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Trichomonas vaginalis acidic phospholipase A_2: isolation and partial amino acid sequence

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Abstract

Sexually transmitted diseases are a major cause of acute disease worldwide, and trichomoniasis is the most common and curable disease, generating more than 170 million cases annually worldwide. *Trichomonas vaginalis* is the causal agent of trichomoniasis and has the ability to destroy *in vitro* cell monolayers of the vaginal mucosa, where the phospholipases A_2 (PLA_2) have been reported as potential virulence factors. These enzymes have been partially characterized from the subcellular fraction S30 of pathogenic *T. vaginalis* strains. The main objective of this study was to purify a phospholipase A_2 from *T. vaginalis*, make a partial characterization, obtain a partial amino acid sequence, and determine its enzymatic participation as hemolytic factor causing lysis of erythrocytes. *Trichomonas* S30, RF30 and UFF30 sub-fractions from GT-15 strain have the capacity to hydrolyze [2-^{14}C-PA]-PC at pH 6.0. Proteins from the UFF30 sub-fraction were separated by affinity chromatography into two eluted fractions with detectable PLA A_2 activity. The EDTA-eluted fraction was analyzed by HPLC using on-line HPLC–tandem mass spectrometry and two protein peaks were observed at 8.2 and 13 kDa. Peptide sequences were identified from the proteins present in the eluted EDTA UFF30 fraction; bioinformatic analysis using Protein Link Global Server charged with *T. vaginalis* protein database suggests that eluted peptides correspond a putative ubiquitin protein in the 8.2 kDa fraction and a phospholipase preserved in the 13 kDa fraction. The EDTA-eluted fraction hydrolyzed [2-^{14}C-PA]-PC lyses erythrocytes from Sprague–Dawley in a time and dose-dependent manner. The acidic hemolytic activity decreased by 84% with the addition of 100 μM of Rosenthal's inhibitor.

Keywords

Trichomonas vaginalis, amino acid sequence, isolation, phospholipases, phospholipases A_2, virulence factors

Introduction

Worldwide, sexually transmitted diseases are a major cause of acute disease, with trichomoniasis being the most common curable sexually transmitted disease, causing more than 170 million cases annually (WHO 2001), 18.5 million of these cases were estimated to be in Latin America (Gülmezoglu and Forna 2000). Trichomoniasis can occur in both genders, predominating slightly more in males. In men, this illness is commonly asymptomatic, but can cause urethritis, prostatitis, cystitis, epididymitis, and sterility. In contrast, women frequently present with symptoms, such as acute or chronic vulvovaginitis and urethritis, but they can also remain asymptomatic. *Trichomonas-induced* urethritis in males is associated with sterility, whereas infection in pregnant women is related to premature rupture of amniotic membranes, premature childbirth, and low birth weight (Sutton *et al.* 2007). In addition, HIV seroconversion occurs more easily in persons with trichomoniasis (Cohn and Clark 2003). *Trichomonas vaginalis* has the ability to destroy *in vitro* monolayers of epithelial cells isolated from human vaginal mucosa by detaching or lysing them (Heath 1981, González-Robles *et al.* 1995, Gilbert *et al.* 2000), or by phagocytosis (Rendon-Maldonado *et al.* 1998, Demirezen 2001).

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Phospholipases A (PLAs) are classified as phospholipase A_1 (PLA A_1) (EC 3.1.1.32) and phospholipase A_2 (PLA A_2) (EC 3.1.1.4), which hydrolyze the first and second ester groups formed by fatty acids and glycerol, respectively (Van den Bosch 1980, Dennis 1983). All the PLAs A_2 enzymes characterized to date depend on the Ca^{2+} concentration and the pH, and their molecular weight ranges from 14 to 16 kDa (Dennis 1983).

PLAs A_2 are the major enzymes from insects, arthropods, and reptilian venoms that cause cytolysis and neurotoxic effects (Dennis 1983). These enzymes are important in the pathogenic mechanisms of the protozoa such as *T. vaginalis* (Vargas-Villarreal *et al.* 2003, 2005, Lubick and Burgess 2004), *Entamoeba histolytica* (Long-Krug *et al.* 1985; Vargas-Villarreal *et al.* 1995, 1998; González-Garza *et al.* 2000), *Trypanosoma cruzi* (Wainszelbaum *et al.* 2001), and *Naegleria fowleri* (Barbour and Marciano-Cabral 2001). We have previously shown that the P30 and S30 subcellular fractions of *T. vaginalis* incubated in presence of human or rat erythrocytes. The enzymes present in this fractions cause damage on the membrane of erythrocytes, this direct hemolytic activity have a maximal activity at pH 6.0 and 8.0 in the presence of 1 mM Ca^{2+}. We have also shown that this hemolytic activity was inhibited by Rosenthal's inhibitor (Vargas-Villarreal *et al.* 2003), and contained particulate and soluble acidic, and alkaline direct and indirect hemolytic activity, which are partially dependent on alkaline or acidic PLA A_1 and PLA A_2 enzymes (Vargas-Villarreal *et al.* 2005). It has been suggested that both the hemolytic activity and PLA A_2 have the same mechanism. The subcellular fraction S30 shows PLA A_2 activity in the pH range 6.0–8.0 (Vargas-Villarreal *et al.* 2005).

