Purification of Balansain I, an Endopeptidase from Unripe Fruits of *Bromelia balansae* Mez (Bromeliaceae)

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A new plant endopeptidase was obtained from unripe fruits of *Bromelia balansae* Mez (Bromeliaceae). Crude extracts were partially purified by ethanol fractionation. This preparation (redissolved ethanol precipitate, REP) showed maximum activity at pH 8.8−9.2, was very stable even at high ionic strength values (no appreciable decrease in proteolytic activity could be detected after 24 h in 1 M sodium chloride solution at 37 °C), and exhibited high thermal stability (inactivation required heating for 60 min at 75 °C). Anion exchange chromatography allowed the isolation of a fraction purified to mass spectroscopy, SDS-PAGE, and IEF homogeneity, named balansain I, with pI = 5.45 and molecular mass = 23192 (mass spectrometry). The purification factor is low (2.9-fold), but the yield is high (48.3%), a common occurrence in plant organs with high proteolytic activity, where proteases represent the bulk of protein content of crude extracts. Balansain I exhibits a similar but narrower pH profile than that obtained for REP, with a maximum pH value ∼9.0 and was inhibited by E-64 and other cysteine peptidases inhibitors but not affected by inhibitors of the other catalytic types of peptidases. The alanine and glutamine derivatives of *N*-α-carbobenzoxy-L-amino acid *p*-nitrophenyl esters was strongly preferred by the enzyme.The N-terminal sequence of balansain I showed a very high homology (85−90%) with other known Bromeliaceae endopeptidases.

Keywords: *Plant endopeptidase; Bromelia balansae; Bromeliaceae; purification*

INTRODUCTION

Proteases, which firmly maintain first place in the world market of enzymes, play an important role in biotechnology, given that proteolysis changes the chemical, physical, biological, and immunological properties of proteins. Hydrolysis of food proteins is carried out for various reasons: improvement of the nutritional characteristics, retarding deterioration, modification of different functional properties (solubility, foaming, coagulation, and emulsifying capacities), prevention of undesired interactions, change of flavors and odors, and removal of toxic or inhibitory factors, among others. Enzymatic hydrolysis is strongly preferred over chemical methods because it yields hydrolysates containing well-defined peptide mixtures and avoids the destruction of L-amino acids and the formation of toxic substances such as lysinoalanine (Lahl and Brown, 1994; Mahmoud, 1994).

Although most proteases used in industrial processes come from microbial sources, some plant cysteine proteinases, namely, papain, bromelain, and ficin, are still preferred in a number of cases (Mantell et al., 1985) and are currently used in the food industry for cheese and beer manufacture, tenderization of meat, production of emulsifiers, and other uses.

Bromeliaceae is a plant family whose members usually produce large amounts of proteinases with no apparent function in plant growth and development (Boller, 1986). To date, 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. hieronymi* (Priolo et al., 1991), and *Pseudananas macrodontes* (Natalucci et al., 1996).

Bromelia balansae Mez (folk name "caraguatá") is a wild acaulescent plant currently not used in agriculture, having with 1 m long thorny leaves. Each plant has a unique infructescence (mean = 30 fruits, average fruit size = 4 × 2.5 cm). The species is native of South America and is propagated by suckers and crowns.

In the present paper the isolation, purification, and characterization of balansain I, the main cysteine proteinase of unripe fruits of *Bromelia balansae* Mez (Bromeliaceae) is reported as part of a program dealing with the isolation of new proteases from regional plants that are currently not used in agriculture, a situation that could have positive effects for local economies.

MATERIALS AND METHODS

Plant Material. Infructescences of *B. balansae* Mez in different stages of development were collected by Dr. Aníbal Amat (Universidad Nacional de Misiones) near Posadas, Argentina. Individual fruits were separated from the in-

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fructescence, carefully cleaned with tap water, and stored at −20 °C according to their different stages of development, until the beginning of the extraction procedure.

Crude Preparations. Crude extracts were obtained by chopping and homogenizing frozen fruits (50 g) for 5 min in an Omni Mixer (Sorval) with 250 mL of cold 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA and 25 mM cysteine. Homogenates (final pH 7.2) were filtered through a two layers of gauze to remove plant debris and then centrifuged for 30 min at 16000*g*. Supernatants were collected, filtered, and immediately frozen at −20 °C until analysis. All operations were carried out at 0−4 °C (López et al., 2000).

