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Hidrolytic Profile and isolation of the Proteolytic Components of Latex from *Araujia angustifolia* Fruits

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SUMMARY. The presence of hydrolases in the latex of *Araujia angustifolia (Asclepiadaceae),* a climbing plant that grows in Argentina, has been studied. The crude extract (CE) obtained by differential centrifugation at 8000 and 15000 rpm of latex from *A. angustifolia* fruits, collected on 0.05 M citric-citrate buffer (pH 4.5) with 5 mM EDTA, exhibited several enzymatic activities. CE showed amydasic activity on L-pyroglutamyl-L-phenylalanyl-L-leucine-p-nitroanilide (PFLNA), proteolytic activity on 1% casein in Tris-HCl pH 8.5, polygalacturonidase activity using 1% polygalacturonic acid in acetic acetate buffer pH 4.5, pectin methyl esterase on 1% pectin in citric phosphate pH 6.8 in the presence of 0.02M Cl_2Ca, and endosterolytic activity on *p*-nitrophenyl esters of N-α-carbobenzoxy-L-amino acids in Tris-HCl buffer pH 8.0. As proteolytic activity was the main hydrolytic activity observed, the proteolytic properties of CE were stablished. The proteases present in CE showed the characteristics of the cysteine proteases: optimum pH at alkaline range, isoelectric point (pI) higher than 8.0, and inhibition of activity by thiol blocking reagents, such as E-64 and iodoacetate. A remarkable thermal stability was also evident in the CE. Three proteases have been detected by IEF and zymogram in the CE and purified by FPLC affording three basic active fractions *(araujiain aI, aII and aIII)* with molecular masses about 23 kDa (SDS-PAGE).

RESUMEN. "Perfil Hidrolítico y Aislamiento de los Componentes Proteolíticos Presentes en el Látex de Frutos de *Araujia angustifolia"*. Se estudiaron las hidrolasas presentes en el látex de *Araujia angustifolia (Asclepiadaceae),* enredadera que crece en Argentina. El extracto crudo (CE) obtenido por centrifugación diferencial a 8000 y 15000 rpm del látex de frutos de *A. angustifolia,* recogido en buffer cítrico-citrato 0,05 M (pH 4,5) con EDTA 5 mM, exhibió varias actividades enzimáticas: amidásica con L-piroglutamil-L-fenilalanil-L-leucina-*p*-nitroanilida (PFLNA) como sustrato; proteolítica sobre caseína al 1% en Tris-HCl pH 8,5; poligalacturonidasa usando ácido poligalacturónico al 1% en buffer acético-acetato pH 4,5; pectín metil esterasa ensayada sobre pectina al 1% en buffer cítrico-fosfato pH 6,8, en presencia de Cl_2Ca 0,02M y endosterolítica empleando los ésteres *p*-nitrofenilados de N-α-carbobenzoxi-L-aminoácidos en buffer Tris-HCl. pH 8,0. Dado que la actividad proteolítica fue la principal actividad hidrolítica observada, se determinaron las características proteolíticas del CE, que contiene proteasas de tipo cisteínico: pH óptimo alcalino, punto isoeléctrico (pI) superior a 8,0 y resultan inhibidas por reactivos bloqueantes de grupos tiólicos, tales como iodoacetato y E-64. El CE mostró notable estabilidad térmica. Se detectaron tres proteasas por IEF y zimograma y luego fueron purificadas por FPLC. Las masas moleculares de las mismas fueron del orden de 23 kDa (SDS-PAGE). Las mismas fueron denominadas *araujiaína aI, aII* y *aIII.*

INTRODUCTION

Hydrolases are involved in essential metabolic processes for growing, development and maintenance of plants. On the other hand, many of these enzymes, mainly proteases, pectinases, xylanases, esterases, polygalacturonidases, and cellulases are of great interest for industrial purposes, owed to the variety of processes in which they can be used, and most of them are usually present in latexes of species belonging to the *Asclepiadaceae* family, also known as milkweed family, since these plants ooze a sticky, white latex when cut [1].

