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Purification and Characterization of Four New Cysteine Endopeptidases From Fruits of *Bromelia pinguin* L. Grown in Cuba

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Abstract *Bromelia pinguin* L. is a plant broadly distributed in Central America and Caribbean islands. The fruits have been used in traditional medicine as anthelmintic, probably owed to the presence of a mixture of cysteine endopeptidases, initially termed pinguinain. This work deals with the purification and characterization of the four main components of that mixture, two of them showing acid pI and the other two alkaline pI. Molecular masses (SDS-PAGE and MALDI-TOF), N-terminal sequence and the reactivity and kinetic parameters versus synthetic substrates (*p*-nitrophenyl-N-α-CBZ-amino acid esters, PFLNA, Z-Arg-Arg-*p*-NA, and Z-Phe-Arg-*p*-NA) of the studied peptidases are given, as well as the N-terminal sequences of the enzymes and the homology degree with other plant endopeptidases.

Keywords *Bromelia pinguin* L. · *Bromeliaceae* · Plant cysteine endopeptidases · Pinguinain

Abbreviations

CBZ	Carbobenzoxy
DTT	Ditiothreitol
E-64	Trans-epoxysuccinyl-L–leucylamido-(4-guanidino)-butane
EDTA	Ethylenediaminetetracetic acid
FPLC	Fast protein liquid chromatography
IEF	Isoelectric focusing
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
PFLNA	L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilida
PPP	Partially purified pinguinain
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
Tris	2-Amine-2-hydroxymethyl-1,3-propanediol
Ucas	Caseinolytic unit
Z-Arg-Arg-NH-Mec	*N*-α-CBZ-L-Arg-L-Arg-7-(4-methyl)-coumarylamide
Z-Arg-Arg-p-NA	*N*-α-CBZ-L-arginyl-L-arginine-*p*-nitroanilida
Z-Phe-Arg-NHMec	*N*-α-CBZ-L-Phe-L-Arg-7-(4-methyl)-coumarylamide
Z-Phe-Arg-p-NA	*N*-α-CBZ-L-phenylalanyl-L-arginine-*p*-nitroanilida

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1 Introduction

Bromeliaceae is a plant family including species that produce proteases in unusual quantities. *Bromelia pinguin* L. ("maya" or "pinguin bromelia") is broadly distributed in Central America and the Caribbean Islands. A

preparation from the fruit pulp has been traditionally used as anthelmintic. Pinguinain is the name given to the proteolytic preparation from fruits of *Bromelia pinguin* L., scarcely studied [1, 2] and recently characterized [3].

The present work deals with the purification and characterization of the main components of the cysteine endopeptidases mixture contained in the fruits of *Bromelia pinguin* L. grown in Cuba.

2 Materials and Methods

2.1 Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, Ohio); iodoacetic acid, L-cysteine, ditiothreitol (DTT), E-64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane), EDTA (ethylenediamine tetracetic acid), Tris (2-amine-2-hydroxymethyl-1,3-propanediol), isoelectric point markers (IEF MIX 3.6–9.3) and Biolyte 3–10 ampholytes were purchased from Sigma Chemical Company (St. Louis, Missouri). Coomassie Brilliant Blue R-250, acrylamide and bisacrylamide were obtained from BioRad (Hercules, California). Low molecular weight markers (Pharmalyte 3–10), SP-Sepharose HP and Q-Sepharose HP were purchased from Amersham Pharmacia (Uppsala, Sweden). All other chemicals were obtained from commercial sources and were of the highest purity available.

2.2 Plant Material

Mature fruits of *Bromelia pinguin* L. (3.6 cm average long; 2.1 cm average wide; 8.3 g average weight), collected in Cienfuegos, Cuba, in August 2004, were stored at –20 °C until the preparation of extracts.

2.3 Protease Extraction and Ethanol Fractionation

Frozen fruit pulp was homogenized in a domestic blender, and the suspension was filtered through double gauze piece to remove plant debris. One volume of cold ethanol was added to one volume of the filtered suspension with gentle stirring, then the suspension was settled for 30 min at –10 °C and centrifuged at 10,290*g* during 20 min at 4 °C. The precipitate was discarded, the supernatant was withdrawn to a beaker and three volumes of ethanol were added, repeating the procedure of settling and centrifugation. The supernatants were discarded, the precipitate was redissolved in one volume of distilled water and the solution was centrifuged at 24,400*g* during 30 min at 4 °C. This preparation, named partially purified pinguinain (PPP), was stored at –20 °C.

