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Purification of a New Endopeptidase Isolated from Fruits of *Bromelia hieronymi* Mez *(Bromeliaceae)*

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SUMMARY. A new plant endopeptidase has been obtained from unripe fruits of *Bromelia hieronymi* Mez *(Bromeliaceae)*. Crude extracts were partially purified by organic solvents fractionation: best results (90% of proteins, 94% of total caseinolytic activity and only 9.9% of soluble sugars) were obtained by adding 4 volumes of cold acetone to the crude extract. This preparation (redissolved acetone precipitate, RAP) showed maximum activity (> 80%) at pH 7.3-10.7, and exhibited high thermal stability (80% of residual activity after heating for 30 min at 60 ºC). Low sodium chloride concentrations (0.2 M) does not affect caseinolytic activity, but diminishes with the increase of salt concentration (52% of residual activity at 2.5 M NaCl). RAP showed also clotting activity and a notably preference for κ casein over α_{s1}, αs_2 and β caseins, properties that could be of interest in the cheese industry. The enzyme was completely inhibited by E-64 and iodoacetic and activated by the addition of cysteine; these results strongly suggest that the isolated protease should be included within the cysteine group, as all the other studied proteases belonging to the family *Bromeliaceae*. IEF-zymogram of RAP showed six bands (pI 5.9 to >9.3), most of them proteolytically actives, but only three of which (pI 6.4, 8.3 and >9.3) proved to be important. Cation exchange chromatography (FPLC) allowed the isolation of the main fraction, named hieronymain I (pI = >9.3, molecular weight = 25 kDa).

RESUMEN. "Purificación de una nueva endopeptidasa aislada de frutos de *Bromelia hieronymi* Mez *(Bromeliaceae)*". Se ha aislado una nueva endopeptidasa a partir de frutos inmaduros de *Bromelia hieronymi* Mez *(Bromeliaceae).* Los extractos crudos fueron parcialmente purificados por fraccionamiento con solventes orgánicos, habiéndose obtenido los mejores resultados al precipitar el extracto crudo con 4 volúmenes de acetona fría (el precipitado retiene el 90% de las proteínas y el 94% de la actividad caseinolítica y contiene sólo 9,9% de azúcares solubles). El precipitado acetónico redisuelto (RAP) mostró máxima actividad (> 80%) a pH 7,3 -10,7 y muestra gran estabilidad térmica (mantiene 80% de actividad residual luego de calentarlo a 60 ºC durante 30 min). Bajas concentraciones de cloruro de sodio (0,2 M) no afectan la actividad caseinolítica, pero ésta disminuye con el incremento de concentración salina (52% de actividad residual cuando la concentración de cloruro de sodio es 2,5 M). El RAP demostró poseer actividad coagulante de la leche y notable preferencia por la fracción κ caseína por encima de las α_{s1}, α_{s2} y β caseínas, características que pueden resultar de interés en la industria quesera. La enzima resultó totalmente inhibida por E-64 y ácido iodoacético y activada por cisteína, lo que sugiere que pertenece al grupo de la endopeptidasas cisteínicas, en el que se encuentran todas las proteasas estudiadas de la familia *Bromeliaceae.* El isoelectroenfoque seguido de zimograma revela la presencia de seis bandas (pI 5,9 a > 9,3), la mayoría de ellas proteolíticamente activas, pero sólo tres (pI 6,4, 8,3 y >9,3) de importancia. Por cromatografía de intercambio catiónico (FPLC) se logró aislar la fracción principal, denominada hieronymina I (pI >9,3 peso molecular 25 kDa).

INTRODUCTION

It is beyond question that the results of research on proteolytic enzymes, or peptidases, are already benefiting mankind in many ways, and there is no doubt that research in this area has the potential to contribute still more in the future. One of the clearest indications of the general recognition of this promise is the vast annual

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PALABRAS CLAVE: Actividad coagulane de la leche, *Bromelia hieronymi, Bromeliaceae,* Fitopeptidasa, Purificación parcial.

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expenditure of the pharmaceutical industry on exploring the involvement of peptidases in human health an disease [1].

Bromeliaceae is a plant family whose members usually produce large amounts of peptidases with no apparent function in plant growth and development [2]. Up 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* [3-7], as well as proteases from fruits of *Bromelia balansae* [8,9], *B. hemispherica* [10,11], *B. palmeri* [10,11], *B. pinguin* [12,13], *B. plumieri* [14], *B. sylvestris* [10,11], and *Pseudananas macrodontes* [15-17].