Fractionation with Organic Solvents. Owing to the high concentration of carbohydrates in crude extracts, 50 mL of crude extract was treated with an equal volume of cold (−20 °C) acetone, the pellet was discarded, and then 4 volumes (200 mL) of acetone was added to the supernatant. In both steps the homogenate was treated with gentle agitation and left to settle for 20 min prior to centrifugation at 16000*g* for 20 min. The final acetone precipitate was redissolved with 50 mL of 50 mM Tris-HCl buffer (pH 8.0) and frozen until further use (Scopes, 1984).

To find a more appropriate solvent for the use of the protease in the food industry (acetone is hard to remove from precipitates and is toxic even in low quantities), ethanol was assayed in the same way as indicated above, affording the corresponding redissolved ethanol precipitates (REP).

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

Proteolytic Activity Assays. The reaction mixture contained 1.1 mL of 1% (w/v) casein solution in 0.1 M glycine−sodium hydroxide buffer (final pH 8.7) containing 25 mM cysteine and 0.1 mL of enzyme solution. The mixture was incubated for 2−10 min, according to enzyme concentration, 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 and 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).

Effect of Inhibitors and Activity Enhancers. The effect of specific inhibitors (Salvensen and Nagase, 1989) on proteolytic activity was determined by measuring the residual activity on casein or azocasein at pH 9.0, after preincubation at 37 °C for 30 min in the presence of the following inhibitors: EDTA (1−5 mM), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; 1−10 μM), iodoacetic acid (10−100 μM), mercuric chloride (0.1, 1.0, and 10 mM), pepstatin A (0.5 μM), 1,10-phenanthroline (1−10 mM), and phenylmethanesulfonyl fluoride (PMSF; 0.1−5 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, 12.5, 25, and 50 mM) to the reaction mixture, and then caseinolytic activity was measured as mentioned.

Thermal Stability. To determine the effect of heating, samples were held for 0, 5, 10, 20, 40, 60, 90, and 120 min at 37, 45, 55, 65, and 75 °C, and then the residual caseinolytic activity was measured as mentioned.

Effect of pH on Enzyme Activity. Proteolytic activity versus pH was measured on 1% casein solution containing 25 mM cysteine within the pH range 5.8−10.0 using 20 mM sodium salts of the following "Good" buffers (Good and Izawa, 1972): MES, MOPS, TAPS, AMPSO, and CAPS.

Effect of Ionic Strength on Enzyme Activity. Stability at different ionic strength conditions was determined by incubating enzyme samples at 37 °C and pH 7.8 for 10 min and 12 and 24 h with different sodium chloride concentrations (0, 0.06, 0.12, 0.25, 0.50, and 1.0 M) and measuring the residual caseinolytic activity as mentioned.

Assays with Synthetic Substrates. The esterolytic activity of the enzyme was tested on the *p*-nitrophenyl esters of the following *N*-α-carbobenzoxy (CBZ)-L-amino acids: *N*-α-CBZ-L-alanine, *N*-α-CBZ-L-asparagine, *N*-α-CBZ-β-benzyl-L-aspartic acid, *N*-α-CBZ-glycine, *N*-α-CBZ-L-isoleucine, *N*-α-CBZ-L-leucine, *N*-α-CBZ-L-lysine, *N*-α-CBZ-L-phenylalanine, *N*-α-CBZ-L-tryptophan, *N*-α-CBZ-L-tyrosine, and *N*-α-CBZ-L-valine.

The reaction mixture contained 1.8 mL of 0.1 M phosphate buffer (pH 8.5), 0.1 mL of substrate solution (1 mM in acetonitrile), and 0.1 mL of enzyme solution. The reaction was carried out at 37 °C, and changes in the absorbance were measured at 405 nm during 2 min. An arbitrary enzyme unit (Ucbz) was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol/min at 37 °C and pH 8.5.

Isoelectrofocusing (IEF) and Zymograms. 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, and the protein sediments were redissolved and precipitated once again with acetone and finally redissolved in half-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 by Coomassie Brilliant Blue R-250.

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 8.7) 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).

Ion Exchange Chromatography. Anion exchange chromatography was performed on a column (1.5 × 30 cm) of DEAE-Sepharose Fast Flow (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) at 4 °C. After the column had been washed with the same buffer, the retained proteins were eluted with 100 mL of a linear sodium chloride gradient (0.0−0.25 M) in the starting buffer.