Proteolytic enzymes, like other natural prod-

KEY WORDS: *Araujia angustifolia, Asclepiadaceae,* Hydrolases, Milkweed family, Plant latex.
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ucts, have been used in empiric medicine and food applications for a long time. Proteases preparations are, on a commercial basis, the most important of the currently produced enzymes. Nowadays peptidases of plant origin are of special interest in medicine and industry because they are active at a very wide range of temperature and pH [2].

Proteases are very frequently in the latex of *Asclepiadaceae*. Nevertheless, studies about these proteases are limited to a few species belonging to the genera *Asclepias* [3-9] and *Calotropis* [10-13]. Studies on proteases from *Asclepiadaceae* species growing in Argentina have been previously reported by our group: *Araujia hortorum* [14,15], *Asclepias fruticosa* [16], *Morrenia brachystephana* [17-19], *M. odorata* [19,20], *Philibertia gilliesi* [21], *Funastrum clausum* [1, 22] and *Asclepias curassavica* [23].

In this paper the proteolytic behavior of the latex from fruits of *Araujia angustifolia* (Hook. et Arn.) Decaisne is informed. As the presence of hydrolases other than proteases has been reported for some latexes [22-28], latex of *Araujia angustifolia* was also assayed for polygalacturonidase, pectin methyl esterase, rhamnogalacturonidase, xilanase and methylcellulase activities.

MATERIALS AND METHODS

Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc., Cleveland, Ohio. AMPSO, CAPS, cysteine,DTT, E-64, EDTA, iodoacetic acid, MOPS, *p*-nitrophenyl esters of N-α-carbobenzoxy-L-amino acids, TAPS and Tris were purchased from Sigma Chemical Company, St. Louis. Coomassie Brilliant Blue R-250, acrylamide, bisacrylamide and low molecular weight markers were obtained from Bio-Rad, Hercules, California. SP-Sepharose , SulfoPropyl-sheparose Fast Flow and Pharmalyte 3-10 were purchased from Pharmacia Biotech, Uppsala. All other chemicals were obtained from commercial sources and were of the highest purity available.

Plant material

Fruits of *Araujia angustifolia* (Hook. et Arn.) Dec. were obtained from plants grown in M. B. Gonnet, Province of Buenos Aires, Argentina. The plant is an attractive, small, tender vine with small green twining stems, evergreen arrow shaped leaves and most unusual white bell shaped flowers with the petals reflexed leaving a pink pointed central structure [29]. Voucher specimens were deposited at the LPE herbarium (Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina).

Preparation of the crude extract (CE)

Latex obtained by superficial incisions of fruits, received on 0.05 M citric-citrate buffer (pH 4.5) containing 5 mM EDTA, was first centrifuged at 8,000 rpm for 30 min at 4 °C. Gums and other insoluble materials were discarded, and the supernatant was centrifuged at 15,000 rpm for 60 min at 4 °C. This new supernatant ("crude extract"), containing soluble proteins, was fractionated and conserved at -20 °C for further studies.

Protein determination

Proteins present in the crude extract were determined by Bradford's method [30] using bovine albumin (Sigma Chem. Co., St Louis, MO) as standard. During chromatographic separation, protein content of eluates was estimated by measuring the absorbance at 280 nm and by the biuret assay method [31].

Hidrolytic activity assays

Esterolytic activity

Measurement of endoesterolytic activity [32] was performed with N-α-carbobenzoxy-*p*-nitrophenyl esters of the following amino acids: Gln, Ala, Asp. The synthetic substrates were obtained from Sigma Chem. Co. (St. Louis, USA). Assays were made at 37 °C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 mM EDTA and 0.6 mM cysteine in the reaction mixture. To 50 µl of crude extract solution, 50 µl of 1 mM substrate acetonitrile stock solution and 1.9 ml of the mentioned buffer solution were added. The absorbance of the released *p*-nitrofenol was followed spectrophotometrically with an Agilent 8453 E UV-visible spectroscopy system at 405 nm every 3 sec for one min. Esterolytic activity was expressed according to an arbitrary enzyme unit (U_{cbz}), defined as the amount of peptidase that released 1 µM of *p*-nitrophenolate per min in the assay conditions. A standard curve of *p*-nitrophenol (15-70 (M) was performed to determine the micromoles of *p*-nitrophenol produced during the reaction.