In the present study, we isolated an acidic PLA A_2 in UFF30 sub-fraction from S30 fraction using affinity chromatography and obtained a partial amino acidic sequence by HPLC–tandem mass spectrometry. In addition, we investigated if the activity of PLA A_2 depended on the hemolytic activity and partial characterization of the homogenous enzyme.

Materials and Methods

Parasites

The *T. vaginalis* strain GT-15 was used in all experiments. The parasites were maintained under axenic conditions by serial subcultivation (Padilla-Vaca and Anaya-Velázquez 1997). In order to have a greater amount of enzyme possible, mass cultures were grown as described elsewhere (Vargas-Villarreal *et al.* 2003, 2005). Briefly, 1 L spinner Flasks (Bellco Glass Inc., Vineland, New Jersey) with 600 mL PEHPS medium (Said-Fernández *et al.* 1988) containing 10% (v/v) bovine serum and Diamond's vitamins–Tween 80 mixture (Diamond *et al.* 1978) were inoculated with 5×10^3 trophozoites of *T. vaginalis*/mL and incubated steadily at 36.5°C for 48 h. The cultures were chilled in ice water for 10 min and centrifuged at 1000 g for 15 min at 4°C. Protozoa were washed twice with 10 volumes of phosphate-buffered saline (PBS; pH 6.0) and processed immediately after in order to obtain trichomonad total extracts (TTE) and the subcellular fractions.

Subcellular fractions

The subcellular fractions were obtained as described elsewhere (Vargas-Villarreal *et al.* 2003, 2005). Briefly, the pellet of freshly obtained *T. vaginalis* trophozoites was washed and resuspended in Hank's balanced salt solution (BBS; 0.7 mM $CaCl_2$, 5.5 mM glucose, 120 mM NaCl, 5.3 mM KCl, 1.7 mM $MgSO_4$, 1 mM Trizma Base). Appropriate concentrations of sodium acetate-acetic acid (1 M) was added to adjust to pH 6. Subsequently, parasites were disrupted with a motor-driven Elvehjem-Potter Teflon/glass homogenizer and centrifuged at 30,000 g for 15 min at 4°C. The resultant TTE was separated into two fractions: the supernatant (S30) and the pellet (P30). A proportion of the S30 fraction (5 mL) was stored at –70°C, and the remaining fraction was poured into a microcentrifuge cartridge filter (Ultrafree-MC of 30,000 Da, Millipore) and centrifuged at 5,000 rpm for 2 h at 4°C. The retained (RF30) and ultrafiltered (UFF30) sub-fractions were separated, and the retained material was resuspended in sodium acetate-acetic acid (0.1 M) buffer adjusting the pH to 6.0. The S30 sub-fractions were distributed separately in 200 µL aliquots. All the above preparations were stored at −70°C until required.

PLA A_2 assays

PLA A_2 activities were determined as previously described (Vargas-Villarreal *et al.* 1995) using 1,2-dipalmitoyl-[2-palmitoyl-1-^{14}C]-phosphatidylcholine/mL (58 mCi/mmol; [2-^{14}C-PA]-PC). All radioactive substrates were purchased from New England Nuclear (Boston, Massachusetts). Each of the radioactive substances (4 µCi per assay) was mixed with 1.0 mL sodium acetate-acetic acid (0.1 M) (pH 6.0), 2 mM $CaCl_2$, 0.2 % Triton X-100, and 0.27 mM phosphatidylcholine. The mixtures were sonicated with an Ultratrip Labsonic System (Lab-Line Instrument Inc., Melrose Park, Illinois), which was operated at 40 W for 60 s. The resultant emulsion was distributed in 0.5 mL aliquots and stored at –70°C until required.

