized and used for further purification steps.

2.4. Protein Content

Protein concentration was determined by the Coomassie blue dye-binding method (Bradford, 1976), using bovine serum albumin as standard. In chromatographic fractions, protein content was determined by measuring the absorbance of eluates at 280 nm.

2.5. Caseinolytic Activity, Inhibition and Activation Assays

The reaction mixture contained 1.1 ml of 1% (w/v) casein solution in 0.1 *M* Tris–HCl buffer (pH 8.0) containing 15 m*M* 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 *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). The same proteolytic determination assay was used in presence of specific inhibitors (Salvesen and Nagase, 2001) E-64 ($100 \mu M$) and iodoacetic acid (0.1 and 1.0 mM) as well as for the observation of activity enhancer effect, which was determined by adding different cysteine amounts to the reaction mixture during the activity determination (Bruno et al., 2003).

2.6. Endopeptidase Activity on Synthetic Substrates

PFLNA (Filippova et al., 1984), Z-Phe-Arg-p-nitroanilide and Z-Arg-Arg-p-nitroanilide (Rowan and Buttle, 1994) were used to determine amidasic activity. The reaction mixture for the former substrate contained 1.5 ml of 0.1 M phosphate buffer (pH 6.5) containing 0.3 M KCl, 0.1 mM EDTA and 3 mM DTT, 0.18 ml of substrate solution and 0.12 ml of enzyme. In the case of the other two substrates the reaction mixture contained 1.4 ml of 0.2 M phosphate buffer (pH 8.0) containing 4 mM EDTA and 8 mM DTT, 0.3 ml of substrate solution and 0.3 ml of the enzyme. In both cases the reaction rate was determined by measuring the absorbance at 420 nm for 3 min, using an UV–visible spectrophotometer Agilent 8453E.

2.7. Effect of pH on Enzyme Activity

Proteolytic activity vs. pH of RAP as well as the new purified endopeptidase was measured on 1% casein solution containing 15 mM cysteine within the pH range 6.0–12.5 using 25 mM sodium salts of the following "Good" buffers (Good and Izawa, 1972): MES, MOPS, TAPS, AMPSO and CAPS. The pH profile of the new endopeptidase was also made within the pH range 6.0–10.0 on Z-Phe-Arg-p-nitro-anilide using 25 mM sodium salts of the same "Good" buffers containing EDTA 4 mM and dithiothreitol 8 mM and measuring the absorbance at 410 nm.

2.8. Isoelectrofocusing and Zymogram

Isoelectrofocusing (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, centrifuged, the protein sediments redissolved and precipitated once again with acetone and finally redissolved in a volume of deionized water as for to contain 1–10 μg of protein. 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.

Zymograms were performed in order to visualize proteolytic activity. 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).

2.9. FPLC Anion-Exchange Chromatography

Exchange chromatography was performed onto a column (Pharmacia XK 16/40, with AK16 adaptors) packed with 33 ml of Q-Sepharose High Performance (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 a broken saline gradient: 20 ml of 0.00–0.1 M and then 160 ml of 0.1–0.3 M sodium chloride.

2.10. FPLC Cation-Exchange Chromatography

The first fraction retained was recromatographied in the same column but packed with 32 ml of SP-Sepharose High Performance (Pharmacia) and equilibrated with 20 mM citrate buffer (pH 5.6) at 20°C. After washing the column with the same buffer, the retained protein was eluted with 90 ml of a linear sodium chloride gradient (0.0–0.15 M) in the starting buffer.

2.11. SDS-PAGE

SDS-polyacrylamide gel electrophoresis 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 Site with E-64

Titration of the active site was performed as described by Barrett and Kirschke (1981) with some modifications. The enzyme ($1.2 \mu M$) was preincubated with the activation buffer (50 mM Tris–HCl, pH 8.0, containing 20 mM cysteine). Fractions ($150 \mu l$) were incubated with $50 \mu l$ of different concentrations ($0 - 2 \mu M$) of E-64 for 30 min at 37°C and the residual activity was then measured on casein as previously described. The enzyme concentration was established by determining both protein content (Bradford, 1976) and molecular mass value (mass spectrometry).

