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Isolation, Purification and Partial Characterization of a New Protease from the Latex of Fruits of *Morrenia brachystephana* Griseb. *(Asclepiadaceae)*

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SUMMARY. Latex obtained from fruits of *Morrenia brachystephana* Griseb. *(Asclepiadaceae)*, received on 0.1 M phospate buffer (pH 6.9) containing 5 mM EDTA and 5 mM cysteine and centrifuged at 16,000 x g for 30 min gives a crude extract that shows high proteolytic activity when assayed on casein in the presence of 12 mM cysteine. The enzyme preparation was strongly and irreversibly inhibited by very low concentrations of sodium iodoacetate (0.01 mM) and mercuric chloride (1 mM), but not fully and reversibly inhibited by 10 mM PMSF, suggesting that the protease belongs to the cysteine type. Maximum activity was reached at alkaline pH (8.0-10.0). The crude extract shows a remarkable thermal stability (80% of residual activity after 2 h at 60 °C). Fractioned acetone precipitation followed by cation exchange chromatography (CM-Sepharose CL-6B Fast Flow) allows the separation of two proteolytically active fractions, the principal of which has a relative molecular mass of 22.1 kDa (SDS-PAGE) and an isoelectric point higher than 9.3 (IEF).

RESUMEN. "Aislamiento, Purificación y Caracterización Preliminar de una Proteasa de Látex de frutos de *Morrenia brachystephana* Griseb. *(Asclepiadaceae)*". El látex proveniente de frutos de *Morrenia brachystephana* Griseb. *(Asclepiadaceae)* recogido sobre buffer fosfatos 0,1 M (pH 6,9) conteniendo EDTA y cisteína 5 mM y centrifugado a 16.000 x g durante 30 minutos constituye un extracto crudo con actividad proteolítica destacada frente a caseína como sustrato, en presencia de cisteína 12 mM. La preparación enzimática fue fuerte e irreversiblemente inhibida por muy bajas concentraciones de iodoacetato de sodio (0,01 mM) y de cloruro mercúrico (1 mM) e inhibida en forma parcial y reversible por PMSF 10 mM, sugiriendo que la proteasa pertenece al tipo cisteínico. La actividad caseinolítica resulta máxima a pH alcalino (pH 8,0-10,0). El extracto crudo muestra una notable estabilidad térmica (80% de actividad residual luego de 2 h a 60 °C). Mediante precipitación acetónica fraccionada, seguida de cromatografía de intercambio catiónico (CM Sepharose CL-6B Fast Flow), se obtienen dos fracciones proteolíticamente activas. La fracción principal (II) tiene una masa molecular relativa de 22,1 kDa (SDS-PAGE) y un punto isoeléctrico superior a 9,3 (IEF).

KEY WORDS: *Asclepiadaceae*. Enzymes, Latex, *Morrenia brachistephana*, Plant proteases.
PALABRAS CLAVE: Asclepiadaceae, Enzimas, Fitoproteasas, Látex, *Morrenia brachystephana*.

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INTRODUCTION

Proteolytic enzymes are still very used in different pharmaceutical preparations [1]. Papain and ficin (both "latex proteases") are (together with bromelain, obtained from stems and fruits of pineapple) the most representative plant proteases. Though proteases are frequently present in plant laticifers, not all the latex-containing species are good producers of proteolytic enzymes [2]. In the *Asclepiadaceae* family the only references on proteases are restricted to a few species of the genus *Asclepias* and *Calotropis* [3-11].

The most recent ethnopharmacological information on the genus *Morrenia* has been provided by Burkart [12], who refered the use of *Morrenia brachystephana* latex for milk clotting and *Morrenia odorata* latex for the extirpation of warts. Popular uses of the latex of both species could be related with the presence of proteolytical enzymes. In the present paper we report the isolation, purification and partial characterization of a protease present in the latex of fruits of *Morrenia brachystephana* Griseb. *(Asclepiadaceae)*. Preliminary efforts on the purification of M. odorata are under way in our laboratory.