The assays were performed by mixing 10 µL of substrate with 10 µL of S30 or the retained (RF30) and ultrafiltered (UFF30) sub-fractions, all containing 150 µg of total proteins, and incubating them at 36.5°C for 1 h in a water bath. In each assay a positive control from *Crotalus adamanteus* and a negative control containing acetate buffer was added to the reaction mixture. The phospholipids hydrolysis reaction was stopped by the addition of 25 µL of rat liver free fatty acids (FFA) (final concentration 1 mg/mL), 1.0 mg egg-yolk lysophosphatidylcholine (LPC)/mL and 0.75 mg egg-yolk phosphatidylcholine (PC)/mL in 5% trichloroacetic acid in *n*-butanol, to bring the final volume of the mixture to 45 µL.

In each experiment the amount of PC, LPC, or FFA in the assay mixtures were determined as follows: 45 µL of the hydrolysis mixture of each sample was placed drop by drop on silica-gel thin layer chromatography plates (60 mesh; Merck, Darmstadt, Germany) 20 × 20 cm, and a plate thickness of 0.25 mm, which were cut to 10 × 10 cm with a glass cutter. The plates were placed into a developing tank with a solvent system formed by chloroform:methanol:acetic acid:water (140:40:16:8 volumes). Chambers were loaded with the chromatographic mobile phase and allowed to saturate during 20 min at room temperature, then sealed hermetically. The plates with samples were placed vertically in the chambers and allowed mobile phase to ascend one centimeter above the upper edge of the chromatoplates. The plates were removed from the chamber and dried with hot air. Later, visualization of spots in the chromatoplates lipid was made to be deposited in a chamber saturated with iodine vapor (Skipsky and Barclay 1969). The spots belonging to PC, LPC and FFA were identified. These lipids were recovered by scalpel scraped of each sample from the plates, and their radioactivity determined (expressed as disintegrations per min, DPM) as described elsewhere (Vargas-Villarreal *et al.* 1995). All determinations were performed in triplicate and presented as mean ± SE. Specific PLA activity (PLAU/mg of total protein/h) was calculated by arbitrarily defining 1 unit of PLA (PLAU) as the hydrolysis of 1 pmol of [2-^{14}C-PA]-PC.

The time curve of PLA A_2 activity was realized at varying times of incubation in the EDTA-eluted fraction (UFF30–EDTA) (0 to 120 min) at 36.5° C, the radioactivity present in the spots corresponding to the chromatoplates eluted hydrolysis products was quantifying as described previously.

Dose-Response Curve of PLA A_2 activity was determined in in the EDTA-eluted fraction (UFF30–EDTA by adding 10 µL of 2X assay mix and 10 µL of varying amounts of the test sample. The mixtures were incubated at 36.5° C for 2 hrs and identified areas DPM for [2-^{14}C-PA]-AGL.

For the determination of the effect of pH on the activity of PLA A_2 were used 10 ml of 2X assay mix and 10 ml of in the EDTA-eluted fraction (UFF30–EDTA (122 ng total protein). The pH of the mixture was adjusted to 4.0, 5.0 and 6.0 with acetate buffer and 7.0, 8.0 and 9.0 with Tris-base.

Affinity chromatography

Analysis by affinity chromatography was based on the method described by Vargas-Villarreal (Vargas-Villarreal *et al.* 1991, 1998). A mini-column (Corning-Sigma) was packed with 3 mL of Sepharose 4B coupled to Rosenthal's reagent (dimethyl-$_{D,L}$-diesteroyloxypropyl-2-hydroxyethyl ammonium; Sigma), a PLA A inhibitor (Vargas-Villarreal *et al.* 1991, 1998). The column was washed with 10 mL of 4 mM $CaCl_2$ dissolved in sodium acetate-acetic acid (0.1 M) buffer (pH 6.0). Then 20 mL of UFF30 was mixed with 2 mL of 4 mM $CaCl_2$ in sodium acetate-acetic acid (0.1 M) buffer and passed through the column. The bound material was successively eluted with: (a) 10 mL of sodium acetate-acetic acid (0.1 M) buffer plus sodium chloride 0.5 M and calcium chloride 4 mM, (b) 50 mM ethylenediamine-tetraacetic acid (EDTA) dissolved in sodium acetate-acetic acid (0.1 M) buffer (pH 6.0), and (c) 0.1 M acetic acid. Fractions of 1 mL were collected at a flow rate of 8 mL/h.

