2: Biochemistry

2.01 Circulating activities of APN and DPPIV in monosodium glutamate-obese and food-deprived rats

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Introduction: Several peptides related to energy balance are susceptible to hydrolysis by neutral aminopeptidase (APN) and dipeptidyl peptidase IV (DDPIV). **Objectives:** To evaluate the involvement of glycemia, biometry and activity levels of plasma APN and DPPIV in obesity and food deprivation. **Methods:** APN, DPPIV, protein, glycemia, body mass, mass of retroperitoneal and periepididymal fat pads, and Lee index were measured in monosodium glutamate obese (MSG) and food-deprived (FD). **Results and Discussion:** Plasma APN was distinguished as sensitive (PSA) and predominantly insensitive (APM) to puromycin, while DPPIV was sensitive (DPPIV-DS) and predominantly insensitive (DPPIV-DI) to diprotin A. Although unchanged in MSG and FD, APM activity levels were closely correlated with body mass, Lee index and mass of retroperitoneal fat pad in FD but not in MSG. Plasma levels of DPPIV-DI activity decreased in MSG-FD and correlated with body mass, Lee index and mass of periepididymal fat pad. The negative correlation between plasma APM and retroperitoneal fat pad is suggestive of downregulation of somatostatin in food deprivation. Reduction of plasma DPPIV-DI levels may be a homeostatic response associated with food deprivation for recovering energy balance in MSG obese rats.

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2.02 Hypothalamic and hippocampal DPPIV activity and CD26 in monosodium glutamate and food-deprived rats

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Introduction: Dipeptidyl peptidases (DPPs) are emerging as a protease family with important roles in the regulation of signaling by peptide hormones. **Objectives:** To study DPPIV/CD26 in soluble (SF) and solubilized membrane-bound (MF) fractions from hypothalamus (HT) and hippocampus (HC) of fasted (FD) and non-fasted monosodium glutamate obese (MSG) and normal rats. **Methods:** Catalytic activity of DPPIV by fluorometry and monoclonal protein expression of CD26 by ELISA. **Results and Discussion:** Compared to controls, MSG and/or FD induced the reduction of diprotin A insensitive (DI) DPPIV activity in SF and MF from HT, as well as in diprotin A-sensitive (DS) DPPIV activity in MF from HC. MSG and/or FD induced an opposite response (increase) of DPPIV-DI activity in MF from HC. The monoclonal protein expression of CD26 in MF by ELISA decreased in HT and increased in HC of MSG and/or FD relative to controls. The existence of DDPIV-like activity with different sensitivities to diprotin A and the identity of the less sensitive as CD26 were demonstrated for the first time in the central nervous system. DPPIV-DI/CD26 activity seems to play a role in the endocrine regulation of energy balance and anxiety, respectively, in the HT and HC.

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2.03 Isolation of bioactive peptides by the action of serinoproteases in venom of *Bothrops jararaca* on endogenous substrates and actions in cell culture and bioassays

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Introduction: Venoms are a rich source of proteolytic enzymes; from the *Bothrops jararaca* venom serinoproteases and metalloproteases are the main enzymes that act on various tissues and proteins present in the victim. Besides the action on tissues, these proteases could generate some compounds that could have specific actions in cells or show other mechanisms towards the generation of bioactive peptides. There are protein precursors of bioactive peptides, but nowadays there is a new class of proteins that in some conditions may generate bioactive peptides; they are called crypteins. **Objective:** Identification of bioactive peptides by the action of the serinoproteases, trypsin and from the venom of *Bothrops jararaca* on endogenous substrates (casein, IgG, hemoglobin, collagen and myoglobin). To biochemically isolate and characterize the peptides obtained and determine the possible effects and biological properties of these peptides through several biological tests "in vitro" and "in vivo." **Methods:** Serinoprotease from *Bothrops jararaca* venom was separated from whole venom using a Sephadex G-100 gel filtration column. The serinoproteases were incubated with the endogenous substrates chosen for a preset time. The endogenous substrates were also incubated with trypsin, as well the serinoproteases from the venom of *Bothrops jararaca*. Directly from the incubations, the remaining proteins and resulting products were observed by gel electrophoresis SDS-PAGE with silver staining and also by means of HPLC (high performance liquid chromatography) profile. The hydrolysates were tested in fibroblast cell cultures. After the test in cell culture, the active hydrolysates were purified by HPLC, and the peaks would then be tested by the same methods in order to identify the active peptides. **Results and Discussion:** In fibroblast cell cultures, a low cell proliferation rate was obtained with hemoglobin and collagen hydrolysates and a significant cytotoxic effect with immunoglobin (IgG) using trypsin as proteolytic enzyme. The results obtained in HPLC showed us that profiles of hydrolysates differed for each type of substrate used. The study suggests that these serinoproteases (trypsin or those from Bothrops jararaca venom) were able to generate peptides with important biological activity; specifically with snake venom, it could be another component of the bite response.