2.4 Ion Exchange Chromatography

Partially purified pinguinain (3.5 mL) was applied onto a SP-Sepharose HP column (Amersham Pharmacia K 19/26), equilibrated with 50 mM acetate buffer (pH 5.5) at 16 °C. Cationic exchange chromatography was carried out in a FPLC equipment (Amersham Pharmacia) and was developed by adding 35 mL of the starting buffer (50 mM acetate buffer, pH 5.5), followed by 140 mL of a sodium chloride lineal gradient (0.00–0.07 M) prepared in the same buffer. The non-retained fractions were concentrated and deionized by acetone precipitation and subsequent centrifugation to 15,600*g* for 20 min at 4 °C. The precipitate was redissolved in 1.5 mL of deionized water. This solution was applied onto a Q-Sepharose HP column (Amersham Pharmacia K 19/26), equilibrated with 50 mM Tris–HCl buffer (pH 8.0) at 16 °C. Anionic exchange chromatography was developed by adding 35 mL of the starting buffer (50 mM Tris–HCl buffer, pH 8.0), followed by 140 mL of a sodium chloride lineal gradient (0.00–0.085 M) prepared in the same buffer.

2.5 Caseinolytic Activity

The reaction mixture contained 1.1 mL of 1% casein solution and 0.1 mL of enzyme solution, both in 50 mM Tris–HCl buffer (pH 8.0), containing 5 mM cysteine. The reaction was carried out at 37 °C and stopped by the addition of 5% trichloroacetic acid (1.8 mL), and then each test tube was centrifuged at 3,970*g* for 20 min and the absorbance of the supernatant was read at 280 nm. An arbitrary enzyme unit ("caseinolytic unit", U_{cas}) was defined as the amount of enzyme that produces an increase of one absorbance unit (1 cm light path) per minute under the assay conditions. For the calculation of specific activity values the protein content was determined by the Bradford's method [4].

2.6 Electrophoretical Techniques

Isoelectric focusing (IEF) was carried out in a Mini IEF Cell (Model 111, Bio-Rad). Enzyme-containing samples were concentrated and deionized by acetone precipitation and subsequent centrifugation to 15,600*g* for 20 min at 4 °C. The precipitate was redissolved in deionized water. Polyacrylamide gels containing wide pH range ampholytes (3.0–10.0) were used. Focusing was developed under

constant voltage conditions in a gradual procedure: 100 V for 30 min, 200 V for 15 min and 450 V for 60 min. The gel was stained with Coomassie Brilliant Blue R-250.

Zymograms were developed in unstained gels contacted for 10–15 min at 55 °C with an agarose gel soaked with 1% casein solution in 0.1 M Tris–HCl buffer (pH 8.0) containing 5 mM cysteine, and then washed twice with distilled water. The agarose gel was then stained with Coomassie Brilliant Blue R-250. Unstained bands evidence proteolytic activity.

SDS-PAGE was performed in a Miniprotean II dual slab cell (Bio-Rad) using 50 mM Tris–HCl (pH 8.25) as reservoir buffer. Enzyme samples were concentrated and deionized as was indicated for isoelectric focusing. After vacuum drying, the precipitate was redissolved in 3 M Tris–HCl (pH 8.45) buffer containing 10 mM SDS, placed in a boiling water bath for 5 min, cooled and then β-mercaptoethanol was added (final concentration approximately 2%). Polyacrylamide gels 12.5% w/v were used. Current was kept constant at 50 mA for 80 min. Gels were stained with Coomassie Brilliant Blue R-250 and by the silver staining procedure [5].

2.7 Molecular Mass Determination

Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry was used for the determination of molecular masses as well as purity of the chromatographic fractions. Samples were mixed with sinapinic acid (matrix) dissolved in 0.1% trifluoracetic acid and processed in a MALDI-TOF Bruker equipment, model Ultraflex Bruker Daltonics, using bovine tripsinogen as standard.

2.8 Activity Assays with Synthetic Substrates

2.8.1 *Nitrophenyl Esters of* N-α-*carbobenzoxy (CBZ) Amino Acid Derivatives*

The following nitrophenyl esters of *N*-α-(CBZ) amino acid derivatives were assayed: L-alanine, L-asparagine, L-aspartic acid, L-glutamine, glycine, L–isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-tryptophan, L-tyrosine, and L-valine. The reaction mixture contained 1.8 mL of 0.1 M Tris–HCl buffer (pH 8.5), 100 μL of substrate solution (0.5 mM in acetonitrile), and 100 μL of enzyme solution. The reaction was carried out at 37 °C and changes in the absorbance were measured at 405 nm for 2 min. In this case one enzyme unit (U_{CBZ}) was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute at 37 °C and pH 8.0.