The fractions obtained with acetic acid were collected in tubes containing 1 mL of sodium acetate-acetic acid (0.1 M) buffer (pH 6.0). The fraction obtained by EDTA elution was desalinated using a filtration cartridge (Millipore). Each one of these fractions was tested for PLA A_2 activity. Fractions that constituted the peak with the highest PLA A_2-specific activity were pooled, divided into 250 µL portions and stored at –70°C until required.

Protein quantification

The protein concentration in the fraction was evaluated according to the methods described by Lowry (Lowry *et al.* 1951), except in the affinity chromatography fraction. In this preparation, the protein concentration was measured spectrophotometrically by assuming that 1 absorbing unit at 280 = 1 mg/mL.

Hemolysis assays

Hemolysis assays were performed by adapting the experimental conditions to the methods described previously to analyze the PLA-dependent hemolytic activity of *T. vaginalis* (Vargas-Villarreal *et al.* 2003). Briefly, rat erythrocyte suspension obtained from total blood by aortic punction. This blood was centrifuged 600×g during 10 min on three times, supernatants and white surface sediment (white blood cells) remaining red blood cells were re-suspended on phosphate buffer pH 7 in two times and finally in sodium acetate-acetic acid with osmolarity on 290 mOsm/Kg and pH adjusted to 6.0. Then, 25 µL of this erythrocyte suspension was placed into 1.5 mL polypropylene centrifuge tubes (Sigma) and mixed with 25 µL of the UFF30–EDTA fraction (containing 30 µg of the total protein and 1 mM $CaCl_2$). For the controls, 25 µL of double-distilled water and 25 µL of sodium acetate-acetic acid (pH 6.0) were mixed with 25 µL of the rat erythrocyte suspension, reaching 100% and 0% hemolytic activity, respectively. Mixtures were incubated for 1 h at 36.5°C. Then, 1 mL of PBS, pH 7.4 (Diamond 1978) was added and the tubes were centrifuged at 600 g for 5 min at 4°C, and the absorbance at 415 nm (A_{415}) was measured with a spectrophotometer (PMQ III, Model MM3, Zeiss, Oberkochen, Germany). Activity was expressed as the percentage hemolysis with respect to the controls.

Specific hemolytic activity (SHA) was reported as hemolytic units per mg of total protein per hour (HU/mg/h) of incubation at 36.5°C. One hemolytic unit was defined as the amount of hemoglobin released from lysed erythrocytes equivalent to 0.05 optical units at 415 nm.

Time-course of hemolytic activity

Assay mixtures containing 25 µL of the erythrocyte suspension in sodium acetate-acetic acid (pH 6.0) were placed into 1.5 mL polypropylene centrifuge tubes (Sigma) and mixed with 25 µL of the UFF30–EDTA fraction (containing 30 µg of the total protein and $CaCl_2$ 1 mM). They were incubated for variable time periods (0–60 min) and were separated and quantified as described previously (Vargas-Villarreal *et al.* 2003).

Dose-response relationship between Rosenthal's inhibitor and hemolytic activity

These determinations were performed using the UFF30–EDTA fraction (0–30 µg of protein) following the methods described to determine the hemolytic activity, and were pre-incubated at 36.5°C for 10 min with various concentrations (0–120 µM) of Rosenthal's inhibitor.

Molecular weight determination of PLAs A_2

A sample of protein obtained from the affinity column was dried under vacuum and resuspended in 100 µL of ammonium bicarbonate (100 mM) and injected into an Alliance 2695 chromatographic system (Waters). Separation of the fractions was obtained by injecting a 10 µL sample into a column Symmetry 300, C-18, 2.1×100 mm, 3.5 microns (Waters). The elution gradient was created using acetonitrile–0.1% formic acid water solution, and the gradient was run in a 5–60% ramp for 30 min at column temperature 50°C. The mobile phase flux during the assay was 0.4 mL/min. The mass spectrometer was programmed to obtain data in MS mode, range from 200 to 3000 of m/z by use of Mass Lynx v 4.0 software. Later, deconvolution was performed using the software MaxEnt3. The positive control used was a phospholipase from *Crotalos adamanteus* and *Apis mellifera*. All assays were completed in triplicate.

Peptide sequencing by mass spectrometry, preparation, and LC/LC/MS/MS analysis of T. vaginalis peptides

Sample preparation

The sample containing 1.0 mg of protein was added to 100 µL of NH_4HCO_3 50 mM and digested using the trypsin protocol as follows.

To reduce the disulfide bonds, 5 µL of dithiothreitol (DTT) was added to the sample and boiled for 10 min. The sample was mixed in a vortex and centrifuged. The sample was then incubated at 65°C for 1 h.