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2.04 Heat shock protein responses in thermally stressed freshwater snails (*Biomphalaria glabrata)*

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Introduction: The synthesis of a major stress protein class (HSP70) is induced by extremes of a wide variety of environmental variables, both physical and chemical. This protein of approximately 70,000 Da is a widespread heat shock protein and is present in various isomorphs in many species. Nevertheless, it is highly conserved among organisms from bacteria to man. Many researchers have tried to institute HSPs as biomarkers of environmental damage, investigating a broad range of organisms. **Objectives:** This work was designed to identify HSP70 stimulus by increases of environmental temperature in snails *Biomphalaria glabrata* reared in the laboratory, as an initial step to establish HSP70 as a biomarker of environmental stress. This species was chosen because of its increasing importance as an experimental model. **Methods:** Snails were maintained at temperatures of 5-10 °C above its environmental temperature for one week and dissected for protein analysis of digestive gland tissue. The proteins were extracted by RIPA buffer and centrifugation at 14,000 x g, and the supernatants were analyzed using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, using the HSP70-directed antibody. The samples were applied to the gels at 200 µg per lane. Protein concentrations were determined by the Lowry method. A 10% SDS-PAGE gel was used to separate the proteins on a BioRad minigel apparatus using Laemmli buffer. The gels were transferred to nitrocellulose membranes using a transfer system (BioRad). Blots were blocked with PBS-milk (5%) and incubated for 1 h with rabbit HSP70 antibody (1:1000 in PBS-milk), rinsed with PBS-milk and incubated with goat anti-rabbit horseradish peroxidase conjugated antibody (1:2000 in PBS-milk) for 1 h. The nitrocellulose membrane was then rinsed and incubated with a developing kit and kept in contact with photographic film to allow visualization. **Results and Discussion:** There was an induction of HSP70 in snails submitted to increased environmental temperature. The stimulus for HSP70 in thermally exposed snails was confirmed by the presence of protein, visualized in the film; no protein was detected in control samples. These results suggest that the stress protein response may be a useful environmental stress indicator.

2.05 A lipocalin sequence signature modulating cell survival, extracellular matrix remodeling and wound healing

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Introduction: Lopap is 20-kDa prothrombin activator with serine protease-like activity found in the bristles of the *Lonomia obliqua* caterpillar. Interestingly, this protein does not have sequence similarity with other proteases, but is considered a member of the lipocalin family. Lipocalins are β-barrel proteins recognized as carriers of small hydrophobic molecules. Some members have been described as modulating cell survival, developmental process and homeostasis. **Objectives:** The aims of this study were to identify the region in Lopap molecule involved in modulation of cell responses, to survey this sequence as a lipocalin signature and to evaluate its potential in extracellular matrix (ECM) remodeling and tissue repair. **Methods:** Synthetic peptides were obtained based on characteristic conserved motifs of lipocalins found in the Lopap primary sequence. The peptides were tested in serum-deprived human umbilical vein endothelial cells (HUVECs) for a screening of their antiapoptotic activity. The peptide that was able to promote cell survival (AP) was analyzed using bioinformatics tools. Primary cultures of human fibroblasts were stimulated with AP and the synthesis of ECM molecules was evaluated by immunocytochemistry. To evaluate the effect of AP *in vivo*, mice were treated in the dorsal area with intradermal injections of AP (0.04-25μg) on one side and saline on the opposite side. Skin fragments of 1 cm^2 were subjected to histological preparation and Picrosirius red coloration to measure the percentage of collagen in the dermis. The potential of AP as a wound-healing agent was evaluated in a rat skin lesion-induced model. The lesions were treated with local applications of AP or saline and the wound closure was monitored. The regenerating tissue was analyzed by histological techniques. In addition, the content of collagen and metalloproteinases (MMPs) were evaluated. **Results and Discussion:** The active peptide had 11 amino acid residues and was located in a β-sheet in the Lopap tertiary structure. Similar sequences were found among other lipocalins and putative proteins. HUVECs treated with AP showed 100% cell viability, in contrast to 65% of non-treated cells. AP-treated fibroblast cultures showed an increase from 2.6±0.1, 4.9±0.5 and 22.7±1.3 to 3.9±0.3, 9.4±0.9 and 36.8±1.9 percent procollagen. tenascin and fibronectin, respectively, in relation to non-treated cultures. Similarly, treatment of mice with AP resulted in a significant increase in collagen in the mouse dermis. A cumulative dose of 0.6 μg induced an increase of 15% in collagen content, which persisted after 12 weeks. Mouse skin lesions treated with the peptide showed an improvement of tissue repair, with a better organization of collagen fibers and the absence of keloid scars. The collagen content was substantially increased in the regenerating tissue of lesions treated with AP. A significant increase in MMP-2 was also observed. All these data reveal the potential of AP in aiding tissue repair. The Lopap-derived sequence identified seems to be a conserved property among lipocalins, promoting cell survival and possibly playing an important role in homeostasis. It should be important in the regulation of the metamorphosis process in *Lonomia*.