2.8.2 *L-pyroglutamyl-L-phenylalanyl-L-leucine*-p-*nitroanilide (PFLNA)*

The reaction mixture 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, 180 μL of substrate solution (2.22 mM in dimethylsulfoxide), and 120 μL of enzyme solution. The reaction was carried out at 37 °C and changes in the absorbance were measured at 410 nm for 2 min. One enzyme unit (U_{PFLNA}) was defined as the amount of enzyme that releases 1 μmol of *p*-nitroaniline per minute at 37 °C and pH 6.5.

2.8.3 *N-α-CBZ-L-arginyl-L-arginine*-p-*nitroanilide (Z-Arg-Arg*-p-*NA)*

Enzyme solution (100 μL) was activated for 2 min with 1.8 mL of 0.2 M phosphate buffer (pH 6.8) containing 4 mM EDTA and 8 mM DTT. Then 100 μL of substrate solution (4 mM in dimethylsulfoxide) was added. The reaction was carried out at 40 °C and changes in the absorbance were measured at 410 nm for 3 min. One enzyme unit (U_{ArgArg}) was defined as the amount of enzyme that releases 1 μmol of *p*-nitroaniline per minute at 40 °C and pH 6.8.

2.8.4 *N-α-CBZ-L-phenylalanyl-L-arginine*-p-*nitroanilide (Z-Phe-Arg*-p-*NA)*

Enzyme solution (300 μL) was activated for 2 min with 1.6 mL of 0.2 M phosphate buffer (pH 6.8) containing 4 mM EDTA and 8 mM DTT, then 100 μL of substrate solution (5 mM in dimethylsulfoxide) was added. The reaction was carried out at 40 °C and changes in the absorbance were measured at 410 nm for 7 min. In this case one enzyme unit (U_{PheArg}) was defined as the amount of enzyme that releases 1 μmol of *p*-nitroaniline per minute at 40 °C and pH 6.8.

2.9 Determination of Kinetic Parameters

Km and Vmax were determined on N-α-CBZ-L-Ala *p*-nitrophenyl ester, PFLNA, and Z-Phe-Arg-*p*-NA. Measurements of the initial rates of reaction were made under the described conditions, using a thermostatized cell. Data collection was made via a microprocessor linked to the spectrophotometer. Values were calculated by the Enzyme Kinetics module of SigmaPlot 9.0.

2.10 N-terminal Sequence

The purified fractions obtained from ion exchange chromatography were submitted to SDS-PAGE and then electrotransferred to a PVDF (polyvinilidene fluoride) membrane [6]. The N-terminal sequence was determined by Edman's automated degradation using an Beckman LF3000 (System Gold, Beckman) peptide sequencer. Protein homology searches were performed using the BLAST network service [7].

3 Results

3.1 Peptidases Extraction and Purification

Results of PPP preparation are shown in Table 1. Most of the proteolytic activity present in the juice was retained in PPP (68.3%), and it was also achieved the elimination of more than 96 % of the sugars (the relationship protein/sugar concentration is 13.4 times higher in PPP). The purification factor could seem low (1.45 times), but it should be kept in mind that proteolytic enzymes are the main protein components present in the juice obtained from the pulp fruit.

Partially purified pinguinain was used to obtain the purified peptidases by means of ion-exchange chromatography. Figure 1 shows the cation-exchange chromatography which provided a major (NR) and a minor (NR1) non retained fraction and two additional fractions which eluted after the application of the saline gradient (B1 and B2).

Figure 2 shows the anion-exchange chromatogram. Fractions NR and NR1 were mixed and loaded onto the column and when the saline gradient was applied two main fractions eluted (A1 and A2), followed by other three minor fractions (A3–A5).

The basic fractions B1 and B2 as well as the main acid fractions, A1 and A2, were selected for further study. A1 and A2 fractions revealed to be pure, but B1 and B2 were a mix of two very similar components (IEF, Fig. 3). The two components in B1 seem to be two forms of one enzyme, a common fact in this kind of endopeptidases [8, 9]. SDS-PAGE of PPP showed that the molecular masses of the main proteolytic components, as confirmed by the corresponding zymogram (data not shown), ranged around 20 kDa (Fig. 4).