Alkylation. The sample was alkylated by adding 4 µL of iodoacetamide solution, mixed by vortexing and centrifuged. The sample was then incubated at room temperature for 1 h. *Neutralization*. The sample was neutralized by adding 20 µL of DTT, mixed by vortexing and centrifuged. The sample was then incubated at room temperature for 1 h.

Digestion. The sample was digested for 18 h at 37°C using a solution of trypsin by maintaining a 1:50 relationship (w/w). The reaction was stopped by placing the sample at –20°C or by adding 10 µL of trifluoroacetic acid. Prior to the injection, the sample was centrifuged for 10 min at 13,000×g.

Solubilization. The digested sample was centrifuged at 11,000×g by 10 min. The supernatant was transferred to an Eppendorf tube and labeled as hydrophilic peptides. The resulting pellet underwent washed with Milli-Q water resuspended in 500 µL of 70% isopropanol and sonicated for 10 min. The sample was centrifuged at 11,000×g by 10 min and the supernatant was transferred to a further Eppendorf tube and labeled as hydrophobic peptides. These samples were stored at –20°C.

LC-MS/MS sequence experiment

The digested peptides were injected into a BioSuit PA-A C18, 2.1×150 mm, 3 micron column and separated using a reverse phase chromatograph (Alliance 2695XE, Waters). The peptides were eluted at the electrospray ionization (ESI) interface of the model mass spectrometer QT of-micro. The control and data acquisition was performed using Mass Lynx version 4.0 software and exported for processing with Protein Lynx Global Server (PLGS) version 2.3 software. The MASCOT and SwissProt databases were used to generate the peptide sequences for the databank and the *de novo* sequence strategy. During the study, bovine albumin was used as a control to optimize the separation conditions, detection and processing method and a PLA A_2 from *Crotallus adamanteus* was used as a positive control.

Chromatographic separation

The chromatographic separation of the digested peptides was performed by injecting 10 µL of sample into a BioSuit PA-A C18, 2.1×150 mm, 3 microns column using an elution gradient of acetonitrile-water 0.1% formic acid, 1–60% in 120 min, and the column temperature was set to 50°C with a flux to 0.150 mL/min.

Detection conditions

The mass spectrometer was calibrated with a glucofibrinopeptide solution in an m/z range from 100 to 1500. The ion corresponds to the charge [(M+2H) 2+], with m/z 785.8426 released through the reference of the source Lock Spray sprayer.

The instrument was used in the DAD acquisition mode in a 100–200 m/z range for peptides and a 50–1500 m/z range for fragments with a 4-charge recognition level of fragmentation and a 10-sec Lock Spray frequency of 30 sec. The ESI interface was set to positive mode, 3500 volts for the capillary,

350°C for the desolvation temperature, and 500 L/h for the nitrogen flow during the 120 min.

Data analysis

The spectra acquired were processed with Protein Lynx Global Server version 2.3 (Waters Corp proprietary software), and then realized in a data bank search looking the Uniprot database. Finally, the search was allocated to find similarity with other PLAs reported. We also ran experiments with the *de novo* sequence algorithm to retrieve any reported peptide sequences supported with the manual spectra review for each assigned sequence.

Results

The trichomonads S30 fraction from GT-15 strain had the capacity to hydrolyze [2-^{14}C-PA]-PC at pH 6.0. When the proteins from the S30 fraction were separated by filtration utilizing ultrafree-MC filter units of 30 kDa, two sub-fractions were obtained: the RF30 (retained) and the UFF30 (ultrafiltered). UFF30 showed PLA A_2 activity, although this was 29% lower than that observed in S30 (Table I). When 40.99 mg of the sub-fraction UFF30 containing 10165 U of PLA activity units were added to a Rosenthal's inhibitor-Sepharose 4B column, 85% of the protein with PLA activity eluted from the column (Fig. 1). Protein bound to the column eluted with 50 mM EDTA dissolved in sodium acetate-acetic acid (0.1 M) buffer (pH 6.0). This fraction contained 9.64% of the total initial activity. The specific activity increased by 25- and 35-fold compared to S30 and UFF30 activity, respectively. A further fraction from the Rosenthal's inhibitor-Sepharose 4B column was eluted with 0.1 M acetic acid and only had 0.8% of the total initial activity, but the specific activity was 22 and 31 times more than S30 and UFF30 activity, respectively. The ad-