The presence of endopeptidases in fruits of *Bromelia hieronymi* Mez *(Bromeliaceae)* had been previously reported [18,19]. In this paper the isolation and partial characterization of the main cysteine proteinase of unripe fruits of *Bromelia hieronymi* Mez *(Bromeliaceae)* is reported, adopting a new purification strategy. The present study is part of a program dealing with the isolation of new proteases from regional plants that are currently not used in agriculture, a fact that could have positive effects for local economies.

MATERIALS AND METHODS

Plant Material

Bromelia hieronymi Mez (folk name "chaguar") is a stoloniferous plant that have 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 x 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 ºC until the beginning of the extraction procedure.

Crude preparations

Crude extracts were obtained by chopping and homogenizing frozen fruits (50 g) for 1 min in an Omni Mixer (Sorval) with 250 mL of cold 0.1M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA and 5 mM cysteine. Homogenates were filtered through a two-folded piece of gauze to remove plant debris, and then centrifuged for 30 min at 16000 x g. Supernatants were collected, filtered, and immediately frozen at -20 ºC until analysis. All operations were carried out at 0-4 ºC [16].

Fractionation with organic solvents

Owing to high concentration of carbohydrates, 5 mL of crude extract were treated with increasing volumes of cold (-20 ºC) acetone and ethanol (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0, respectively) with gentle agitation and left to settle for 20 min prior to centrifugation at 16000 x g for 30 min. The final acetone precipitates were redissolved with 5 mL of the extraction bauffer and frozen until further use [20].

Protein and carbohydrate content

Protein concentration was determined by Coomassie Blue dye-binding method [21], 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 [22].

Proteolytic activity assays

The reaction mixture contained 1.1 mL of 1% (w/v) casein solution in 0.1M Tris-HCL buffer (final pH 8.0) containing 15 mM cysteine and 0.1 mL of 1:6 dilution of enzyme solution. The mixture was incubated for 2 min, according to enzyme concentration, at 37 ºC 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 x 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 [15].

Effect of inhibitors and activity enhancers

The effect of specific inhibitors [23] on proteolytic activity was determined by measuring the residual activity on casein at pH 8,0 after preincubation at 37 ºC for 30 min in the presence of E-64 (100 µM), and iodoacetic acid (0.1 and 1.0 mM). Controls were prepared by pre-incubating 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 y 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, 30, 40, 60, 90, and 120 min at 37, 55, 60, 65, and 75 ºC and then the residual caseinolytic activity was measured as mentioned.

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 to 12.5 using 25 mM sodium salts of the following "Good" buffers [24]: MES, MOPS, TAPS, AMPSO and CAPS.

Effect of temperature on enzyme activity

Progress curves for different temperatures (37, 45, 55, 65 y 75 ºC) were made by measuring the caseinolytic activity along time (1, 2, 4, 8, 12, and 16 min) as indicated above.

Effect of ionic strength on enzyme activity

Caseinolytic activity at different ionic strength conditions was determined by incubating enzyme samples at 37 ºC and pH 8.0 with different sodium chloride concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 2.5 M) and measuring the residual caseinolytic activity as mentioned.

Isoelectrofocusing and zymograms

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 ºC) acetone, centrifuged, 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 by Coomassie Brilliant Blue R-250.

In order 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, containing 15 mM cystein) and then washed twice with distilled water. Unstained IEF gels were contacted for 7 min at 60 ºC with the agarose gel. Proteolytic activities became visible as clear bands on the stained agarose gels [25].

FPLC cation-exchange chromatography

Cation exchange chromatography was performed on a column of SP-Sepharose FF equilibrated with 50 mM Tris-HCl buffer (pH 8.25) at 21 ºC. After washing the column with the same buffer, the retained proteins were eluted with 80 mL of a linear sodium chloride gradient (0.0-0.15 M) in the starting buffer.

SDS-PAGE

SDS-polyacrylamide gel electrophoresis of enzyme samples was performed in a Miniprotean III Cell (Bio-Rad) according to Laemmli [26]. 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.

Clotting activity

Clotting activity was measured using skim milk powder (San Regim, SanCor, Santa Fe, Argentina); the milk substrate was dissolved (10% w/v) at 37 ºC in with 0.01M $CaCl_2$ (pH 6.0); 0.5 ml of this substrate were mixed with 0.1 ml of enzyme. One unit of milk clotting activity (CU) was defined as the reciprocal of the time (min) necessary to start milk clotting [9].

Hydrolysis of caseins

Hydrolysis was carried out at 45 ºC and pH 8.0 with an enzyme/substrate relationship of 4.5 Ucas/g of casein. Hydrolysates were subjected to denaturing electrophoresis in tricine gels composed of a stacking gel (4%), a separating gel (10%) and a resolution gel (16,5 %T), which is especially suitable to resolve the mixture of peptides produced. The electrophoretic profiles were analyzed by densitography [27].