Amidasic activity

Amidasic activity was measured according to the method of Filippova *et al.* [33] with some modifications. A stock solution of 4 mM PFLNA in DMSO was prepared, as well as a 0.1 M phosphate buffer, pH 6.5, containing 0.3 M KCl, 10^{-4} M EDTA and 0.003 M DTT. The reaction

mixture contained 1.5 ml 0.1 M phosphate buffer pH 6.5, 0.3 M KCl, 10^{-4} M EDTA, 0.003 M DTT, 0.18 ml substrate and 0.12 ml enzyme. The *p*-nitroaniline released at 37 °C was detected spectrophotometrically at 410 nm. An arbitrary enzyme activity unit (U_{PFLNA}) was defined as the amount of protease that released one micromol of *p*-nitroaniline per min in the assay conditions.

Pectin methyl esterase activity

An 1% pectin suspension in citric phosphate 55mM buffer, pH 6.8 containing Cl_2Ca 0.02M was used as substrate in order to detected PME activity [34]. The assay was performed at 37 °C by incubation of 600 µl of this suspension with 300 µl of CE until gelificaction. The results were reported like detectable (+) or not detectable (–) activity.

Carboximetilcellulase, poligalacturonidase and xilanase activities

These activities were assayed on the following substrates: 1% carboximetilcellulose, 1% poligalacturonic acid and 1% xilane solutions in citric-phosphate buffer 0.1 M, pH 5 at 37 °C.After one hour of incubation, reducing sugars released by the CE were determined by the Nelson Somogyi method [35].

Rhamnogalacturonidase activity

Rhamogalacturonic acid (0.5% in 50 mM acetic acid-acetate buffer, pH 4.5) was the substrate used to test the presence of rhamnogalacturonidase activity in the CE. The assay was made on Petri dishes containing the gelled substrate, upon which samples were placed and incubated for 4 h at 37 °C; the existence of enzyme activity was evidenced by a white halo on the gel surface by adding rhutenium red dye [36].

Proteolytic (caseinolytic) activity

Proteolytic assays were made using casein (Hammarsten type; Research Organics, Cleveland, OH) as substrate. The reaction mixture was prepared by mixing 0.1 ml of enzyme extract with 1.1 ml of 1% casein containing 12 mM cysteine, in a 0.1 M Tris-HCl buffer (pH 8.0). The reaction was carried out at 45 °C and stopped 2 min later by the addition of 1.8 ml of 5% TCA. Each test tube was centrifuged at 3,000 x g for 30 min and the absorbance of the supernatant measured at 280 nm. An arbitrary enzyme unit (caseinolytic unit, U_{cas}) was defined as the amount of protease which produces an increment of one absorbance unit per min in the assay conditions [37].

Proteolytic characterization of Crude Extract

pH profile of the proteolytic activity

The effect of pH on enzyme activity of the crude extract was measured with casein (pH range 6.4 to 10.5) using 10 mM sodium salts of the following "Good" buffers [38]: MES, MOPS, TAPS, AMPSO and CAPS (Sigma Chem. Co., St Louis, MO).

Inhibitors effect

The action of inhibitors of cysteine proteases was evaluated by incubating the crude enzyme preparation for 15 and 30 min at 37 °C with 5 mM sodium iodoacetate and 10 µM E-64. The residual caseinolytic activity after each incubation assay was measured as indicated above.

Thermal effect

Progress curves for different temperatures (37 °C, 45 °C, 50 °C, and 60 °C) were made by measuring the caseinolytic activity along the time (5, 10, 15, 20, and 30 min) for the crude extract [39].