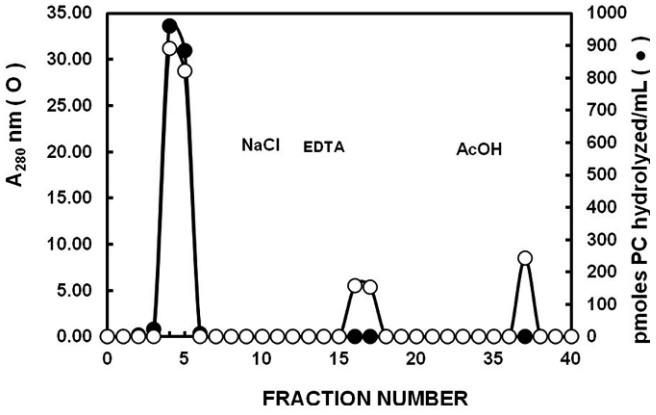

Fig. 1. Purification of phospholipase A_2 on Rosenthal's inhibitor-Sepharose 4B column. Unbound proteins were eluted with loading buffer. The column was successively developed with the following solutions: loading buffer containing 0.5 M NaCl, 50 mM EDTA and 0.1 M acetic acid. Arrows indicate where elutions with the different buffer solutions were initiated. The flow rate was 14 ml/h and fractions of about 1 mL were collected. The A_{280} mm (open circles) and the phospholipase activity (solid circles) are shown

Table I. Purification of phospholipase A_2 from the S30 of *Trichomonas vaginalis*: yield and specific activity of fractions

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg/h)	Purification (fold)	Yield %
S30	62.63	22120	353	0	100
RF30	20.84	1980	95	0.2	8.9
UFF30	40.99	10165	248	0.7	46
NaCl	0	0	0	0	0
EDTA	0.17	1504	8816	25	6.8
Acetic-acid	0.01	81	7714	21.8	0.3

*Specific activity is expressed as pmoles of palmitic acid hydrolyzed by mg of protein per h at 37°C.

Table II. Peptidic sequences obtained from the UFF30–EDTA-eluted fraction previously digested by trypsin

Protein	Sequence	Gen	Molecular weight	Product
8-kDA	KDSTLHLVLLR	TVAG_184140	8758	Putative
		TVAG_184150	8758	Ubiquitin
		TVAG_184180	8758	
		TVAG_174190	8758	
13-kDA	LETMTVAELANNSVRLFVK	TVAG_265110	13853	Conserved PLA
		TVAG_602280	13432	

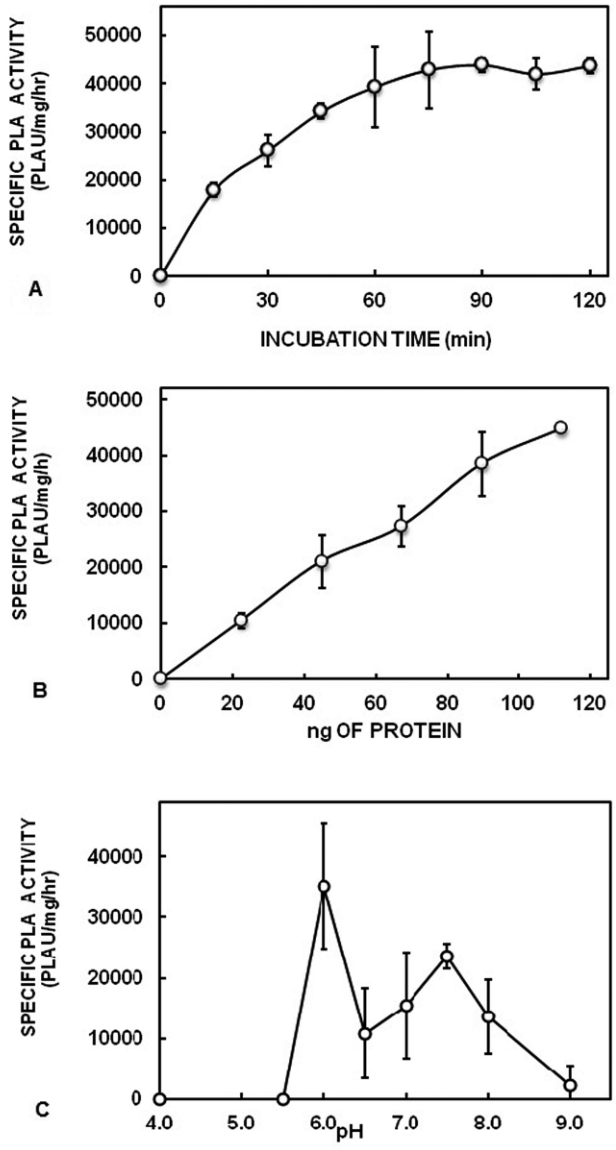

Fig. 2. Time course (A), doses (B) and pH (C) of fraction UFF30–EDTA with phospholipase A_2 activity. Symbols correspond to the mean ± SE PLA units/mg/h of [^{14}C]-palmitic acid released by the action PLA A_2 activity of the UFF30–EDTA