3.2 Molecular Masses (MALDI-TOF) of the Main Purified Fractions

The four purified peptidases, that is, fractions A1, A2, B1 and B2, appeared homogenous by MALDI-TOF and showed very close molecular masses: 23234.637, 23473.779, 23639.814 and 23.547.890 Da, respectively (Fig. 5); this is very frequent among plant peptidases

Table 1 Purification of *Bromelia pinguin* juice to obtain partially purified pinguinain (PPP)

Step	Protein (mg/mL)	Activity (Ucas/mL)	Specific activity (Ucas/mg)	Sugars (mg/mL)	Ratio (protein)/(sugars)	Yield (%)	Purification (times)
Juice	3.71	18.04	4.89	388.67	0.0095	100	1.000
PPP	1.73	12.32	7.11	13.64	0.1268	68.26	1.45

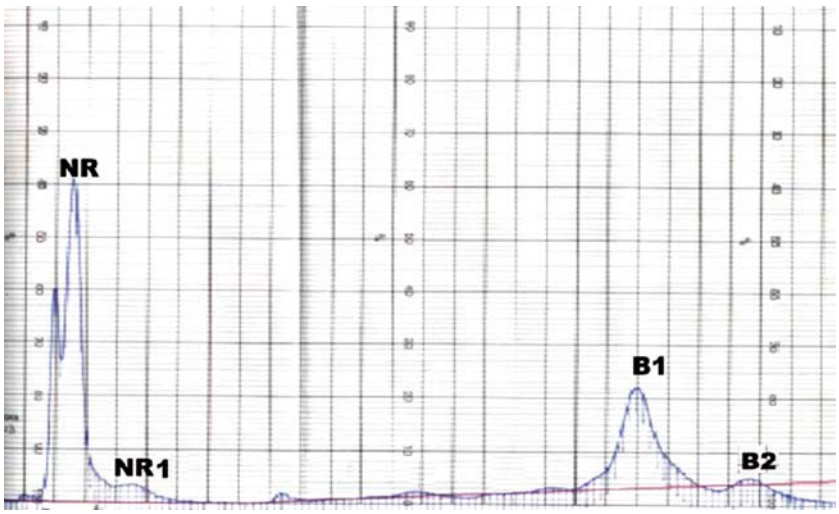

Fig. 1 Chromatogram of PPP on SP—Sepharose HP column (FPLC), equilibrated with acetate 50 mM; pH 5.5 buffer to 16 °C. It was eluted with a lineal gradient of sodium chloride 0.00–0.07 M dissolved in the buffer. Fractions B1 and B2 and the main not retained fraction (NR), as well as a minor non retained fraction (NR1) are shown

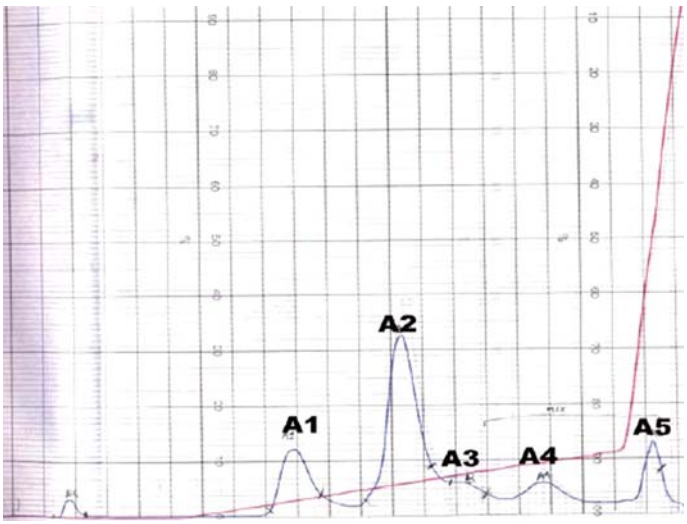

Fig. 2 Chromatogram of NR + NR1 on Q—Sepharose HP column (FPLC), equilibrated with Tris HCl 50 mM; pH 8.0 buffer to 16 °C. Five fractions (A1–A5) were eluted with a lineal gradient of sodium chloride 0.00–0.085 M dissolved in the buffer

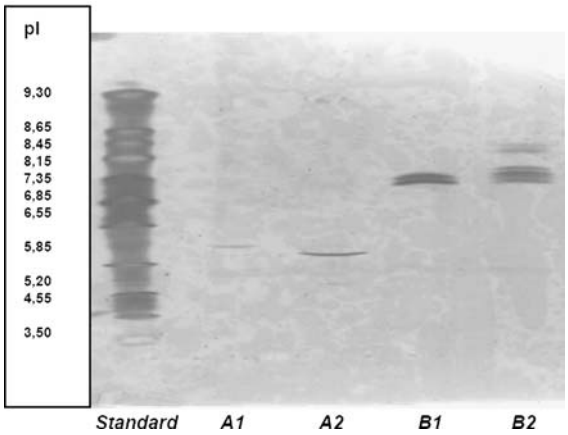

Fig. 3 Isoelectrofocusing of major fractions from partially purified pinguinain on polyacrylamide gel containing ampholites of wide pH range (3.0–10.0). Silver nitrate staining