MATERIAL AND METHODS

Plant Material

Morrenia brachystephana Griseb. (argentine folk names: "tasi", "doca") is a pubescent vine with hastates, acuminated leaves, 4-6 cm long. Flowers are small, white-greenish, organized in umbelliferous inflorescences. Fruits are ovoidal follicles (6-7 cm long, 2.5-3 cm wide), showing a waved surface [13]. Latex was obtained from plants grown in the surroundings of Rosario, Province of Santa Fe, Argentina.

Preparation of the crude extract

Latex was obtained by superficial incisions of fruits and gathered on 0.1 M phosphate buffer (pH 6.9) containing 5 mM EDTA and 5 mM cysteine, at 0-4 °C. The resulting suspension (9% latex, v/v) was diluted (1:5) with the same buffer, homogenized and centrifuged for 30 min at 4 °C and 16,000 x g. The precipitate - gums and insoluble materials- was discarded, and the supernatant containing the soluble protein fractionated and conserved at -20 °C for further studies.

Proteolytic activity assays

Casein (Hammarsten type, Research Organics) was the proteolytic substrate used in all the assays. The reaction mixture was prepared with 1.1 ml of 1% casein containing 12 mM cysteine and 0.1 ml of enzyme extract, both in a 0.1 M Tris-HCl buffer (pH 8.5). The reaction was carried out at 37 °C and after 2 min was stopped by the addition of 1.8 ml 5% trichloroacetic acid. Each test tube was centrifuged at 3,000 x g for 20 min and the absorbance of the supernatant read at 280 nm. An arbitrary enzyme unit ("caseinolytic unity", U_{cas}) was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the assay conditions.

Protein and carbohydrate content

Proteins were determined according to Bradford [14], using bovine albumin (Sigma Chem. Co., St Louis, MO) as standard. In all chromatographic procedures

protein concentration was estimated by measuring the absorbance of eluates at 280 nm. Carbohydrate content was estimated using the method of Dubois *et al.*[15].

Effect of pH on proteolytic activity

The dependence of enzyme activity of crude preparations on pH was measured using casein as substrate within the pH range 6.0 to 11.0 using 0.01 M sodium salts of the following "Good" [16] buffers: MES, MOPS, TAPS, AMPSO and CAPS (Sigma Chem. Co., St Louis, MO).

Effect of activators and inhibitors

The action of different activators and inhibitors of proteases was evaluated on proteolytic activity by incubating the crude enzymatic preparation for 10 and 30 min at 37 °C with different chemicals: 12 mM cisteyne, 0.1 mM and 1 mM mercuric chloride, 10 mM phenylmethylsulphonylfluoride (PMSF), 0.01 mM and 0.1 mM sodium iodoacetate, and 2% (w/v) sodium dodecylsulphate (SDS). The residual caseinolytic activity after each incubation assay was measured as indicated above.

Thermal stability

Thermal stability of crude enzyme preparation was evaluated by measuring residual caseinolytic activity at 39 °C (pH 8.5) after incubation of extracts for 10, 20, 30, 60, and 120 min at 40 °C, 50 °C, 60 °C and 70 °C.

Acetone fractionation

One volume of the crude extract was successively treated with one to three volumes of cold (-20 °C) acetone with gentle agitation. The suspensions were settled for 15 min at -20 °C and then centrifuged at 16,000 x g for 15 min. In order to choose the most suitable purification procedure prior to chromatography, each precipitate was redissolved in 55 mM citric acid-phosphate buffer (pH 6.2) and its caseinolytic activity, as well as its protein and carbohydrate content, were determined.

Isoelectric focusing (IEF)

A Mini IEF Cell (Model 111, Bio-Rad) was employed to carry out isoelectric focusing of the proteases. Samples were concentrated and deionized by acetone precipitation and further centrifugation at 11,000 x g for 20 min; the precipitates were redissolved with deionized water and the treatment repeated twice. Polyacrylamide gels (10%) containing broad pH range ampholytes (Pharmalyte 3-10, Pharmacia) were used. Focusing was carried out under constant voltage conditions in a stepped fashion: 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.