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2.06 Purification of coagulation factor VIII using chromatographic methods

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Introduction: Factor VIII (FVIII) is a plasma glycoprotein essential in the coagulation process, whose deficiency causes hemophilia A. Treatment consists in infusions of FVIII recombinant protein or concentrates obtained from plasma, none of them produced in Brazil. Here, we continue the studies to develop a process to purify FVIII employing solely chromatographic methods. We propose the use of ion-exchange as the first step of FVIII purification because it is inexpensive and suitable for a scaling up method. Besides, based on previous results, ion-exchange can be used by direct application of plasma. We have previously used the Q-Sepharose resins, which are strong anion-exchange matrices made from 6% agarose. To improve the recovery of FVIII activity, we have tested ANX-Sepharose Fast Flow (FF). This resin is composed of 4% agarose and, therefore, has high capacity for binding large molecules. Being a weak anion-exchange, this resin also shows an alternative selectivity. Another aspect of this project is the analysis of the FVIII fraction collected from chromatographic separations by immunoblotting. In order to prepare an in-house FVIII standard, we have used a modified method from the GE Healthcare validated process, consisting of gel filtration, cation-exchange and finally gel filtration. **Objectives:** 1. To test the ANX-Sepharose FF as the first step for FVIII purification. 2. To prepare an in-house FVIII standard. **Methods:** 1. We studied the ANX-Sepharose FF capacity of binding FVIII as well as the profile of the collected samples. We also analyzed the FVIII containing fraction in a Sepharose 6 FF gel filtration column. 2. FVIII was purified by direct application of plasma to Sepharose 4FF column, followed by the application of the FVIII-containing fraction to SP-Sepharose FF and desalting in the G25 fine column. All collected fractions were analyzed for protein content (Bradford), FVIII and protein C activity (chromogenic methods), and presence of factors IX and X (Western blotting). **Results and Discussion:** The FVIII binding capacity of ANX-Sepharose FF is much higher than that observed for Q-Sepharose. We applied up to 15 column volumes (CV) of plasma to the ANX-Sepharose FF column and no FVIII was detected in the flow-through fraction, while with Q-Sepharose resins the amount of plasma applied was 5 CV. After the application of 15 CV of plasma, solid residues were clearly visible on the top of the column. FVIII coeluted with protein C and coagulation factors IX and X. The recovery of the FVIII activity in the eluate was between 50 to 60% and was comparable with that found for Q-Sepharose columns. Finally, application of the ANX column eluate to Sepharose 6FF also gave a similar profile compared to that observed using Q-Sepharose column eluate. Two peaks were eluted containing FVIII activity, one in the void volume and a second peak coeluting with lower-molecular-mass proteins.

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2.07 Purification and characterization of a hypothermic component from the venom of *Phoneutria nigriventer*

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Introduction: The neural control of body temperature can be modulated by several toxic substances. Arthropod venoms, from scorpions and spiders for example, are a rich source of potent toxins responsible for a significant number of non-lethal human envenomations. The evenomation by the spider "*Phoneutria nigriventer*" shows several systemic manifestations including vomiting, sweating, hyperthermic or hypothermic state, mainly in children. **Objectives:** The biochemical characterization of "*Phoneutria nigriventer*" venom profile on temperature regulation of young anesthetized rats. **Methods:** The crude venom lyophilized and its "pool II" purified using gel filtration (Sephadex G-50) and reverse phase HPLC; was administered intraperitoneally in young rats, anesthetized with a solution of ketamine hydrochloride (40 mg/kg)/ diazepam (5 mg/kg). The body temperature was recorded by rectal probe acquisition system (3x20mm) and registered on software Contemp® (mod.IPM94) every 0.5 min up to 3 h. **Results and Discussion:** The data of the crude venom indicated a marked hypothermic state in young rats in the first hour of the assay. In the second hour, the venom induced a reduction in hypothermic effect to compensate for the hemodynamic changes, and in the 3rd hour the temperature fell significantly to above the control level. The preliminary data obtained from pool II demonstrated similar effects mainly in the 3rd hour. The results suggest that the hypothermic effect involves a neurotoxin activity on the thermoregulation of young rats. Purification of the other pools is under way to determine their activity on the temperature regulation of young rats.

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2.08 Anti-*leishmania* molecule in the extract of the body of *Otostigmus* sp.