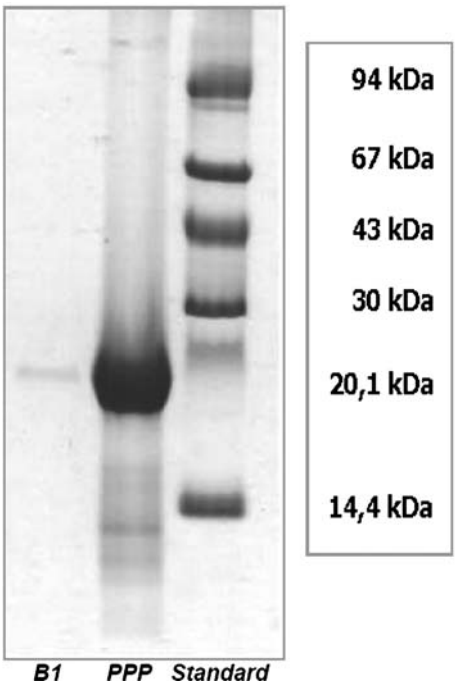

Fig. 4 SDS-PAGE electrophoresis of B1 and PPP on 12.5% W/V polyacrylamide gel using 50 mM Tris–HCl (pH 8.25) as reservoir buffer. The gel was stained with Coomassie Brilliant Blue R-250

isolated from fruits of the *Bromeliaceae* family and it could be attributed both to minor changes in the protein sequence and/or changes in the charge of the molecules [10]. Not withstanding, the present values of molecular masses were different from those previously reported [1, 11, 12], probably owed to the use of less accurate procedures.

3.3 Activity Assays with Synthetic Substrates

3.3.1 p-*Nitrophenyl Esters of N-α-CBZ Amino Acids*

As happened when PPP was assayed against the same substrates [3], A1, A2 and B1 fractions showed higher preference for the L-Ala derivative, but not in the same way. B1 was the less specific, as the preference for the Phe, Asp, Gln, Leu and Tyr derivatives is more than 80% of that showed for the Ala derivative. On the contrary, following the Ala derivative, A1 preferred the Asn, Gly, Tyr and Lys derivatives, while the order in A2 was Gly, Tyr and Gln (Fig. 6). The behavior of the peptidases assayed against these type of substrates revealed marked differences on the preference for the amino acid located in P1 position, which is almost inspecific in the case of B1 but much more specific for A1 and A2. It is also a common fact the low affinity of all the peptidases assayed for the Pro derivative, what could be explained by the particular configuration of this residue, which makes resistant the peptidic bond to the action of endopeptidases.