SDS-PAGE. SDS−polyacrylamide gel electrophoresis was performed in a Miniprotean II cell (Bio-Rad) according to the method of Laemmli (1970). The 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. The molecular weight markers (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).

N-Terminal Sequence. A sample of the main purified fraction obtained from ion exchange chromatography was adsorbed on a PVDF membrane (Millipore) and washed several times with deionized water. The N-terminal sequence was determined by Edman's automated degradation using a Beckman LF3000 protein sequencer equipped with a PTH-amino acid analyzer System Gold (Beckman). Protein homology searches were performed using the BLAST network service (Altschul et al., 1997).

Mass Spectrometry. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for the determination of the molecular mass, as well as the degree of purity 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, sinapic acid) in 0.1% trifluoroacetic acid in 2:1 water/acetonitrile and a protein solution of a concentration in the range of 1−10 μM. From this mixture, 1 μL was spotted on

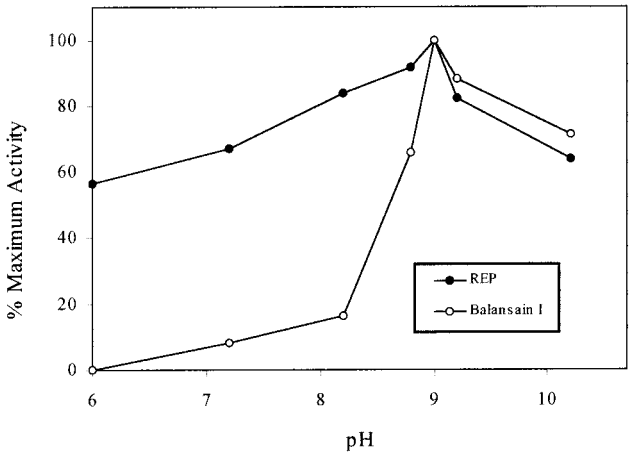

Figure 1. Effect of pH on proteolytic activity. Proteolytic activity was measured on 1% casein solution containing 25 mM cysteine. Data points represent the mean value of four determinations, and each experiment was repeated twice.

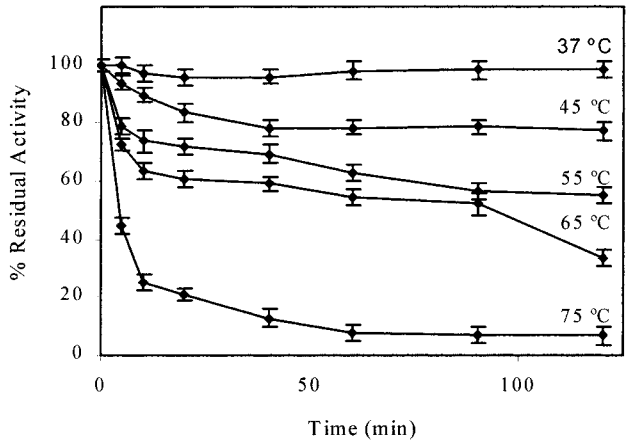

Figure 2. Thermal stability of REP. Data points represent the mean value of five determinations, and each experiment was repeated twice.

the sample slide and allowed to evaporate to dryness. Proteins of known molecular mass were used as standards for mass calibration.

RESULTS AND DISCUSSION

Crude extracts from fruits in different stages of development were obtained and their proteolytic activities determined. Enzyme activity was practically not affected by prolonged storage at −20 °C (up to 180 days). Extracts obtained from unripe fruits showed maximum total and specific caseinolytic activities of 17.0 Ucas/g of fruit and 35.4 Ucas/mg of protein, respectively, and were chosen for further studies.

As crude extracts contained phenolic compounds, which could oxidize and irreversibly react with proteins, a fractionation was carried out with organic solvents (acetone and ethanol). Fractionation with both solvents provided similar enzyme preparations: the redissolved acetone precipitate contained 83% of proteins, 86% of total caseinolytic activity, and 61% of soluble sugars, whereas the redissolved ethanol precipitate contained 83% of proteins, 92% of total caseinolytic activity, and 55% of soluble sugars, with respect to crude extracts. On the basis of these results and taking into account the potential use of the enzyme preparation in food technology, the REP was selected for enzyme purification and characterization.