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Introduction: Arthropods constitute one of the oldest groups of organisms, and also show a wide distribution in different ecosystems and habitats. This fact leads to the question about which factors allowed such an ecological success in these organisms. Knowing that most of the environments where they are found show a high presence of microorganisms and pathogens, it can be affirmed that part of the success of arthropods in colonizing these environments are their defense mechanisms and immune systems. One of the main components of the defense mechanism in vertebrates and invertebrates are the peptides with immune functions, which control the growth and invasion of the different pathogens. The defensive role of a variety of antibiotic peptides in multicellular organisms is increasingly recognized, but the characterization of the peptides with anti-parasitic function, had not begun until recent times. Also, the majority of this research has focused on the hydrophobic peptides, ignoring the hydrophilic compounds. For all these reasons, it is important to study this subject, not only to understand the success of these invertebrates and their defense mechanisms, but also to find alternatives to fight infectious and also parasitic diseases that affect humans. This is why the purification and characterization of these peptides and the knowledge of the function of their immune systems are even more interesting. **Objectives:** The aim of this study was the separation and analysis of highly hydrophilic compounds to identify the antimicrobial factors in the extract of the body of the Brazilian myriapod *Otostigmus* sp. **Methods:** The bodies of the animals were first subjected to maceration and acid extraction and then fractionated in two steps. First, using C18 Sep Pak column cartridge, the hydrophilic and hydrophobic fractions were separated. The hydrophilic fraction was concentrated in a vacuum centrifuge, reconstituted in ammonium acetate (pH 6.7) and loaded onto an Asahipak GS-320 column, using an isocratic gradient of ammonium acetate (pH 6.7) for the second purification step. The column effluent was monitored by absorbance at 225 nm and the anti-parasitic and antimicrobial activity was determined by liquid growth inhibition assay. **Results and Discussion:** After the HPLC separation, antimicrobial activity was detected against *Escherichia coli*. The same fraction also showed anti-parasite activity against *Leshmania* spp. The first analysis with mass spectrometry showed that the fraction that showed that anti-parasitic and antimicrobial activity had a low molecular weight. As a hydrophilic fraction, its mechanism of action must be different from the mechanisms of action of hydrophobic molecules, which will be important in understanding the mechanism of action of these substances and learning how they interact with the membrane. The purification and characterization of these fractions is still in progress.

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2.09 The effect of a recombinant Kunitz type inhibitor (r-KTI) from *Amblyomma cajennense* on cell survival and its molecular target

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Introduction: A recombinant protein, showing factor-Xa inhibitor activity and characterized as a Kunitz-type inhibitor was obtained from a cDNA library of salivary glands of the tick *Amblyomma cajennense*. The protein did not show cytotoxicity against normal cells (fibroblasts and HUVECs), but it was able to induce death of tumor cells (human melanoma SK Mel-28, pancreatic adenocarcinoma Mia Paca-2) *in vitro*. At a later stage, we also investigated the influence of this rKTI on the control of cell cycle phases and gene expression in tumor cells. **Objectives:** The aim of this study was to evaluate the mechanism of the action of the rKTI inducing tumor cell death. **Methods:** Cell cycle and cytotoxicity studies were performed by flow cytometry. Changes in gene expression of Sk Mel-2 and Mia-Paca-2 were evaluated by DNA microarray using the CodeLink platform Whole Human Genome Bioarray. Proteasomic catalytic activity was assessed by fluorimetry and poly-ubiquitinylated tumor cell proteins by immunoassay. **Results and Discussion:** rKTI induces cell cycle arrest at G0/G1 and apoptosis in tumor cells. Analysis of gene expression by micorarray showed several genes altered in Mia Paca-2 and SK Mel-28, among them 24 common genes for both cell lines. The results showed that the rKTI inhibits the trypsin and chymotrypsin-like catalytic activity of proteasome. The total poly-ubiquitinylated cancer cell proteins were increased after treatment. Altogether, our results suggest that among the common altered genes after rKTI treatment, the majority are related to cell cycle control and cell death by the apoptotic route. The alteration in the *PSMB2* gene (which codes for a proteasome β-2 subunit) corroborated the alterations observed in the inhibition of catalytic activity and consequent increase in poly-ubiquitinylated proteins, indicating that the proteasome is a cellular target of the rKTI in inducing cell death.

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2.10 Characterization of digestive lipase from hematophagous vectors