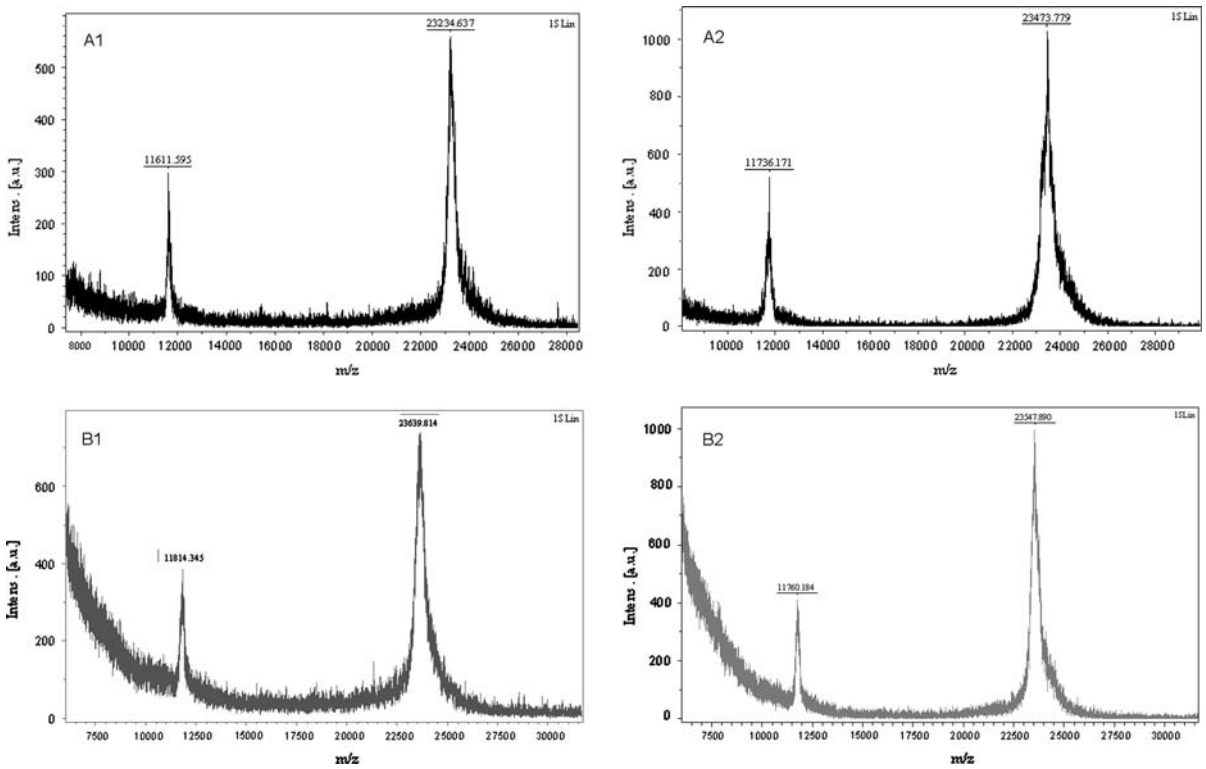

Fig. 5 MALDI-TOF mass spectra of A1, A2, B1 and B2, obtained in a Bruker equipment, nitrogen laser detector (337 nm), lineal positive ions mode, acceleration voltage 20 kV, sinapinic acid matrix

3.3.2 PFLNA

B1 did not show activity against PFLNA. The activity of A2, A1 and B2 against PFLNA was 333.5, 140.5 and 143.7 μmol/min, respectively, a behavior that resembles those of bromelain and papain [13]. It is noteworthy that the activity of PPP is much lesser than those of the purified fractions (2.8 μmol/min), suggesting the action of some unknown factors in the activity of the crude mix of proteases.

*3.3.3 Z-Arg-Arg-*p*-NA*

None of the fractions showed activity against this substrate. This behavior is different from that of stem bromelain, which exhibited good activity against a similar substrate (N-α-CBZ-L-Arg-L-Arg-7-(4-methyl)-coumarylamide, Z-Arg-Arg-NH-Mec), but is similar to that of ananain and fruit bromelain, which were inactive against this substrate [2, 14]. The assay revealed that the main components of PPP showed no affinity for basic residues in P2 position.

*3.3.4 Z-Phe-Arg-*p*-NA*

B1 and B2 were active on this substrate and both showed low activity values (4.92 and 6.83 U_{PheArg} for B1 and B2, respectively). This behavior resembles that of ananain, that not exhibited very high activity against a similar substrate: N-α-CBZ-L-Phe-L-Arg-7-(4-methyl)-coumarylamide, Z-Phe-Arg-NHMec [2, 14]. The assay revealed that B1 and B2 have higher affinity for aromatic residues in P2 position that for basic residues, a characteristic feature of cysteine endopeptidases of the family C1.

3.4 Determination of Kinetic Parameters

3.4.1 N-α-(CBZ)-L-Ala

Results of kinetic behavior of fractions A1, A2 and B1 are shown in Table 2. The esterolitic reaction rate followed the order A2 > A1 > B1. According to these results, the most efficient enzyme was A1, followed by A2 and B1. Do not exist similar data on this substrate for other endopeptidases belonging to the family *Bromeliaceae*.

3.4.2 PFLNA

Results are showed in Table 3 for A1 fraction. The Km value is very similar to that of stem bromelain and also to that of papain [13], which would have similar affinity for this substrate.