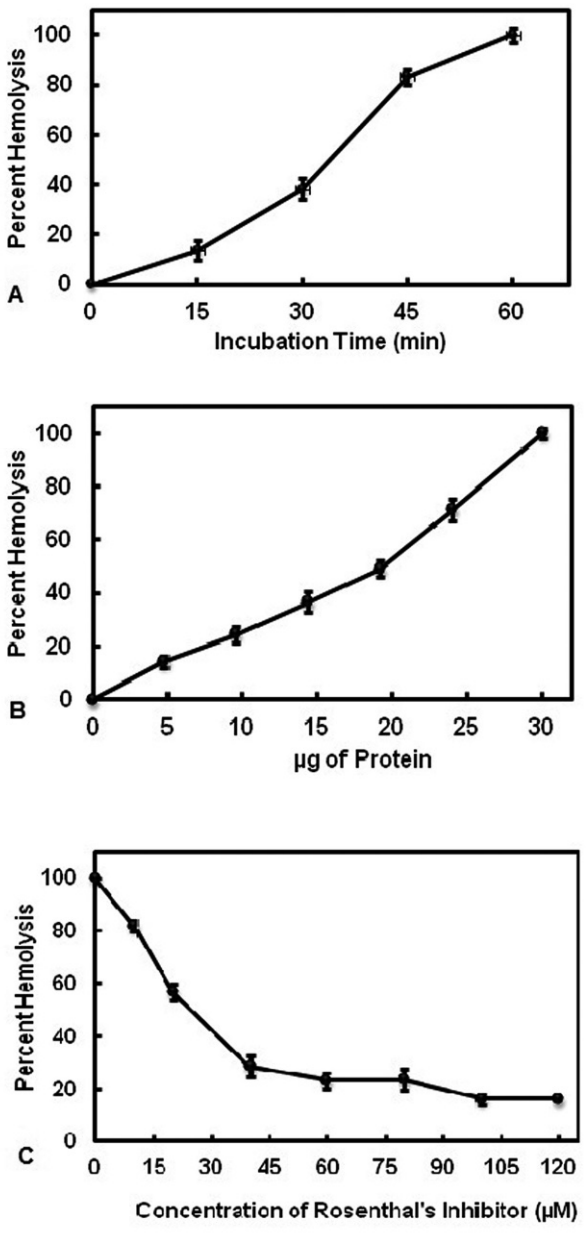

Fig. 3. Time course (frame A), protein dependence (frame B) and effect of Rosenthal´s inhibitor (frame C) on the hemolytic activity of the phospholipase A_2 obtained fraction UFF30–EDTA. Each point corresponds to the average ± SE of three determinations.

ministration of sodium chloride (0.5 M) does not cause the elution of any protein with PLA activity.

The EDTA-eluted fraction (UFF30–EDTA) was analyzed by HPLC using on-line HPLC–tandem mass spectrometry. Only two protein peaks were obtained, at 8.2 and 13 kDa (Data not showed). The sequences of the peptides from the proteins present in the affinity chromatography fraction obtained with EDTA after digestion with trypsin and analyzed by HPLC/MS are shown in Table II. Bioinformatic analysis using Protein Link Global Server charged with *T. vaginalis* protein database suggests that eluted peptides are related with a putative ubiquitin protein in the 8.2 kDa fraction and a phospholipase preserved in the 13 kDa fraction.

The PLA A_2 activity was determined in the EDTA-eluted fraction (UFF30–EDTA). In this assay, using the samples containing 112 ng of the UFF30–EDTA at pH 6.0, we demonstrated that the hydrolysis of [2-^{14}C-PA]-PC increased in function with increased incubation time; we observed linear increments in the first 60 min and then maximal activity was maintained in the following 60 min (Fig. 2A). The PLA activity of UFF30–EDTA at pH 6.0 was increased linearly according to the protein concentrations (0–112 ng) (Fig. 2B). There were two peaks in the hydrolysis activity of the UFF30–EDTA fraction, one at pH 6.0 and the other at pH 7.5 (Fig. 2C).