Thermal stability

To determine the effect of heating, samples were held for 0, 5, 10, 15, 20, and 30 min at 37, 45, 50, and 60 °C and then the residual caseinolytic activity was measured as mentioned.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to Shägger and von Jagow [40] with tricine cathodic buffer in 10% polyacrilamide gels on both crude extract non inhibited and inhibited with sodium iodoacetate (0.25 mL of crude extract plus 25 µl of 50 mM sodium iodoacetate on ice bath for 30 min). Potential was kept constant at 40 mV for the stacking gel and at 150 mV for the resolution gel. The gels were treated with Coomassie Brilliant Blue R-250. Molecular weight markers (SDS-PAGE Molecular Weight Standards, Low Range, Bio Rad) were used to estimate the molecular weights of the differente componentes of the crude extract.

Isoelectric focusing (IEF) and Zymogram

IEF was developed on immobilized pH gradient gels of polyacrylamide (10%) in the pH range from 3 to 10 (Biolyte 3-10 carrier ampholytes, Bio-Rad, Hercules, CA, USA) in a Mini IEF Cell (Model 111, Bio-Rad). Samples were concentrated by acetone precipitation and further centrifugation at 11,000 x g during 20 min. Deionization was performed redissolving the precipitates in deionized water and repeating

the whole treatment twice. IEF of proteases was carried out according to this conditions: 100 V for 30 min, 200 V for the following 15 min and 450 V for the last 60 min. Then, gels were fixed and stained with Coomassie Brilliant Blue R-250.

To confirm the proteolytic activity of the bands, IEF unstained gels were contacted for 30 min at 55 °C with an agarose gel imbibed in 1% casein solution containing 12 mM cysteine [41] and then stained with Coomassie Brilliant Blue R-250.

Chromatographic Purification of the Crude Extract

Ion exchange chromatography

The purification of the proteolytic components was carried out by cation exchange chromatography. One ml of the crude extract containing 1.5 mg of protein were loaded onto a Pharmacia XK 16/40 column having AK16 adaptors, packed with SP-Sepharose Fast Flow and equilibrated with 0.055 M Tris-HCl (pH 7.4). The chromatography was developed in an FPLC equipment (Pharmacia) by washing with the equilibrating buffer and further elution of the bound material with a step gradient of sodium chloride (0-0.5, 0.5-0.8, and 0.8-2.0 M) in the same buffer. Cation exchange chromatography was monitored spectrophotometrically by absorbance measurement at 280 nm. Caseinolytic activity, protein and peptide content (biuret test) were tested on the eluted fractions.

Gradient electrophoresis

The active fractions obtained from cation exchange cromatography (inhibited and non inhibited with sodium iodoacetate) were submited to a denaturing SDS polyacrylamide gradient electrophoresis (10-16%) with a sucrose gradient to amplify the resolution (aids gradient formation and avoids polyacrylamide diffusion) performed in a Miniprotean III (Bio-Rad), according to Laemmli [42], at 30 mA during stacking and at 150 mA during the electrophoresis run. Gels were stained with Coomassie Brilliant Blue R-250 and silver nitrate. The following molecular weight markers (Bio-Rad) were employed: α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), egg albumin (43.0 kDa), bovine seroalbumin (67.0 kDa), and phosphorilase b (94.0 kDa).

RESULTS AND DISCUSSION

Characterization of CE

The protein content of CE obtained from latex of *A. angustifolia* fruits was 1.5 mg/mL and showed the following hydrolitic activities: protease, amidase, esterase, polygalacturonidase and pectin methyl esterase, but not rhamnogalacturonidase, xylanase nor methylcellulase activities could be detected.

Endoesterolytic activity of the crude extract (CE) was determined on N-α-CBZ-amino acid-*p*-nitrophenyl esters, exhibiting higher preference for the glutamine, alanine and aspartic derivatives, in decreasing order (33.9, 15.5, and 9.55 U_{cbz}, respectively).

As proteolytic activity was the main catalytic activity observed, the proteolytic properties of CE were stablished. Maximum activity was achieved in the presence of 12 mM cysteine. The highest activity (4-fold) was obtained when the crude extract was prepared with 5 mM EDTA and 5 mM cysteine.