Ion exchange chromatography

The enzyme preparation (5 ml) was loaded onto a CM-Sepharose Fast Flow column (Pharmacia K 15/30) equilibrated with 55 mM citric acid-phosphate buffer (pH 6.2). Cation exchange chromatography was developed by adding 60 ml of the starting buffer, followed by 200 ml of sodium chloride linear gradient (0.0-1.0 M) prepared in the same buffer.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Miniprotean II Cell (Bio-Rad) according to Laemmli [17]. Samples were precipitated with 3 volumes of acetone, redissolved in the sample buffer containing β-mercaptoethanol and boiled for 3 min, and then loaded on 14% polyacrylamide gels (stacking gel: 5% polyacrylamide). Current was kept constant at 35 mA for 90 min. Gels were stained by Coomassie Brilliant Blue R-250.

Zymogram

In order to confirm proteolytic activity, unstained IEF gels were contacted for 45 min at 50 °C with an agarose gel imbibed with a 1% casein solution [18], which was then stained by Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Morrenia brachystephana Griseb. belongs to a botanic family *(Asclepiadaceae)* whose members usually develop secretory tissues (laticifers) which frequently include proteolytic enzymes. Latex obtained from fruits of this species was gathered on neutral buffer and centrifuged, obtaining a "crude extract" devoided of gums and other insoluble materials.

For industrial applications, enzyme purity is usually of secondary importance to cost [19, 20]. For these reasons, the effect of pH and thermal stability of the enzyme was assayed on the crude extract before starting with the purification procedure. The enzyme extract shows maximum proteolytic activity within an alkaline range of pH (Figure 1), a property that makes it specially valuable in processes carried out in weak alkaline media.

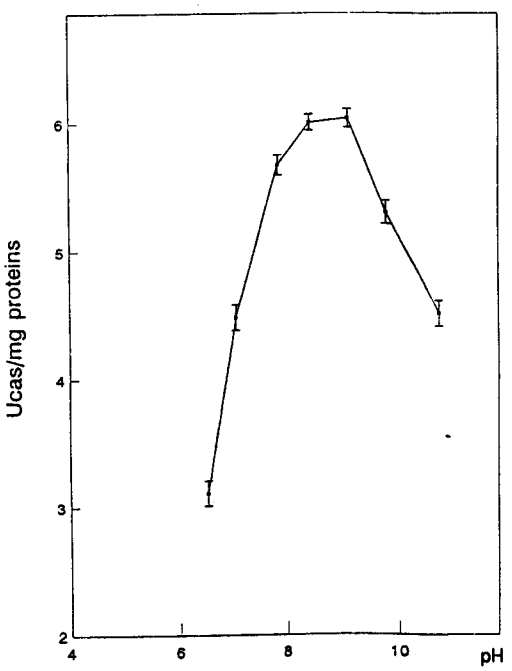

Figure 1. Effect of pH on activity of the crude extract of Morrenia brachystephana latex. pH values are those of the reaction mixtures. Data points represent the mean value of five determinations and each experiment was repeated three times.

The enzyme activity was notably enhanced by the addition of 12 mM cysteine, but it was completely inactivated by incubation with 1mM Cl_2Hg for 10 min and strongly inhibited during 30 min with 0.01 mM sodium iodoacetate. On the other hand, incubation with 10 mM PMSF for 30 min depressed activity to 46% of the initial value, but inhibiton was partially reverted when the reaction was carried out in the presence of 12 mM cysteine (Table 1). This results suggests that -SH groups could be implied in the catalytic mechanism of the enzyme; if so, the proteases present in the latex of *Morrenia brachystephana* fruits would belong to the cysteine type.