2.13. Mass Spectrometry

The purity degree as well as the molecular weight of purified endopeptidase was determined by mass spectrometry (MALDI-TOF-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 $1-10 \mu M$ protein solution. From this mixture, $1 \mu l$ was spotted on 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 basic local alignment search tool (BLAST) network service (Altschul *et al.*, 1997).

2.15. Kinetic Studies

The kinetic parameters of the hydrolysis of the Z-Phe-Arg-*p*-nitroanilide were determined spectrophotometrically by following the rate of the release of *p*-nitroaniline. From the product concentrations vs. time curves, instantaneous rates were calculated at several different substrate concentrations. K_m, and V_{max} were calculated by regression analysis using the nonlinearized form of the Michaelis–Menten equation (Sigma Plot 8.02). k_{cat} (V_{max}/enzyme concentration) was estimated using the functional enzyme concentration obtained on the basis of site active titration.

3. RESULTS AND DISCUSSION

The crude extract of unripe fruits of *Bromelia hieronymi* contained 0.6 mg/ml of proteins and showed a high caseinolytic activity (6.5 Ucas/ml). The RAP retained 79.5% of the initial activity and 76% of the original protein content, showing maximum caseinolytic activity (higher than 80%) between pH 7.3 and 10.7. The analysis of RAP by IEF-zymogram revealed the presence of six protein bands (pI = 5.9, 6.4, 7.6, 8.3 and two higher than 9.3), all of them proteolytically active. Properties of the most basic endopeptidase (hieronymain I) have been previously reported (Bruno *et al.*, 2003); the present paper describes the purification and characterization of hieronymain II, a new endopeptidase of medium pI value.

Anion chromatography of RAP provided two active retained fractions (Fig. 1); the first fraction eluted was rechromatographied on a cationic exchanger, affording a pure enzyme, named hieronymain II (Fig. 2). The purification scheme is presented in Table 1. Yielding of hieronymain I was low (6.4%), almost one third of the amount obtained during the purification of hieronymain I (Bruno *et al.*, 2003). The purification factor is 1.59-fold, of the same order of hieronymain I, 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).

The enzyme was irreversibly inhibited by E-64 and iodoacetate, and activated (6 times) by 20 mM cysteine, suggesting it belongs to the cysteine-type proteases, as all other endopeptidases isolated from Bromeliaceae species (Rawlings and Barrett, 2004).
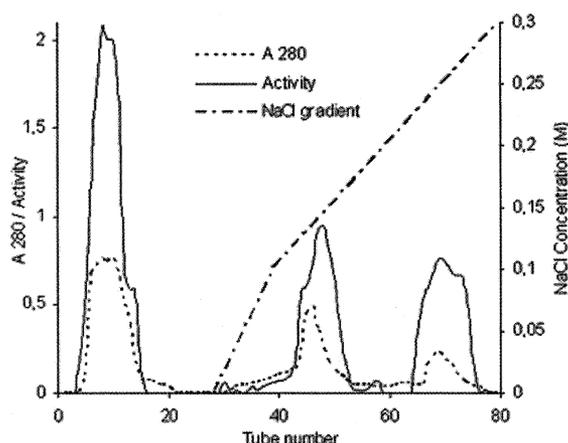
Fig. 1. Anion exchange chromatography (Q-Sepharose High Performance) of RAP. Column diameter: 1.6 cm; column height: 16.5 cm; starting buffer: Tris–HCI 50 mM, pH 8.5; elution saline gradient: 20 ml of 0.0–0.1 M and then 160 ml of 0.1–0.3 M sodium chloride in the starting buffer. Flow rate, 1.0 ml min^{-1}. Fractions of 3.2 ml were collected.