For industrial applications enzyme purity is usually of secondary importance to cost (Illanes, 1994). Crude extracts or only partially purified enzymes are currently used in these cases, and then these preparations need to be characterized. Hence, the effect of pH, temperature, and ionic strength on proteolytic activity was determined. REP exhibited maximum activity (>90%) between pH 8.8 and 9.2 (Figure 1). The preparation is very stable even at high ionic strength values (no appreciable decrease in proteolytic activity could be detected after 24 h in 1 M sodium chloride solution at 37 °C; data not shown). As can be seen in Figure 2, no activity loss was observed when REP was incubated at 37 °C during a period of 120 min, whereas at 45 °C almost 80% of activity remained. The enzyme was almost completely inactivated by heating for 60 min at 75 °C.

IEF of REP followed by zymogram analysis showed two bands with caseinolytic activity (Figure 3, lanes 1

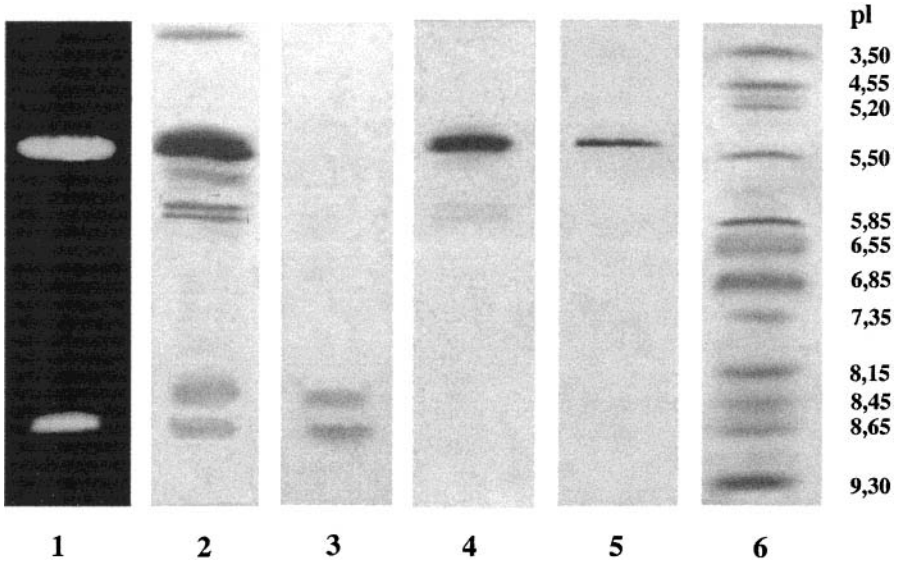

Figure 3. IEF and zymogram: (lane 1) zymogram of REP; (lane 2) IEF of REP; (lane 3) IEF of unretained chromatography fraction; (lane 4) IEF of active retained chromatography fraction; (lane 5) IEF of balansain I; (lane 6) IEF markers (Sigma IEF mix 3.6−9.3 IEF marker).

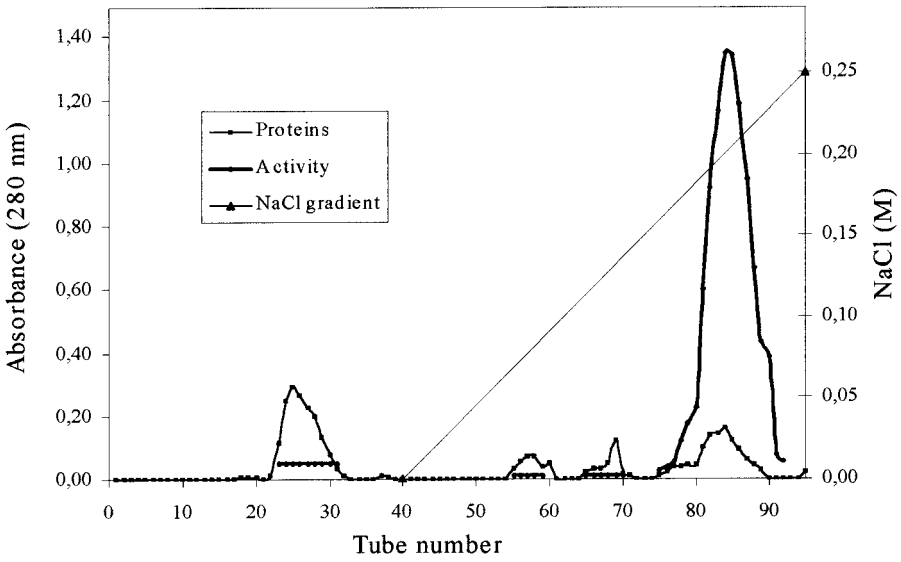

Figure 4. Ion exchange chromatography of REP. Sample volume = 20 mL; flow rate = 0.56 mL min^{-1}; fraction volume = 1.8 mL.