The crude preparation showed maximum activity (higher than 95%) between pH 6.7 and 8.5 (Fig. 1). Inactivation assays with iodoacetate and E-64 suggested the possible cysteinic nature of the proteases present in the crude extract. Thus, 95 % of proteolytic activity was lost when iodoacetate and E-64 were added to the incubation mixture (Fig. 2). The activation and inhibition results would indicate dependence of the proteolytic activity upon the presence of active-SH groups in the enzymes present in the crude extract. This behavior was previously shown by proteases from other *Asclepiadaceae* species [5,7,9,15,18].

Fig. 3 shows the electrophoretic pattern of the crude extract with and without sodium iodoacetate, where it can be seen that the sample treatment prior electrophoresis (heating in presence of β-mercaptoethanol) affords degradation products provoked by heating in a highly reducing medium when the crude extract is not

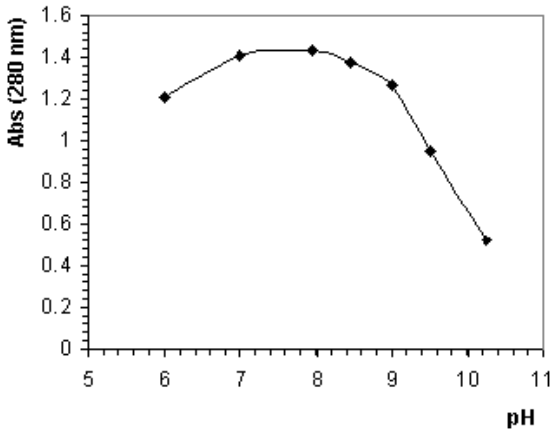

Figure 1. Effect of pH on proteolytic activity of crude extract.

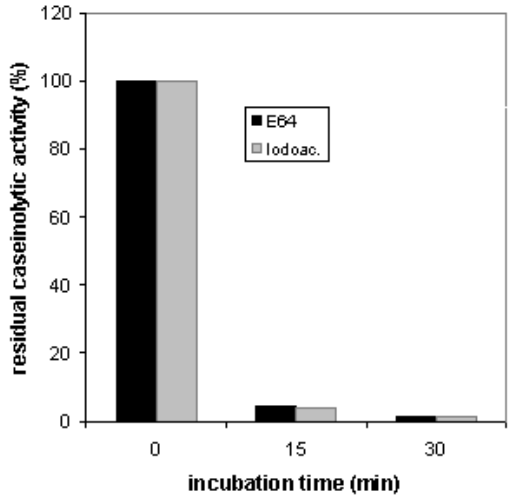

Figure 2. Effect of different protease inhibitors on crude extract.

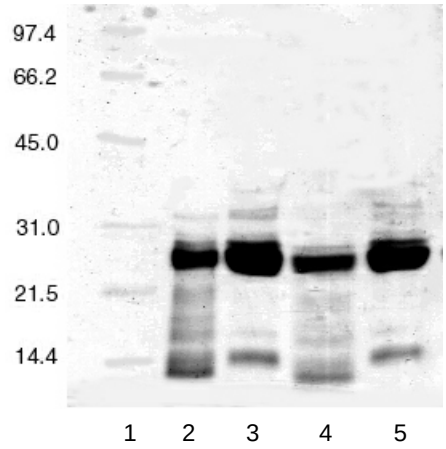

Figure 3. SDS-PAGE. Lane 1: Molecular weight Bio Rad markers: Phosphorylase b, 97.4 kDa; Serum albumin, 66.2 kDa; Ovalbumin, 45.0 kDa; Carbonic anhidrase, 31.0 kDa; Trypsin inhibitor, 21.5 kDa; and Lysozyme, 14.4 kDa. Lanes 2 and 4: crude extract; Lane 3 and 5: crude extract inhibited with sodium iodoacetate.

previously inhibited. This behavior suggests that cysteinic proteases should be inhibited before SDS-PAGE procedure.