Optimum pH activity of hieronymain II on casein was achieved between pH 7.5 and 9.0, a narrower range than that of the RAP, which is attributed to the higher purity of the sample. The pH profile of hieronymain II is more acid than that of hieronymain I (Fig. 3). When Z-Phe-Arg-p-nitroanilide was used as substrate, optimum pH activity (more than 90% of maximum activity) of hieronymain II was achieved between pH 7.4 and 8.4 (data not shown).

The enzyme appeared homogeneous by MS (MALDI TOF-TOF) and SDS-PAGE, although IEF revealed the presence of two isoforms, a frequent fact in endopeptidases of the Bromeliaceae family (Rowan *et al.*, 1990). The molecular mass (MS) was 23,411 Da (Fig. 4) and the value obtained by densitogram analysis from SDS-PAGE (Fig. 5) was 25.0 kDa. The IEF-zymogram is shown in Fig. 6, where two bands at pI 8.3 and 7.6 can be

Fig. 2. Cation exchange rechromatography (SP-Sepharose High Performance) of the first fraction eluted from anion exchange chromatography. Column diameter: 1.6 cm; column height: 16 cm; starting buffer: citrate 20 mM, pH 5.6; elution saline gradient: 90 ml of a linear sodium chloride gradient (0.0–0.15 M) in the starting buffer. Flow rate, 1.0 ml min^{-1}. Fractions of 4.0 ml were collected.

Fig. 3. Effect of pH on activity. 25 mM sodium salts of the following "Good" buffers were used: MES (pH 5.5–6.7), MOPS (pH 6.5–7.9), TAPS (pH 7.7–9.1), AMPSO (pH 8.3–9.7) and CAPS (pH 9.7–11.1). Data points represent the mean value of five determinations and each experiment was repeated three times.

Table 1. Purification Scheme

Step	Activity (Ucas)	Protein (mg)	Specific activity (Ucas/mg)	Purification (n-fold)	Yield (%)
Crude	80.3	7.48	10.7	1	100
RAP	63.8	5.70	11.2	1.05	79.5
Anionic chromatography	6.0	0.41	14.6	1.36	7.5
Hieronymain II	5.1	0.30	17	1.59	6.4
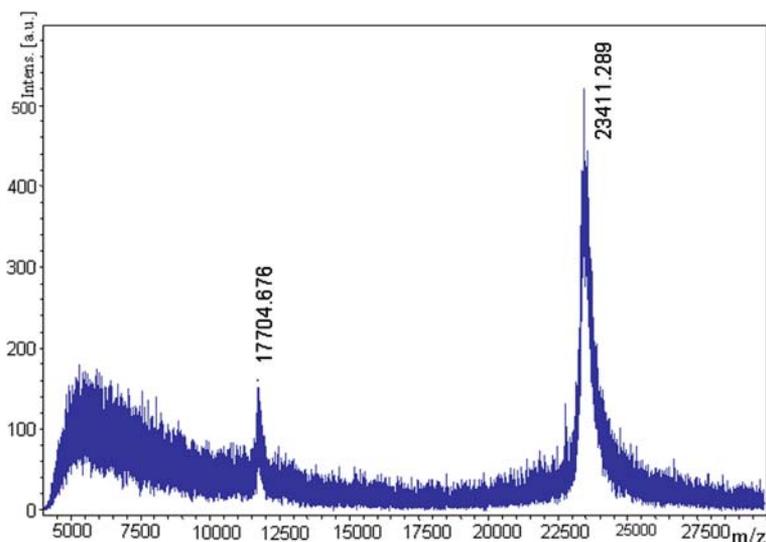
Fig. 4. Mass spectrometry of hieronymain II.

seen, which can be interpreted as chemical modifications with no significant molecular weight variation but with slightly charge variations, affording different pI bands, resembling the two forms of stem bromelain (Harrach *et al.*, 1998)

The N-terminal sequence of the new enzyme was compared to those of other thirteen plant cysteine proteinases (Table 2). The hieronymain II N-terminus shows a great deal of sequence similarity to those of the other Bromeliaceae cysteine endopeptidases: the only difference between hieronymain II and macrodontain I, macrodontain II, stem bromelain,

fruit bromelain and fastuosain is the replacement of Val in position 10 for Asp. It seems clear that the Bromeliaceae endopeptidases are more closely related to each other than to other members of the papain family, suggesting relatively recent evolutive divergence (Rowan and Buttle, 1994).