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Introduction: Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols resulting in the release of fatty acids. Lipases are enzymes found in animals, plants, fungi and bacteria. Several Arthropoda such as mosquitoes and ticks are vectors of plant, animal and human diseases, and they are public health problems. The mosquito *Aedes aegypti* (Insecta) and the tick *Amblyomma cajennense* (Arachnida) are important Arthropoda vector in Brazil of dengue fever and Brazillian spotted fever, respectively. **Objectives**: To characterize the lipid digestion in *A. aegypti* (adult and larva) and *A. cajennense*. In order to do that, we measured the lipase activity in midgut homogenates from these animals. Properties such as pH effect, the effect of Ca^{+2}, acid precipitation and chromatographic separation were determined for these lipases. **Methods**: Midgut from *A. aegypti* (adult and larva) and *A. cajennense* females were isolated and homogenized in Milli Q water or homogenized in 100 mM sodium acetate pH 3.5 buffer in a Potter-Elvehjem homogenizer. Lipase activity was measured using 4-methylumbelliferyl oleate (MUO) as substrate. Homogenate samples were submitted to different chromatographic steps (cation and anion exchange chromatographu). *A. aegypti* adult and larva homogenates were initially applied to a Hitrap Q column equilibrated in 20 mM Tris buffer, pH 7.0. Proteins were eluted in a NaCl gradient (0-1 M) in the same buffer. Lipase-active fractions from *A. aegypti* adult chromatography were pooled, desalted and applied to a Resource Q column. Protein was eluted using a linear gradient of 0-0.5 M NaCl in 20 mM Tris-HCl buffer, pH 7.0. **Results and Discussion**: Lipase activity was measured in midgut homogenate samples from *A. aegypti* (adult and larva) and *A. cajennense.* Absolute and specific activities were as follows: *A. aegypti* (adult) showed 2.1 mU/animal (4.5 mU/mg); *A. aegypti* (larva) showed 6.1 mU (100 mU/mg) and *A. cajennense* 112 mU (8.9 mU/mg). Acidic precipitation tested to *A. aegypti* adult midgut homogenate was not efficient in order to purify lipase due to low activity recoveries differently from what was observed to *A. cajennense* (1.5 x purification). Lipase activities from these samples were not affected by Ca^{+2}. Optimum pH of lipase activities were: *A. aegypti* (adult), 9.0; and *A. cajennense,* a range of 8 to 9.5). Separation after a cation-exchange chromatography (Hitrap S column) indicated only one activity of lipase in samples of midgut homogenate from *A. cajennense.* Lipase assay of anion-exchange chromatography (Hitrap Q column) fractions from *A. aegypti* adult sample indicated one activity of lipase eluted with 0.36 M NaCl, and from *A. aegypti* larva two activities of lipase were eluted with 0.12 M and 0.2 M NaCl. Lipase-active fractions from *A. aegypti* adult were pooled, desalted and applied to a Resource Q column and two distinct activities were eluted (0.22 M NaCl and 0.26 M NaCl). It was observed that there were important differences between lipase from *A. aegypti* (adult and larva) indicating different enzymes involved in lipid digestion in larva and adult probably due to different feeding habits. Optimum pH from lipases studied are in the range of 8.0 to 9.5. *A. cajennense* and *A. aegypti* (adult) had distinct interaction with ion exchange chromatography, and the isolation of these enzymes is being tested.

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2.11 rLosac, the factor X activator from *Lonomia obliqua*: comparative studies between normal and Gla-domainless factor X

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Introduction: Losac is the first factor X (FX) activator purified from *Lonomia obliqua* bristles. The mechanism of recognition and cleavage of FX by Losac is still unknown. On the other hand, this mechanism is well known for RVV-X, the factor X activator from *Daboia russelli* venom. RVV-X activates FX through the recognition of the Gla domain and then by the cleavage of the FX heavy chain by the RVV-X metalloprotease domain, and this interaction is Ca^{+2}-dependent. **Objectives:** To obtain the recombinant Losac with enzymatic activity on FX and to compare its activity with RVV-X. **Methods:** The *E. coli* BL21 (DE3) strain was transformed with the recombinant plasmid (pAE-Losac). This system is designed for expression of rLosac with a 6x-His tail in the N-terminus. We evaluated the activation of human FX (345 nM) using different concentrations of rLosac and RVV-X in chromogenic assays (0.2 mM S-2765) and also through the degradation of normal and deglycosyled FX by SDS-PAGE. The specificity of Losac activity on FX was evaluated assaying its activity on several other substrates such as prothrombin (chromogenic assays), fibrinogen and fibrin (SDS-PAGE and fibrin plates, respectively). The capacity of rLosac to activate factor X in plasma was assayed by plasma recalcification time. **Results and Discussion:** rLosac and RVV-X activated FX in a dose-dependent manner. Only Losac was able to activate deglycosylated FX although this activation was less when normal FX was used. Apparently, the protein is specific for FX since it does not activate prothrombin, does not cleave fibrinogen or fibrin. The normal plasma recalcification time (195.6s ± 37.67) was shortened after 2 min of incubation with rLosac (70.08s ± 4.80) or RVV-X (60.9s ± 2.04). Moreover, when we incubated Losac plus plasma for 10 min, the plasma recalcification time induced by rLosac was faster than that induced by RVV-X (11.6s ± 4.27 and 54.06s ± 6.15, respectively), compared with the control (148.6s ± 25.9). All these results demonstrate that the FX activation by rLosac is different than that induced by RVV-X. The exact mechanism of FX recognition by rLosac is yet to be determined. We are currently working to understand the mechanism by which rLosac recognizes FX.

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2.12 Acid protein digestion in the spider *Nephilengys cruentata*