As it can be seen in Figure 2, the enzyme crude extract shows a remarkable thermal stabililty: after 2 h at 40 °C caseinolytic activity remains practically un-

Inhibitors	Concentration	Residual proteolytic activity, %	
		10 min	30 min
None	–	100.0	100.0
Mercuric chloride	0.1 mM	20.0	19.0
Mercuric chloride	1 mM	0.0	0.0
Sodium Iodoacetate	0.01 mM	10.9	2.4
Sodium Iodoacetate	0.1 mM	4.6	0.0
PMSF	10 mM	59.5	45.9
PMSF + cysteine	10 mM + 12 mM	68.7	73.0
SDS	2% (w/v)	3.2	0.0

Table 1. Effect of inhibitors on proteolytic activity. Data are the mean value of five determinations and each experiment was repeated twice.

changed, and the residual activity is notably high even after 2h at 60 °C (80%) and is still high at 70 °C (45% of remaining activity after 2h).

The crude extract contains soluble sugars (58.6 mg/ml) which were parcially eliminated when mixed with one volume of cold (-20 °C) acetone, that also removed a protein mixture with low-specific activity (specific activity 0.59 U_{cas}/mg). The addition of a second volume of acetone to the first supernatant afforded a precipitate devoided of sugars and very rich in proteolytically active proteins. When a new volume of acetone was added to the second supernatant, a third precipitate also devoided of soluble sugars and containing a minor quantity of proteins with high specific activity was obtained (Table 2). On the basis of this data, acetone fractionation was used as the first purification step. Best results were obtained by adding one volume of cold (-20 °C) acetone to the supernatant and discarding the precipitate (20% of proteins, only 5.3% of activity); further addition of two volumes of acetone to the remaining supernatant provided a partially purified preparation ("acetone precipitate") devoided of soluble sugars and containing: 71.7% of proteins, which retained 84.2% of the original proteolytic activity.

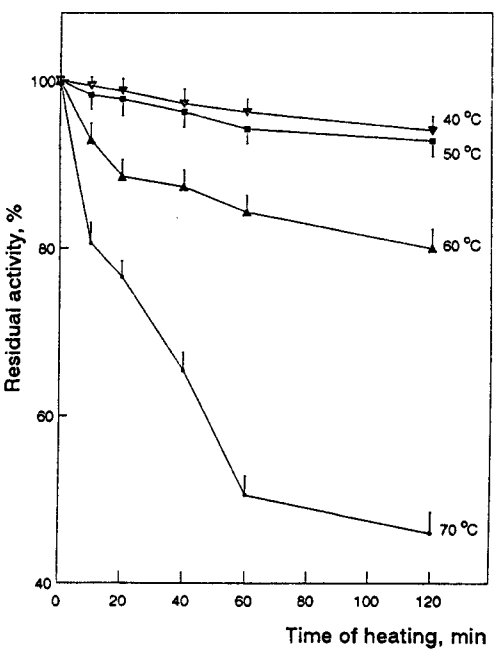

Figure 2. Thermal stability of the crude extract of Morrenia brachystephana latex. Activity on casein at pH 8.3 after 2 min at 39 °C was taken as 100%. Data points represent the mean value of five determinations and each experiment was repeated twice.

Sample	Soluble sugars (mg/ml)	Protein (µg/ml)	Caseinolytic activity (U_{cas}/ml)	Specific activity (U_{cas}/mg)
Crude extract	58.6	1,186	2.63	2.22
Acetone precipitate (1 vol)	16.7	236	0.14	0.59
Acetone precipitate (2 vol)	–	644	1.81	2.82
Acetone precipitate (3 vol)	–	226	0.46	2.03

Table 2. Fractioned acetone precipitation of the crude extract of *Morrenia brachystephana* latex.

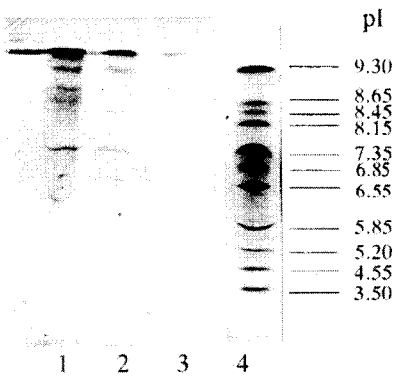

Figure 3. Isoelectric focusing. Lane 1: crude extract; lane 2: acetone precipitate; lane 3: fraction II; lane 4: IEF Sigma markers.