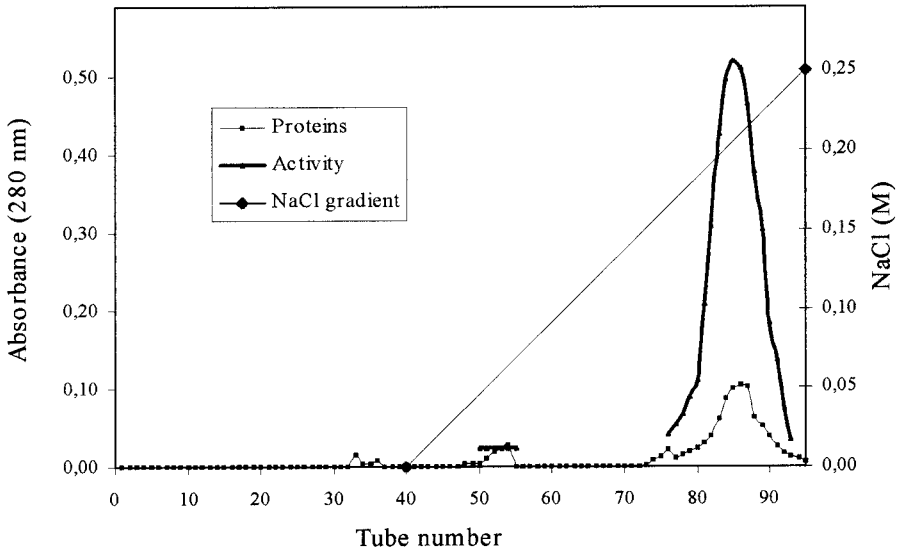

Figure 5. Ion exchange rechromatography. Sample volume = 16.2 mL; flow rate = 0.56 mL min^{-1}; fraction volume = 1.8 mL.

Table 1. Purification Scheme of Balansain I

step	activity (Ucas)	protein (mg)	specific activity (Ucas/mg)	purification (*n*-fold)	yield (%)
crude extract	340.0	9.6	35.4	1.0	100.0
REP	312.8	8.0	39.1	1.1	92.0
active fraction, NaCl gradient	234.6	2.3	102.0	2.9	69.0
balansain I	164.2	1.6	102.6	2.9	48.3

and 2) and very different isoelectric points. On the basis of these results, ion exchange chromatography was selected for further purification steps. Anion exchange chromatography of REP (Figure 4) afforded two proteic components (Figure 3, lane 3), only one of them showing proteolytic activity. The application of a sodium chloride linear gradient allowed the separation of another three protein fractions (Figure 3, lane 4), of which only the last one exhibited caseinolytic activity. This active fraction was rechromatographed under the same conditions (Figure 5), yielding a fraction (balansain I) of which the purity is evidenced by IEF patterns (Figure 3, lane 5), mass spectrometry (Figure 6), and SDS−PAGE (Figure 7).

Table 2. Effect of Different Chemicals on Proteolytic Activity of Balansain I

chemical	concn	residual activity (%)
1,10-phenantroline	10.0 mM	98
cysteine	25.0 mM	250
E-64	1.0 μM	0
EDTA	5.0 mM	110
iodoacetic acid	100 μM	11
mercuric chloride	10.0 mM	0
pepstatin A	0.5 μM	100
PMSF	5.0 mM	23[a]

[a] Inhibition was fully restored by cysteine addition.

The purification scheme is presented in Table 1. The purification factor of balansain I is very low, 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).