E-64 and cysteine endopeptidases have been found to react each other on an equimolecular
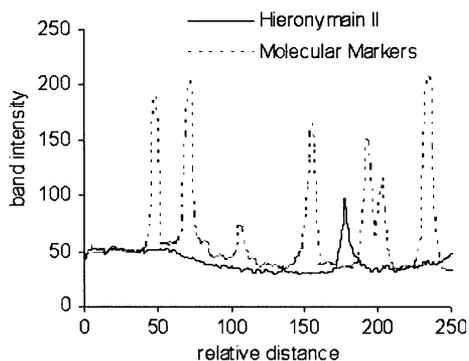
Fig. 5. SDS-PAGE of hieronymain II. Molecular weight standards (Bio-Rad): phosphorylase B (97.4 kDa), serum albumin bovine (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase bovine (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Ten micrograms of sample were applied to the gel.
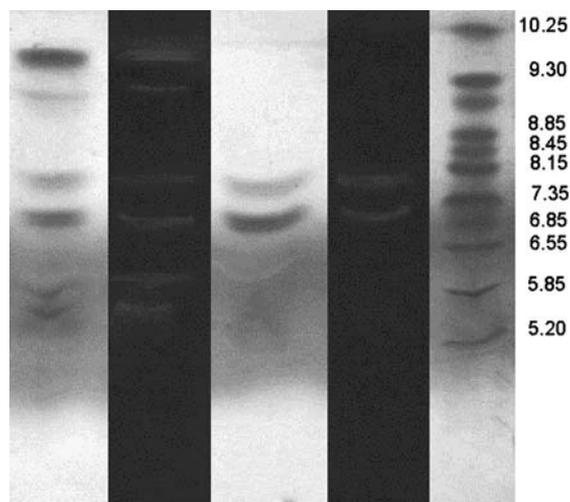
Fig. 6. Isoelectric focusing and zymogram. Isoelectrofocusing (IEF) was developed on 5% polyacrylamide gels containing broad pH range ampholytes (Biolyte 3-10, Bio-Rad) in a Mini IEF Cell (Model 111, Bio-Rad). Lane 1, RAP; lane 2, zymogram of RAP; lane 3, hieronymain II; lane 4, zymogram of hieronymain II; lane 5, pI markers.

Table 2. Comparison of N-terminal Sequences of Hieronymain II and other Plant Endopeptidases

Protease/source	N-Terminal sequence	% Identity	Reference
Hieronymain II	A V P Q S I D W R V Y G A V	100.0	
Macrodontain II	A V P Q S I D W R D Y G A V	92	López *et al.* (2001)
Macrodontain I	A V P Q S I D W R D Y G A V	92	López *et al.* (2000)
Bromelain	A V P Q S I D W R D Y G A V	92	Ritonja *et al.* (1989)
Fastuosain	A V P Q S I D W R D Y G A V	92	Cabral *et al.* (2004)
Fruit bromelain	A V P Q S I D W R D Y G A V	92	Muta *et al.* (1994)
Balansain I	A V P E S I D W R D Y G A V	86	Pardo *et al.* (2000)
Comosain	– V P Q S I D W R N Y G A V	86	Napper *et al.* (1994)
Ananain	– V P Q S I D W R D S G A V	78.6	Lee *et al.* (1997)
Mesembryanthemum sp.	– V P R S I D W R V K G A V	78.6	Firsthoefel *et al.* (1998)
Hieronymain I	A L P E S I D W R A K G A V	71.4	Bruno *et al.* (2003)
Chymopapain	– Y P Q S I D W R A K G A V	71.4	Watson *et al.* (1990)
Vicia sativa	– V P S S I D W R N K G A V	71.4	Shutov and Vaintraub (1987)
CC-IV (*Carica candamarcencis*)	– Y P E S I D W R K K G A V	64.3	Walreavens *et al.* (1993)