As can be seen in Fig. 4, CE is not very stable at higher temperatures, as it lost 50% of its proteolytic activity after 15 min of incubation at different temperatures. However, caseinolytic activity increased with temperature: maximum proteolytic activity was obtained at 60 °C for the crude extract (Fig. 5). These results suggested a protective role played by the substrate or probably by other proteins present in the crude extract.

When CE was analyzed by IEF (Fig. 6), showed it was composed by at least eight fractions,

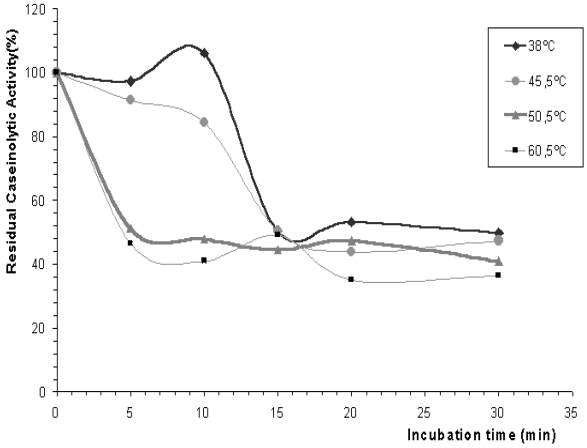

Figure 4. Thermal stability of crude extract.

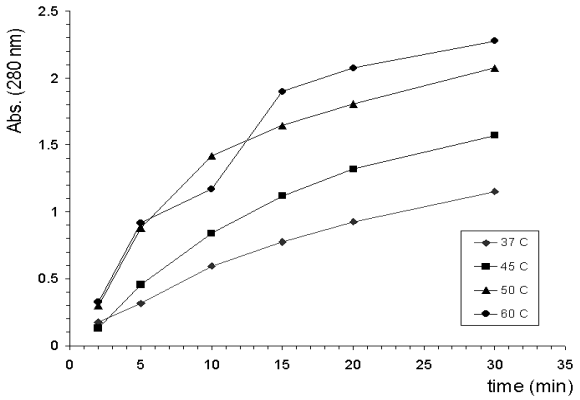

Figure 5. Heat effect on crude extract proteolytic activity.

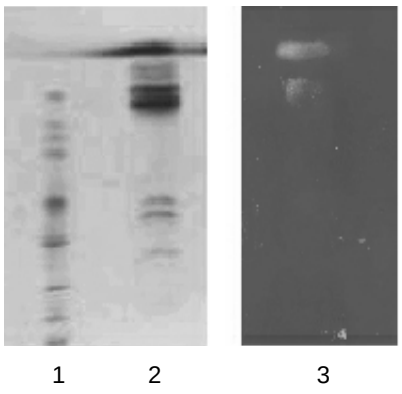

Figure 6. Isoelectric focusing. (IEF). Lane 1: IEF Bio Rad markers; Lane 2: crude extract. Lane 3: Zimograme.

three of them proteolytically active (pI values 8.5, 8.9 and higher than 9.3). This basic nature was also observed in the proteases isolated from the latex of *Asclepias glaucescens* [8,9], *Morrenia brachystephana* [17] and *Morrenia odorata* [20].

Purification of CE

Purification of CE by FPLC (SP-Sepharose 55 mM, Tris-HCl buffer, pH 7.4, NaCl linear gradient 0-0.5) afforded seven fractions (Fig. 7). The non retained fraction as well as fractions I-III showed pectin methyl esterase activity; in addition, fractions I, II and III (named *araujiain aI, aII* and *aIII*, respectively) exhibited proteolytic, amidolytic and esterolytic activitites. Fractions IV-VI are presumably low molecular weight peptides (biuret assay positive, Bradford assay negative).