RESULTS AND DISCUSSION

Crude extracts 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 fractionation was carried out with organic solvents (acetone and ethanol). Best yield was obtained by adding 4 volumes of solvent. Fractionation with both solvents provided similar enzyme preparations: the redissolved acetone precipitate contained 90% of proteins, 94% of total caseinolytic activity and 9.9% of soluble sugars, while the redissolved ethanol precipitate contained 75% of proteins, 88% of total caseinolytic activity and 3.6 % of soluble sugars, with respect to crude extracts. On the basis of these results, the redissolved acetone precipitate (RAP) was selected for enzyme purification and partial characterization.

For industrial applications enzyme purity is usually of secondary importance to cost [28] . 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. RAP 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 ºC during 120 min or at 55 ºC during 40 min, while at 60 ºC after 30 min remained 80% of initial activity. The enzyme was almost completely inactivated by heating for 20 min at 75 ºC. Enzyme activity increases with temperature but at 75 ºC the reaction rate notably diminishes after 2 min because of enzyme denaturation (Fig. 3). Low sodium chloride concentrations (0.2 M) does not affect caseinolytic activity, but diminishes with the increase of salt concentration (52 % of residual activity at 2.5 M NaCl).

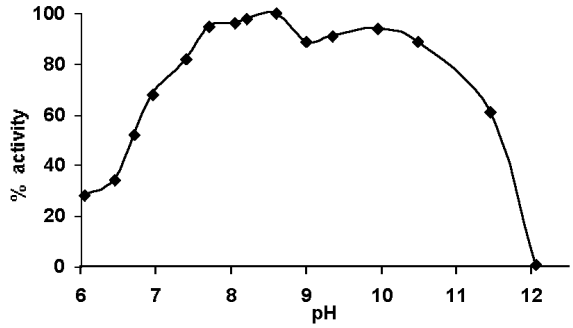

Figure 1. Effect of pH on proteolytic activity. Proteolytic activity was measured on 1% casein solution containing 15 mM cysteine. Data points represent the mean value of four determinations and each experiment was repeated twice.

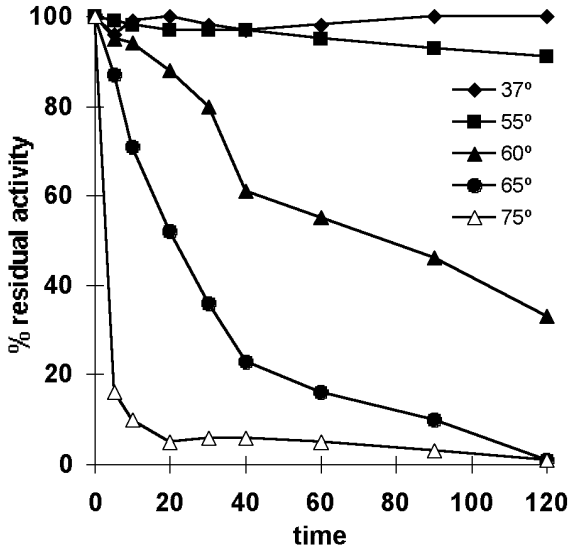

Figure 2. Thermal stability of RAP. Data points represent the mean value of five determinations and each experiment was repeated twice.

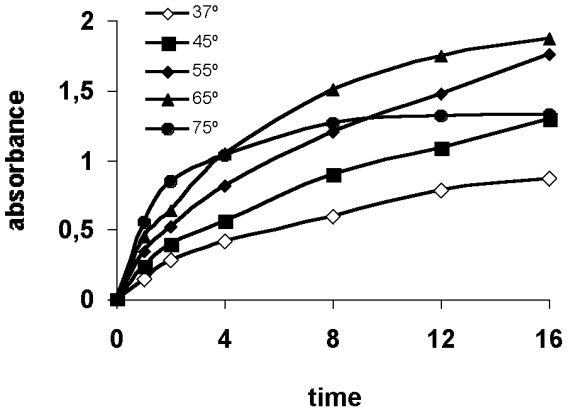

Figure 3. Enzyme activity of crude extract as a function of temperature. Enzyme activity was determined on casein at pH 8.

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 (1.34 times in relation to the same preparation without the addition of cysteine). These results strongly suggest that the enzyme preparation contains cysteine-type proteases, as all the other studied proteases belonging to the family *Bromeliaceae.*

RAP exhibited an interesting milk clotting activity (2.4 CU). The electrophoretic analysis of the casein hydrolysates (Fig. 4) showed a notably preference of RAP for κ casein (involved in milk coagulation), while the β-casein fraction, related to bitterness, showed a slower degradation kinetics, a behavior that could be of interest in the cheese industry.