Shared amino acids are underlined.

basis, so this inhibitor can be used for titration of the enzyme active site (Barrett and Kirschke, 1981). In the titration assay (Fig. 7) the residual enzyme activity gave a straight line (residual activity vs. E-64 concentration) that intersected the abscissa at $2.0\,\mu M$ E-64, corresponding to 56.5% of active enzyme, a value almost equal to that obtained for hieronymain I (Bruno *et al.*, 2003), indicating that almost half of both enzymes are in inactive form.

The kinetic parameters k_{cat} and K_m are generally useful for the study and comparison of the
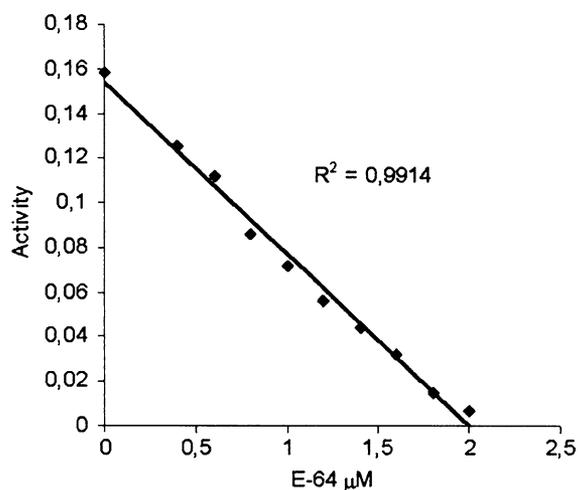
Fig. 7. Titration of active site with E-64. The enzyme $(1.2\,\mu M)$ was preincubated with the activation buffer (50 mM Tris–HCl, pH 8.0, containing 20 mM cysteine). Fractions (150 µl) were incubated with 50 µl of different concentrations (0–2 µM) of E-64 for 30 min at 37°C and the residual activity was then measured on casein as previously described.

kinetic efficiency of enzymes, but either parameter alone is insufficient for this task. The best way to compare the catalytic efficiencies of different enzymes or the turnover of different substrates by the same enzyme is to compare the ratio k_{cat}/K_m for the two reactions. This parameter, sometimes called the specificity constant, is the rate constant for the conversion of substrate to product (Nelson and Cox, 2005). Kinetic enzymatic assays of hieronymain II were made on Z-Phe-Arg-*p*-nitroanilide ($K_m = 0.72\,\mathrm{m}M$, $k_{cat} = 1.82\,\mathrm{seg}^{-1}$, $k_{cat}/K_m = 2.54\,\mathrm{seg}^{-1}$ mM^{-1}), but no detectable activity could be found on Z-Arg-Arg-*p*-nitroanilide, showing a similar substrate preference that ananain, one of the proteases isolated from pineapple stem (Rowan *et al.*, 1990). On the other hand, the enzyme exhibited very low activity on PFLNA, a typical substrate for cystein endopeptidases (Filippova *et al.*, 1984).

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). *Nucleic Acids Res.* **25:** 3389–3402.

Barrett, A. J., and Kirschke, H. (1981). *Meth. Enzymol.* **80:** 535–561.

Bradford, M. B. (1976). *Anal. Biochem.* **72:** 248–254.

Bruno, M. A., Pardo, M. F., Caffini, N. O., and López, L. M. I. (2003). *J. Protein Chem.* **22:** 127–134.

Cabral, H., Rahal, P., Tajara, E. H. S., Leopoldino, A. M., and Bonilla-Rodriguez, G. O. (2004). *GenBank*, accession AY563270, Direct Submission.