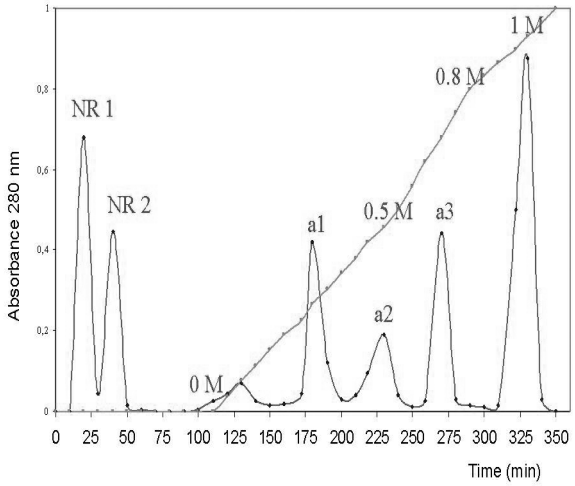

Figure 7. Cation exchange chromatography (SP-Sepharose Fast Flow, column Pharmacia Biotech XK 16/40; Elution buffer 50mM Tris-HCl; Gradient: sodium chloride 0-0.5M , 0.5-0.8M. Flow rate: 0.5 ml/min. NR1 and NR2, non-nretained fractions; a1, a2 and a3 proteolytic active fractions.

Homogeneity of *araujiain aI, aII* and *aIII* could be confirmed by means of SDS-PAGE gradient gel with sucrose, revealed with silver stain (Fig. 8), where it can also be seen that the purified fractions are not susceptible to autodigestion. Figs. 3 and 8 allows to stablish that the molecular masses of the proteases contained in the latex of *Araujia angustifolia* are about 23 kDa, results that are of the same order of those obtained for other proteases from *Asclepiadaceae:* Mr of *Asclepias syriaca* proteases are 21 and 23 kDa [8,9], proteases from *Asclepias glaucescens* have a Mr of 23 kDa [10,11], the four calotropins isolated from *Calotropis gigantea* show molecular masses ranging from 23 to 27 kDa [12,14], two proteases obtained from *Morrenia brachystephana* present molecular masses of 25.5 and 26 kDa [19] and the proteases isolated from *Morrenia odorata* shown Mr of 24.2 and 25.8 kDa [22].

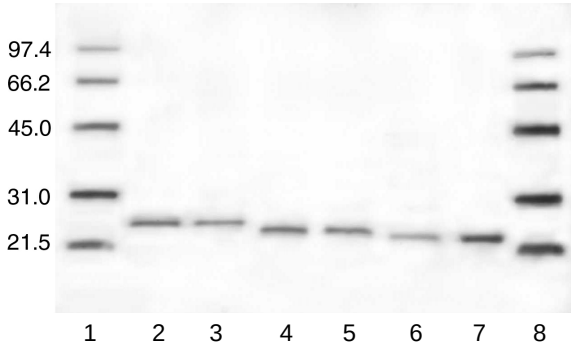

Figure 8. Gradient Electrophoresis. Lanes 1 and 8: Molecular weight Bio Rad markers: Phosphorylase b, 97.4 kDa; Serum albumin, 66.2 kDa; Ovalbumin, 45.0 kDa; Carbonic anhidrase, 31.0 kDa; Trypsin inhibitor, 21.5 kDa; and Lysozyme, 14.4 kDa. lanes 2, 4 and 6: *araujiain aI, aII* and *aIII*, respectively; Lanes 3,5 and 7, *araujiain aI, aII* and *aIII* inhibited with sodium iodoacetate, respectively.

CONCLUSIONS

The crude extract (CE) prepared by clarification of latex obtained from *Araujia angustifolia* fruits exhibited a number of hydrolytic properties (protease, amidase, esterase, polygalacturonidase and pectin methyl esterase activities), reinforcing the prevailing opinion on the role of latex as a defence mechanism in plants.

By considering the CE hydrolytic profile, proteolytic activity appears as the most remarkable feature, owed to the presence of at least three cysteine proteases (named *araujiain aI, aII,* and *aIII)* of different pI (8.5, 8.9 and > 9.3) but very close molecular masses (about 23 kDa), a very common fact in the proteases isolated from latexes of the relatively few species of *Asclepiadeaceae* studied up to date. Previous reports on anthelmintic and other antiparasitic activities attributed to plant cysteine proteases as well as no evidence of parasite attacks to *A. angustifolia* fruits suggested the realization of anti-parasite assays that are currently underwent.

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