Balasain I showed a molecular mass of 23192 (mass spectrometry, Figure 6) and a molecular weight of 24.4 kDa (SDS-PAGE, Figure 7), which are of the same order of the cysteine proteases from fruits of other species of Bromeliaceae (Boller, 1986). Balansain I exhibits a similar but narrower pH profile than that obtained for

Table 3. N-Terminal Amino Acid Sequences of Some Cysteine Plant Endopeptidases

Plant proteases	N-terminal sequence	Reference	% homology
Balansain I (*Bromelia balansae*)	AVPESIDWRDYGAVTSVKNQG		
Stem Bromelain (*Ananas comosus*)	AVPQSIDWRDYGAVTSVKNQN	Ritonja *et al.*, 1989	90.5
Ananain (*Ananas comosus*)	VPQSIDWRDSGAVTSVKNQG	Lee *et al.*, 1997	85.7
Comosain (*Ananas comosus*)	VPQSIDWRNYGAVTSVKNQG	Napper *et al.*, 1994	85.7
Macrodontain I (*Pseudananas macrodontes*)	AVPQSIDWRDYGAVNEVKNQG	López *et al.*, 2000	85.7
Papain (*Carica papaya*)	IPEYVDWRQKGAVTPVKNQG	Cohen *et al.*, 1986	66.7

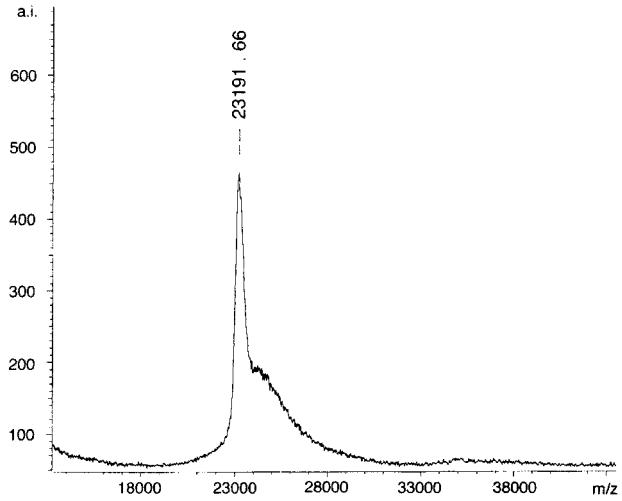

Figure 6. Mass spectrometry of balansain I.

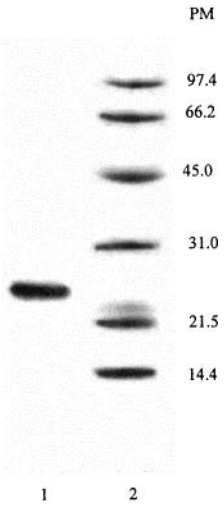

Figure 7. SDS-PAGE of balansain I: (lane 1) balansain I; (lane 2) low molecular weight markers (Bio-Rad).

REP, with a maximum pH value of ~9.0 (Figure 1) and a p*I* = 5.45 (Figure 3, lane 5), a behavior also shown by other proteases from fruits of Bromeliaceae (Barret et al., 1998).

The proteolytic activity of balansain I was inhibited by E-64 and iodoacetic acid, but such activity was not affected by characteristic inhibitors of proteinases other than cysteine proteinases (pepstatin A, EDTA, and 1,10-phenanethroline). The enzyme was completely inhibited by mercuric chloride and partially by PMSF, but in both cases the activity was fully restored by cysteine (Table 2). The addition of cysteine increased proteolytic activity up to a maximum value when cysteine concentration was raised to 25 mM (2.2 times in relation to the same preparation without the addition of cysteine). These results strongly suggest that the protease isolated should be included within the cysteine group, as are all the other studied proteases belonging to the family Bromeliaceae.

When balansain I was assayed on *N*-α-CBZ-L-amino acids, higher endoesterasic relative activities were obtained for the alanine and glutamine derivatives, followed by those of glycine, tyrosine, and leucine (about one-third) and at a lesser extent (one-tenth) by those of valine and tryptophan (data not shown).

The N-terminal sequence of balansain I (21 amino acids) was compared with those of papain and the other known Bromeliaceae endopeptidases (Table 3). Only two different residues can be observed between balansain I and stem bromelain and three between balansain I and the other Bromeliaceae peptidases, but the difference is higher for papain. This is in agreement with the proposal of Rowan and Buttle (1994) that the Bromeliaceae endopeptidases are more closely related to each other than to other members of the papain family.

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