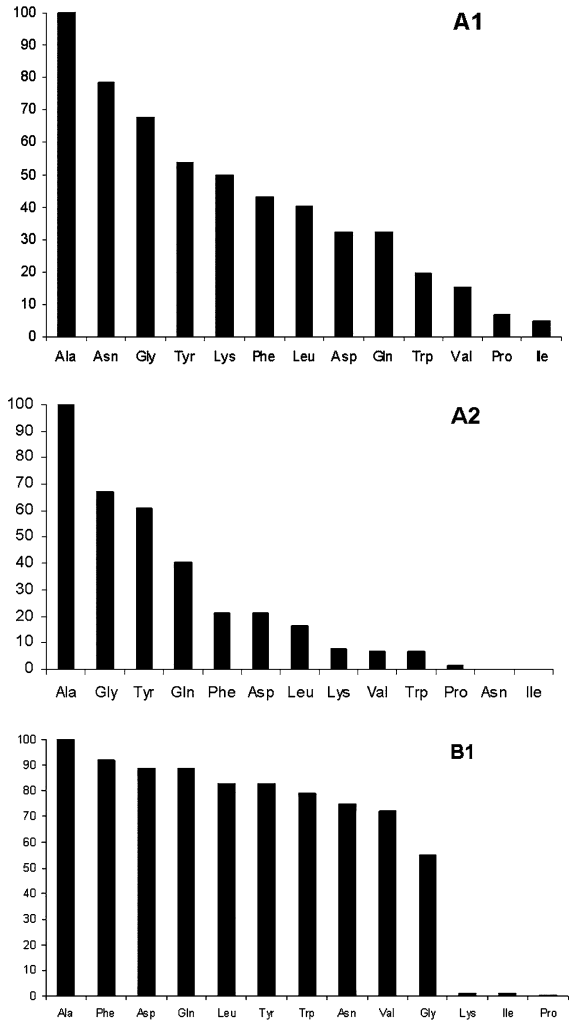

Fig. 6 Relative activity (activity on the alanine derivative was taken as 100%) of A1, A2 and B1. Protein concentration: 1.8 μg/mL in 0.1 M Tris–HCl buffer (pH 8.5) at 37 °C vs. 0.05 mM *p*-nitrophenyl-N-α-CBZ-L-amino acid esters in acetonitrile. Absorbance was read at 405 nm

3.4.3 Z-Phe-Arg-p-*NA*

Results of enzyme kinetic studies of A2 are shown in Table 4. Km value was much higher than those of stem and fruit bromelain, and ananain, against a similar substrate, Z-Phe-Arg-NH-Mec [2, 14], which could be indicative of the higher affinity of A2 for Z-Phe-Arg-cromophoric substrates. The catalytic efficiency, although it was not high, was similar to that of stem and fruit bromelains in the reaction with Z-Phe-Arg-NH-Mec [2, 14]. As was explained before for B1 and B2, A2 showed highest affinity for aromatic residues in P2 position that for basic residues, a characteristic feature for cysteine endopeptidases of family C1.

3.5 N-Terminal Sequence

The N-terminal sequences of the main endopeptidases from *Bromelia pinguin* L. are included in Table 5 (24 residues for A1, 23 residues for A2 and 20 residues for B1 and B2) and compared with those of other plant endopeptidases. Identity % values among the four enzymes studied is notably high (95%), as there is only one different amino acid residue (aspartic acid in position 9 is changed by arginine in the two basic components, B1 and B2).

Another remarkable feature is the great deal of sequence similarity to those of the other *Bromeliaceae* cysteine endopeptidases (balansain I, profastuosain, stem bromelain, ananain, comosain and macrodontain I and II, isolated from *Bromelia balansae, Ananas comosus, Bromelia fastuosa* and *Pseudananas sagenarius*, respectively), which could be interpreted as that the *Bromeliaceae* endopeptidases are more closely related to each other than to other members of the papain family, suggesting relatively recent evolutive divergence [2].

Conservation of certain motifs in the N-terminal sequence of these peptidases must be emphasized. The conservation of a Pro2 residue has been considered to prevent proteolysis of the amino terminal by aminopeptidases action, since the bond Xaa-Pro is inaccessible for this type of enzymes [24]. On the other hand, the Gln19 participates in the catalysis of the hydrolysis of the peptide-bond, whose side-chain amide stabilizes the oxyanion of the tetrahedral intermediate [25]; its absence would suggest an important variation in this mechanism, as happens in CCZ, where the change of Gln19 for Arg can be the key to locate it in another family inside the clan CA. Other highly conserved motifs in *Bromeliaceae* endopeptidases are SIDWR, GAV and VKN (residues 4–8, 11–13 and 16–18, A1 numbering). This extremely higher conservation degree in the N-terminal sequence among the proteases of *Bromeliaceae* in particular, and in cysteinyl peptidases in general, should result in that the tertiary structure of the

Table 2 Enzyme kinetic studies of the major proteolytic components of *Bromelia pinguin* on *p*-nitrophenyl-*N*-α-carbobenzoxy-L-alanine ester

	Km (μM)	Vmax (M s^{-1})	Enzyme (μM)	kcat (s^{-1})	kcat/Km (mM^{-1} s^{-1})
Pinguinain A1	233.1	1.949	0.062	31.435	134.856
Pinguinain A2	507.3	7.845	0.155	50.613	99.769
Pinguinain B1	182.1	1.365	0.182	7.500	41.186