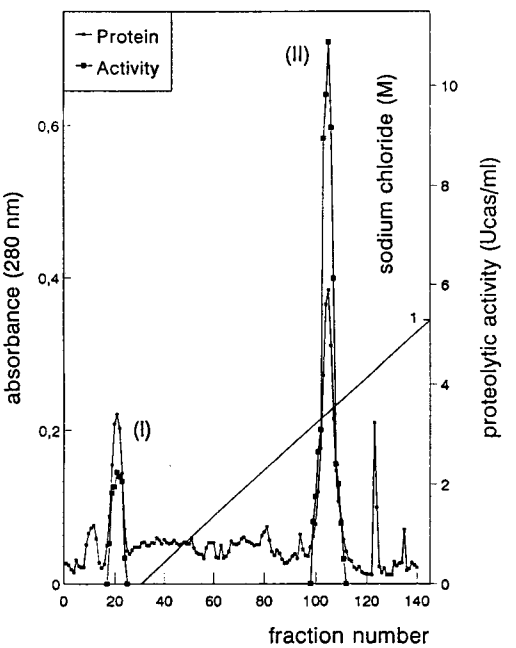

Figure 4. Cation exchange chromatography (CM-Sepharose CL-6B Fast Flow, column Pharmacia K 15/30). Elution buffer: 0.055 M citric acid-phosphate. Gradient: sodium chloride 0.0-1.0 M. Flow rate: 17 $cm.h^{-1}$. Fraction volume: 1.8 ml.

Application of IEF to the crude extract as well as to the acetone precipitate showed that most of the protein fractions were located near the cathode, evidencing their basic nature (Figure 3). Based in this observation, in the next purification step a cation exchange chromatography was used (Figure 4): the elution of the column with the starting buffer afforded a protein fraction with low proteolytic activity (fraction I), and another protein fraction with very high proteolytic activity eluted (fraction II) when the sodium chloride gradient reached 0.66 M concentration. Table 3 shows the progress of the purification procedure.

IEF of fraction II confirmed its basic nature (pI > 9.3, Figure 3) and the zymogram (data not shown) corroborated its proteolytic behavior. The SDS-PAGE analysis (Figure 5) revealed that the principal active fraction had a relative molecu-

Sample	Protein (μg/ml)	Caseinolytic activity (U_{cas}/ml)	Specific activity (U_{cas}/mg)	Purification (fold)	Yield (%)
Crude extract	1,186	2.63	2.22	1.00	100.0
Acetone precipitate	850	2.22	2.61	1.18	84.2
Purified fraction	316	1.55	4.91	2.21	59.0

Table 3. Purification of the proteolytic component present in the latex of *Morrenia brachystephana*.

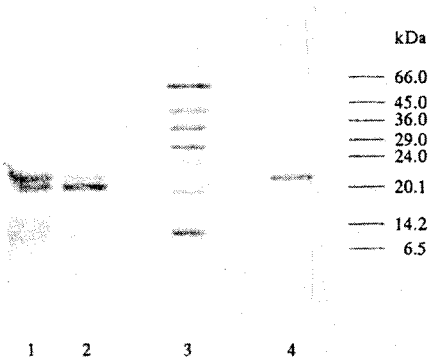

Figure 5. SDS-PAGE. Lane 1: crude extract; lane 2: acetone precipitate; lane 3: Molecular Weight Sigma markers (MW 6,500 - 66,000 kit, Sigma); lane 4: fraction II.

lar mass of about 22.1 kDa, which is in good agreement with the results obtained when other proteases from *Asclepiadaceae* were studied: the M_r of *Asclepias syriaca* proteases are 21 and 23 kDa [6,7], proteases from *Asclepias glaucescens* [8,9] have a M_r of 23 kDa and a pI higher than 9, and the four calotropins isolated from *Calotropis gigantea* show molecular masses ranging from 23 to 27 kDa [10-11].

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