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Introduction: Peptidases form the largest enzyme gene family in vertebrates. According to the Merops database, they comprise 641 genes in humans and 677 in the mouse. Spiders are efficient predators of insects. Protein digestion in spiders has been sporadically studied and there are few reports in the literature. Our group characterized protein digestion in the hepatopancreas of the spider *Nephilengys cruentata*. Studies with natural protease substrates and specific inhibitors indicated two distinct activities: an alkaline activity already characterized as an astacin-like enzyme and an acidic activity. **Objectives:** Classification, isolation and characterization of the digestive acidic peptidases in the spider *Nephilengys cruentata*. **Methods:** Adult females were collected and maintained fasting for two weeks. Afterward, cannibalism among spiders was favored. Fed females were dissected and the isolated hepatopancreas was homogenized in Milli-Q water. Partial isolation was obtained with a combination of two cation-exchange chromatography steps, a Hitrap S and a Resource S both equilibrated in 0.05 M citrate-phosphate buffer, pH 5.0, and eluted with a linear (0 – 1 M) NaCl gradient. The activity was measured at 30° C with 10 μM Z-Phe-Arg-MCA as substrate diluted in 0.1 M citrate-phosphate buffer containing 3 mM cysteine and EDTA. Activity was measured after native PAGE using Z-FR-MCA as substrate. **Results and Discussion:** Chromatographic separation and specific assay using Z-FR-MCA as substrate showed two different peptidases in acidic conditions: an aspartic endopeptidase, inhibited by pepstatin and a cysteine-endopeptidase inhibited by E-64, MMTS and stabilized in the presence of cysteine. Activity measured after native electrophoresis on a 7.5% polyacrylamide gel resulted in one activity band on Z-FR-MCA and gelatin as substrates. Assays using quenched fluorescent substrates showed highest activity on the substrate Abz-F-R-Q-EDDnp, indicating that cysteine-endopeptidase is the major acidic peptidase, probably related to a cathepsin-L-like enzyme hydrolyzing preferentially substrates with a Phe residue at P2. Thus, this cathepsin-L-like activity was characterized. This enzyme showed a pH optimum of 3.6 and a molecular weight of 30.4 kDa determined by SDS-PAGE from partially isolated samples. It was mainly stable in acidic conditions (pH 3.0 to 6.0) at 4°C and 30°C and showed a half-life of 13 min at 60°C. E-64 showed a K_D of 55 nM, which is in agreement of data for other arthropod cathepsin-L-like enzymes. This enzyme will be isolated to homogeneity for detailed specificity studies, in order to characterize its ability to hydrolyze spider silk.

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2.13 Batch mode cultivation of CHO-EPO cells in suspension to establish purification process

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Introduction: Natural erythropoietin (EPO) is present in picomolar amounts in biological samples, making its purification very difficult. In 1977, Miyake et al. succeeded in isolating and purifying the human hormone EPO from the urine of patients with aplastic anemia. This small amount of EPO obtained after a 7-step procedure was the starting point for the full characterization of the molecule. Currently, recombinant DNA techniques allows the hormone be produced through the cultivation of genetically modified cells. It is a glycosylated protein of 165 amino acids with a molecular weight of 34 to 39 kDa; approximately 40% of its weight consists of neutral sugars and sialic acid. Responsible for the maturation of erythrocytes, it is used in the correction of anemia in patients with chronic renal failure. Regarding its production processes, recombinant EPO is obtained from mammalian cells preferentially cultivated with serum-free medium. Thus, the proteins from the cells are the main source of contaminants to be eliminated, besides hydrolysates present in the medium and degradation products of the product itself. **Objectives**: To establish the steps for EPO purification from supernatants obtained with cells adapted to growth in suspension and serum-free medium in batch mode cultivation. **Methods:** CHO cells transfected with the human gene coding for EPO were grown in suspension with EX-CELL™ 302 medium (JRH Biosciences) in a 8 L spinner-flask in a batch mode. Dissolved oxygen, pH and temperature were monitored online. The controller (Corning Life Science) was used to regulate the level of DO (60%) through injection of gas mixture in the headspace of the spinner-flask. The supernatant of the culture was clarified by centrifugation, and then purified in 3 chromatography steps. The first consisted of an affinity resin. EPO was eluted with a high salt concentration. The eluate was diafiltrated through a 10,000 cutoff cartridge. The second and third chromatography steps consisted of ion-exchange resins. At the end the diafiltration step, buffer exchange was needed. **Results and Discussion:** Affinity chromatography allows the elimination of impurities in addition to concentrating the sample about 10 times with a yield of 78%. In the subsequent chromatography with anion exchanger matrix, an additional removal of contaminants was observed with a yield of 27%. The subsequent column filled with a cation exchanger matrix resulted in the elimination of contaminant molecules larger than EPO and final yield of 20%. The analysis of isoelectric focusing demonstrated the presence of 9 isoforms, showing two bands more acidic when compared with the internal standard used by the laboratory, meaning an improvement in capturing isoforms with higher EPO biological activity. The process did not result in a purification profile necessary for clinical use; however, it must be considered that batch cultivation is not suitable for EPO production, as all the contaminants are concentrated and defective forms of EPO accumulate due to protease action. To cultivate suspension cells is very different from adherent cells and the downstream process must be adjusted for this new environment. We are now starting perfusion mode cultivation of suspension cells expecting to work with fewer contaminants in the supernatant.