Table 3 Enzyme kinetic studies of Pinguinain A1 on PFLNA

Km (μM)	Vmax (M s^{-1})	Enzyme (μM)	kcat (s^{-1})	kcat/Km (mM^{-1} s^{-1})
293.7	0.04436	0.103	0.431	1.467

Table 4 Enzyme kinetic studies of Pinguinain A2 on Z-Phe-Arg-p-NA

Km (μM)	Vmax (M s^{-1})	Enzyme (μM)	kcat (s^{-1})	kcat/Km (mM^{-1} s^{-1})
672.9	0.5695	0.230	2.476	3.680

Table 5 N-terminal sequences of cystein endopeptidases

Protease	Species	N – terminal sequence	% identity	Residues
Pinguinain A1	*Bromelia pinguin*	VPESIDWRDYGAVTSVKNQGEXIY		24
Pinguinain A2	*Bromelia pinguin*	VPESIDWRDYGAVTSVKNQGSXG	95.2	23
Balansain I (Pardo et al., 2000)	*Bromelia balansae*	AVPESIDWRDYGAVTSVKNQG	95.2	21
Pinguinain B1	*Bromelia pinguin*	VPESIDWRRYGAVTSVKNQG	95.0	20
Pinguinain B2	*Bromelia pinguin*	VPESIDWRRYGAVTSVKNQG	95.0	20
Fastuosain (Cabral et al., 2006)	*Bromelia fastuosa*	VPQSIDWRDYGAVTSVKNQG	95.0	20
Stem bromelain (Ritonja et al., 1989)	*Ananas comosus*	VPQSIDWRDYGAVTSVKNQ	94.7	19
Comosain (Rowan et al., 1990)	*Ananas comosus*	VPQSIDWRNYGAVTSVKNQG	90.0	20
Ananain (Lee et al., 1997)	*Ananas comosus*	VPQSIDWRDSGAVTSVKNQG	90.0	20
Macrodontain I (López et al., 2000)	*Pseudananas macrodontes*	VPQSIDWRDYGAVNEVKNQG	85.0	20
Macrodontain II (Brullo, 2003)	*Pseudananas macrodontes*	VPQSIDWRDYGAVNEVKNQ	84.2	19
CCX (Mynott et al., 1998)	*Ananas comosus*	VPQSIDWRDYGAVNEVKN	83.3	18
CCZ (Mynott et al., 1998)	*Ananas comosus*	VLPDSIDWRQKGAVTEVKNRG	71.4	21
Papain (Kamphuis et al., 1984)	*Carica papaya*	IPEYVDWRQKGAVTPVKNQG	70.0	20
Hieronymain I (Bruno et al., 2003)	*Bromelia hieronymi*	ALPESIDWRAKGAVTEVKRQDG	66.7	22

Unidentities are underlined

N-terminal domain is conserved mainly as a group of α-helixes [26, 27].

4 Discussion

By homogenizing fruits of *Bromelia pinguin* L., followed by ethanolic precipitation and ion exchange chromatography (FPLC), the four main proteolytic components were purified. The major components were A1 and A2, with acid pI, which were also more active than the other two components with basic pI, named B1 and B2. Molecular masses were determined by MALDI-TOF and ranged from 23,234.637 to 23,639.814 Da. The names Pinguinain A1, Pinguinain A2, Pinguinain B1, and Pinguinain B2 are proposed for these new endopeptidases.

When assayed against N-α-CBZ-*p*-nitrophenyl-L-amino acid esters, Pinguinain A1, Pinguinain A2 and Pinguinain B1 preferred the L-Ala derivative. With the exception of Pinguinain B1, the other three components were active on PFLNA, but not with high activity. This fact reveals that the presence of an aromatic, voluminous and not very polar amino acid, in position P2, is not an important requirement for the activity of major cysteine endopeptidases of the fruit of *Bromelia pinguin* L.

None of the obtained fractions showed activity versus the substrate Z-Arg-Arg-*p*-NA, similar behavior that exhibited stem bromelain and ananain. Pinguinain A2, Pinguinain B1 and Pinguinain B2 showed activity versus Z-Phe-Arg-*p*-NA, although not very high, as was reported for fruit bromelain. These results corroborate that cysteine endopeptidases from the fruit of *Bromelia pinguin* L. do not show particular affinity for an aromatic nor for a basic residue in the position P2.

N-Terminal sequences were determined for the four peptidases studied. Although the sequences not include the cysteine of the active site, a notably homology was observed with well-known endopeptidases of *Bromeliaceae*. On this way, a change should be suggested in the actual classification for pinguinain [28], since in the first place is not an single enzyme, and in second place it is not identical to fruit bromelain.

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