Filippova, I. Yu., Lysogorskaya, E. N., Oksenoit, E. S., Rudenskaya, G. N., and Stepanov, V. M. (1984). *Anal. Biochem.* **143:** 293–297.

Firsthoefel, N. R., Cushman, M. A. F., Ostrem, J. A., and Cushman, J. C. (1998). *Plant Sci.* **136:** 195–206.

Good, N. E., and Izawa, S. (1972). *Meth. Enzymol.* **24:** 53–68.

Harrach, T., Eckert, K., Maurer, H. R., Machleidt, I., Machleidt, W., and Nuck, R. (1998). *J. Protein Chem.* **17:** 351–361.

Laemmli, U. K. (1970). *Nature* **227:** 680–685.

Lee, K., Albee, K., Bernasconi, R., and Edmunds, T. (1997). *Biochem. J.* **327:** 199–202.

López, L. M. I., Sequeiros, C., Natalucci, C. L., Brullo, A., Maras, B., Barra, D., and Caffini, N. (2000). *Protein Express. Purif.* **18:** 133–140.

López, L. M. I., Sequeiros, C., Trejo, S. A., Pardo, M. F., Caffini, N. O., and Natalucci, C. L. (2001). *Biol. Chem. Hoppe-Seyler* **382:** 875–878.

Muta, E., Okamoto, Y., and Ota, S. (1994). DDBJ, Accession D38531, Direct Submission.

Napper, A. D., Bennett, S. P., Borowski, M., Holdridge, M. B., Leonard, M. J. C., Rogers, E. E., Duan, Y., Laursen, R. A., Reinhold, B., and Shames, S. L. (1994). *Biochem. J.* **301:** 727–735.

Natalucci, C. L., Brullo, A., López, L. M. I., Hilal, R. M., and Caffini, N. O. (1996). *J. Food Biochem.* **19:** 443–454.

Nelson, D. L., and Cox, M. M. (2005). *Lehninger Principles of Biochemistry*. 4th edn., W.H. Freeeman & Co., New York 207.

Pardo, M. F., López, L. M. I., Canals, F., Avilés, F. X., Natalucci, C. L., and Caffini, N. O. (2000). *J. Agric. Food Chem.* **48:** 3795–3800.

Rawlings, N. M., and Barrett, A. J. (2004). In: Barrett, A. J., Rawlings, N. M., and Woessner, J. F. (eds.), *Handbook of Proteolytic Enzymes*, 2nd edn, vol. II, Academic Press, London, pp. 1051–1071.

Ritonja, A., Buttle, D. J., Rawlings, N. D., Turk, V., and Barrett, A. J. (1989). *FEBS Lett.* **258:** 109–112.

Rowan, A. D., Buttle, D. J., and Barrett, A. J. (1990). *Biochem. J.* **266:** 869–875.

Rowan, A. D., and Buttle, D. J. (1994). *Meth. Enzymol.* **244:** 555–568.

Salvesen, G. S., and Nagase, H. (2001). In: Beynon, R., and Bond, J. S. (eds.), *Proteolytic Enzymes*, 2nd edn, Oxford University Press, Oxford, pp. 105–128.

Scopes, R. K. (1984). *Protein Purification. Principles and Practice*, Springer-Verlag, N.Y. 219.

Shutov, A., and Vaintraub, I. (1987). *Phytochemistry* **26:** 1557–1566.

van derHoorn, R. A., and Jones, J. D. (2004). *Curr. Opin. Plant Biol.* **7:** 400–407.

Walreavens, V., Jaziri, M., vanBeeumen, J., Schnek, A. G., Kleinschmidt, T., and Looze, Y. (1993). *Biol. Chem. Hoppe-Seyler* **374:** 501–506.

Watson, D. C., Yaguchi, M., and Lynn, K. R. (1990). *Biochem. J.* **266:** 75–81.

Westergaar, J. L., Hackbarth, C., Treuhaft, M. W., and Roberts, R. C. (1980). *J. Immunol. Meth.* **34:** 167–175.