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2.14 Toxicity of both gallic acid and the free phenolic acid fractions from pomegranate pulp (*Punica granatum, L*): effects on growth and apoptosis in MDCK cells

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Introduction: Antioxidants from natural origins, including phenolic acids, due to their expected potential to prevent a chronic pathologic process that results from a burst of free radical excess, are the targets of this type of study. **Objective:** The present study evaluated the effect of antioxidants on cell viability, by determining both growth and apoptosis in MDCK cells. **Methods**: The extract obtained from pomegranate pulp (*Punica granatum,L)* with phenolic compounds were evaluated by the Folin-Ciocalteau method, and its antioxidant activity was measured by the ß-carotene/linoleic acid and DPPH methods. The cells were treated with gallic acid (GA), as a control of antioxidant activity, and free phenolic acid fractions (FFAp) from pomegranate extract, at concentrations of 150, 75, and 15 µg, for 24 h at 37°C. Also, the TUNEL test (Roche®; T[d] T- mediated dUTP nick end-labeling) was used to determine the apoptosis level of these cells treated with FFAp (10 µg), with the same cited conditions. It was observed that cell growth was reduced when treated with gallic acid using 150 µg of this antioxidant, compared with control cells. **Results and Discussion**: Cell growth and viability was 31.37%, compared with 65.86% for control cells. These results were better (70.05%) using lower doses such as 75 µg of GA. Also, the cells treated with FFAp fractions revealed that growth increased (196.58%) with a dose of 15 µg, compared with doses of 150 µg. This showed higher (78.56%) cell viability compared with control cells (65.85%). According to the TUNEL test, these cells treated with FFAp (10 µg) showed more protection when compared with the control group that showed a low cell apoptosis level, and the cells treated with 200 ug of gallic acid showed a higher apoptosis level. These data suggest that, at appropriate doses, the pomegranate extract is an antioxidant with protective action on cell growth.

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2.15 Effect of aqueous extract of pequi (*Caryocar brasiliensis*, Camb) on antioxidant enzymes in liver

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Introduction: The induction of antioxidant enzymes by chemoprotective agents was shown to be an effective strategy for protecting organisms against deleterious effects such as tissue oxidation. The phenolic compounds present in fruits can prevent free radical formation and protect tissues against peroxidation. *Pequi* is a very popular tropical fruit and it is consumed as part of the meals among the population from the central west and northeast areas of Brazil. **Objective:** The objective of this study was to evaluate the antioxidant activity of the aqueous extract of pequi fruit pulp and to determine its effect on the expression of the protective enzymes superoxide dismutase (SOD) and catalase (CAT) in rats. **Methods:** Antioxidant activity was measured by the ß-carotene/linoleic acid and DPPH methods. Rats (eight in each group) were orally administered 100 mg/kg aqueous pequi extract for 30 consecutive days alongside the control group. Afterward, these animals were sacrificed and the livers were extracted for enzyme evaluation. SOD was determined by the McCord and Fridovich (1969) method and CAT by the method reported by Beuter (1975). Enzyme expression was evaluated by RT/PCR techniques using specific primers for SOD and CAT. **Results and Discussion**:The phenolic compounds in dried pequi pulp fruit amounted to 209 mg/100 g with antioxidant activity of 80% (50 ppm) measured by ß-carotene/linoleic acid and 260.37 mg/mL by DPPH. Superoxide dismutase and catalase showed respectively 40% and 20% more activity in the group that received the pequi extract than in the control group. SOD and CAT gene expression, detected by RT/PCR in liver, revealed that there were no changes in SOD, but that CAT gene expression was 50% higher than in control. These results provide a perspective for the contribution of pequi pulp fruit as a functional food.

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2.16 Aqueous extract of pequi (*Caryocar brasilienses*, Camb): phenolic composition, antioxidant activity and influence on MDCK cell growth

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Introduction: Pequi is a tropical fruit that is an abundant crop in the central west and northeast regions of Brazil. This fruit is very popular in Brazil and is normally consumed with rice and chicken meals. The phenolic compounds present in many foods, including fruits, vegetables and spices, exhibit antioxidant properties. **Objective**: The objective of this study was to evaluate the aqueous extract from the pequi fruit pulp, its phenolic composition, its antioxidant activity and its effect on MDCK cultured cells. **Methods**: The phenolic compounds were determined by the Folin-Ciocateau method. The antioxidant activity was measured by the ß-carotene/linoleic acid and DPPH methods. MDCK cells were grown at 37ºC in L15 medium in six 24-well plates. After seven days, the cell monolayers were exposed to 20 ppm and 40 ppm 30 and 60 min, after which the viable cells were evaluated. The phenolic compounds in dried pequi fruit were totaled 209 mg/100 g. The antioxidant capacity (AC) with 50 ppm was higher than 80% in ß-carotene bleaching and 260.37 mg/mL in the DPPH method. **Results and Discussion:** The percentages of viable MDCK cells after 30 min and 60 min with 20 ppm of pequi aqueous extract were 90% and 97%, respectively in contrast to 84% and 15% of the control cells. With 40 ppm of the extract these results were 73% and 50%, respectively. These data suggest that the antioxidant activity of phenolic compounds in the pequi fruit can influence both the growth and protection of MDCK cells.