Isoelectrofocusing of RAP followed by zymogram analysis showed six bands (pI = 5.9, 6.4,

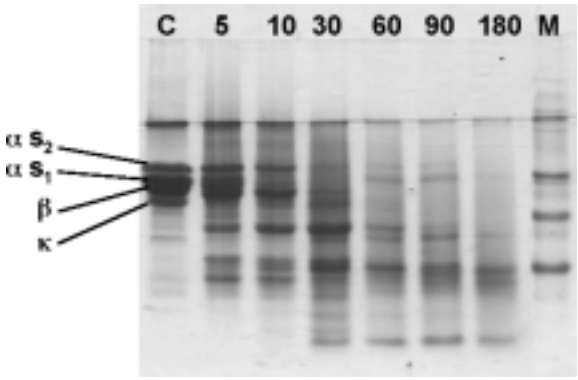

Figure 4. Electrophoretic analysis of casein hydrolysates. C: caseins; 5, 10, 30, 60, 90, 180: casein hydrolylsates corresponding to 5, 10, 30, 60, 90, and 180 min.; M: molecular weight markers

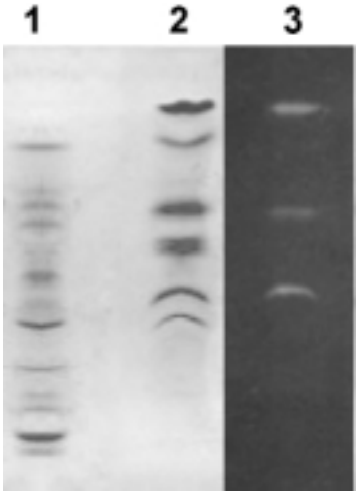

Figure 5. Isoelectrofocusing and Zymogram. Lane 1, IEF markers (Sigma IEF mix 3.6-9.3 isoelectric focusing marker); Lane 2, IEF of RAP; Lane 3, zymogram of RAP.

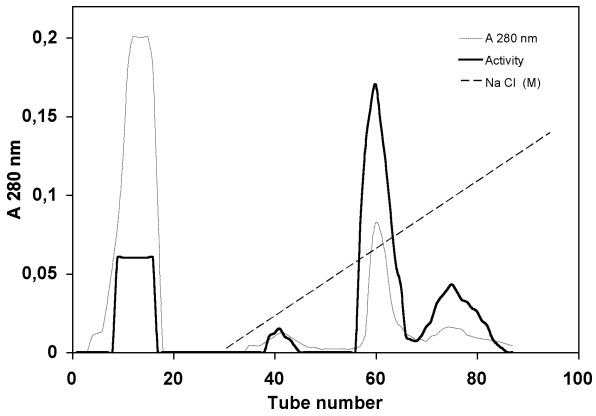

Figure 6. FPLC Ion-Exchange Chromatography of RAP. Sample volume 2 mL; flow rate 0.56 mL.min^{-1}; fraction volume 1.8 mL.

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. 5). On the basis of these results, cation exchange chromatography was selected for the next purification step.

FPLC Cation exchange chromatography of RAP (Fig. 6) allowed the separation of the main proteolytic component (pI > 9.3, MW = 25 kDa), named hieronymain I, which purity is evidenced by SDS-PAGE (Fig. 7).

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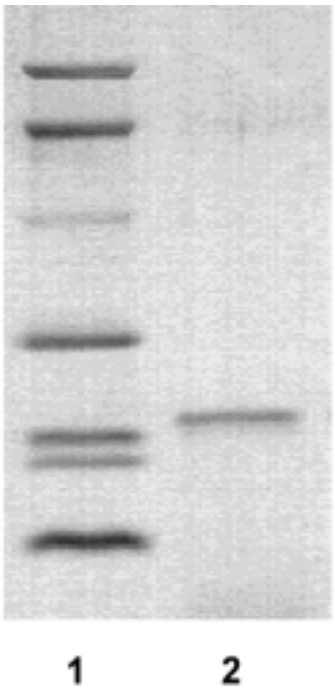

Figure 7. SDS-PAGE of hieronymain I. Lane 1, low molecular weight markers (Bio-Rad): lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase bovine (31.0 kDa), ovalbumin (45.0 kDa), serum albumin bovine (66.2 kDa), and phosphorylase B (97.4 kDa). Lane 2, *hieronymain I.*

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