Finally, we demonstrated the hemolytic activity in the UFF30–EDTA fraction. It was capable of lysing 25 µL of rat erythrocytes at pH 6.0; 100% hemolytic activity was reached at 1 h of incubation using 30 µg of UFF30–EDTA protein (Fig. 3A). The hemolytic activity produced by the UFF30–EDTA fraction assayed on rat erythrocytes at pH 6.0 increased linearly according to the protein concentrations (0–112 ng) (Fig. 3B). Fig. 3C shows that the hemolytic activity of the UFF30–EDTA fraction diminished as a function of the concentration of Rosenthal's inhibitor. In the presence of 100 µM of Rosenthal's inhibitor at pH 6.0, the hemolytic activity of the UFF30–EDTA fraction diminished by 83.76% with respect to the untreated control (Fig. 3C). The inhibition range was 0–84%, considering all the experimental conditions.

Discussion

Knowledge of the virulence factors of parasites allows for the development of diagnostic devices and therapies to treat diseases caused by these microorganisms. Recently, our group has identified PLAs in amoebas and other parasites such as *Giardia lamblia*. We have also identified a PLA present in trichomonas, which acts in alkaline pH. However, the pH of the vagina is acidic and therefore it is more useful to identify one or more PLAs that are active in an acidic environment.

Previously, isolated fractions from trichomonas with PLA A_2 activity came from the P30 and S30 sub-cellular fractions. In this study, we have characterized the sub-fraction from S30, named UFF30. While *E. histolytica* have PLA A_2 activity only in P30 sub-cellular fraction *T. vaginalis*, has PLA A_2 activity in the S30 and P30 fractions. This explains the fact that amoebae only cause contact dependent hemolysis while trichomonas causes damage by both routes: contact and non-contact dependent. Thus, trichomonas has soluble fractions with PLA A_2 activity. In this context, we separated the fractions with proteins with molecular weights greater than 30 kDa, termed the RF30 sub-fraction, and from the fractions with proteins with molecular weights lower than 30 kDa, termed the UFF30 sub-fraction. Both sub-fractions have PLA A_2 activity. However, we decided to explore the UFF30 sub-fraction because the molecular weights of the described PLAs were lower than 15 kDa.

Although the fraction UFF 30 fraction comes from S30 a portion of the specific activity is lost during filtration. As the trials were standardized for the sample volume and the amount of protein, then this loss of activity is real. We think that the filtration process can remove some important cofactors for enzyme activity, as well as the possible protein denaturation and enzyme inactivation caused by the filtering process.

Additionally, the RF30 sub-fraction has a lot of proteins and other elements that can bind PLAs peptides. Thus, the activity present in this fraction may be caused by polymeric or bonded PLAs.

When comparing the specific activity of each of these two sub-fractions with the specific activity found in fraction UFF 30, it is highly likely that the activity corresponds with the highest molecular weight PLAs previously characterized. The activity present in fraction RF30 may have been caused by polymeric PLAs or PLAs bonded with other polymers or molecules as previously described.

For separation, we used a Sepharose affinity column coupled with Rosenthal's inhibitor. After the PLA was bound to the column, several steps were developed to detach the PLA elution of the column. During the first elution step, we achieved a high ionic strength by adding NaCl to remove the inhibitor molecules nonspecifically, leaving only Rosenthal's PLAs. EDTA was then added to the column to sequester the calcium and detach the calcium-dependent PLAs. Finally, acetic acid was added to elute the PLAs that were not calcium dependent.

As expected, the fraction that eluted with NaCl showed no PLA activity, while the fractions eluted with EDTA and acetic acid showed a high PLA activity. With these data, we characterized the fraction that eluted with EDTA in order to demonstrate that this fraction contains the PLA activity. This was decided because previously characterized PLAs are calcium-dependent.

A large proportion of the sample that passes through the affinity column was eluted in the elution front. This can be explained by two possibilities; the first is caused by an overload of the column that is unlikely because the saturation capacity of the column is greater. The second possibility is the existence of isoforms that do not bind the inhibitor Rosenthal but that do have enzymatic activity.

As the main purpose of this work was identifying the amino acid sequence with PLA activity in the S30 fraction, the UFF30–EDTA sub-fraction was trypsin digested and sequenced. The two peaks obtained corresponded to 8.2 and 13.0 kDa protein fractions, which were analyzed using Protein Link Global Server program. The results showed that the 8.2 kDa protein fraction corresponded to a putative ubiquitin with similar molecular weight as previously described for ubiquitin proteins (Keeling and Doolittle 1995). The 13.0 kDa protein fraction corresponded to PLA conserved sequences, as previously described in other parasites. However, this sequence has not been found in trichomonas.

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