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2.17 Trypsin-inhibitors against insect peptidases in the spider *Nephilengys cruentata*

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Introduction: Some insects are agricultural pests and vectors of several diseases. Development of control strategies is important to increase world food production and reduce the incidence of diseases such as dengue fever. Spiders are efficient predators of insects. Prey digestion starts extraorally by the digestive juice produced in the hepatopancreas. Data in literature indicate that spider digestive juice (SDJ) could have peptidase inhibitors (PIs) to control ingested prey's proteases. **Objectives:** To investigate the presence of PIs in both SDJ and hepatopancreas homogenate (HP) from the spider *Nephilengys cruentata*. **Methods:** SDJ and HP samples were used as a source of PIs. Chromatographic separations in anion exchange (AEX) and gel filtration (GF) columns of these samples were performed and fractions were tested against different insect and mammalian peptidases. *Periplaneta americana*, *Musca domestica*, *Aedes aegypti* female, *Aedes aegypti* larva and *Gryllus sp* midgut homogenates were used as sources of insect trypsins. As mammalian trypsin source, commercial bovine trypsin was tested. SDJ samples were submitted to GF chromatography (SuperdexG-75) in 20 mM Tris-HCl, 0.5 M NaCl buffer, pH 7.0. Identification of inhibitory fractions was done with a mixture of 12.5 µL of chromatographic fractions and 12.5 µL of enzyme source. These mixtures were pre-incubated for 30 min at 30°C. Afterward, 0.83 mM benzoyl-Arg-p-nitroanilide (BApNa) in 0.1 M Tris-HCl, 0.25 M NaCl buffer, pH 8.5, was added to mixtures as trypsin substrate. Fractions with decreased trypsin activity were pooled, desalted and applied to an AEX chromatography, using a Resource Q column equilibrated with 0.02 M Tris-HCl buffer, pH 7.0, and eluted with a linear NaCl gradient (25 mL; 0 - 1 M). Fractions with trypsin-inhibitory activity were pooled, desalted and applied to the same Resource Q column equilibrated in the same conditions and eluted with a linear NaCl gradient (25 ml; 0- 0.5 M). Fractions with trypsin-inhibitory activity were individually submitted to SDS-PAGE. HP was boiled for 5 min and centrifuged for 10 min at 13,000 rpm. The supernatant was submitted to AEX chromatography, using a Hitrap Q column, equilibrated with 0.02 M Tris-HCl buffer, pH 7.0, and eluted with a linear NaCl gradient (0-1 M). Fractions with trypsin-inhibitory activity were pooled, desalted and applied to a Resource Q column equilibrated and eluted in the same conditions described before. Fractions with trypsin-inhibitory activity were individually submitted to SDS-PAGE. **Results and Discussion:** Measures of inhibitory activities from *N. cruentata* SDJ and HP on different insect trypsins indicated that SDJ (30 to 100% inhibition) and HP (76 to 96 % inhibition) were able to inhibit insect trypsins. The isolation of inhibitory activity from SDJ using a combination of GF and AEX chromatography was demonstrated by a SDS-PAGE showing the enrichment of a protein of 13 kDa, corresponding to the inhibitory activity. The isolation of inhibitory activity from HP using a combination of AEX chromatographic steps was shown by SDS-PAGE indicating a partial purification of different molecules with molecular masses of 14 to 30 kDa. SDJ and HP possess efficient trypsin inhibitors with low molecular masses which will be kinetically characterized and used to bioassay against *A. aegypti* being added to their diet.

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2.18 The effect of *Bothrops jararaca* antithrombin on cell migration induced by carrageenan in mice

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Introduction: Antithrombin is an important inhibitor of several coagulation serine proteases, including factors X_a, IX_a, XI_a and thrombin. Besides, a large number of recent studies have shown that human antithrombin has anti-inflammatory actions, which are independent of its effects on coagulation. **Objective:** The aim of this work was to investigate the effects of *B. jararaca* antithrombin (BjAT) on cell migration induced by carrageenan (cg) in mice. **Methods:** Antithrombin was purified from *B. jararaca* plasma using a HiTrap Heparin HP column. BjAT (20 μg/100 μL i.v.) or saline (100 μL) was administered 1 h before intraperitoneal injection of cg (300 μg/200 μL) or saline (sal) (200 μL) in male Swiss mice (18-22 g). After 4 h of cg injection or sal, cell migration to the peritoneal cavity was evaluated. Total peritoneal cell counts were carried out using a Neubauer hemocytometer, and differential counts were preformed with smears stained with panchromatic stain. A total of 100 cells were counted by optical microscopy. **Results and Discussion:** Pre-treatment with BjAT diminished cg-induced cell influx into the peritoneal cavity, when compared with the group pretreated with sal (sal+cg). The decrease in cell migration in animals pretreated with BjAT was 41% (sal+cg: 4.66 ± 0.56, BjAT+cg: 2.74 ± 0.31; $p<0.05$). A significant decrease of 82% was observed for polymorphonuclear cells in animals pretreated with BjAT (sal+cg: 3.50 ± 0.81 , BjAT+cg: 0.60 ± 0.09; $p<0.05$). The results demonstrate that BjAT significantly inhibited the migration of polymorphonuclear cells to the peritoneal cavity. Thus, we suggest that BjAT possesses anti-inflammatory properties.

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2.19 Characterization of digestive carbohydrases and isolation of α-fucosidase from *Amblyomma cajennense*

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Introduction: *Amblyomma cajennense* is one of the most important tick species in the Brazilian fauna, and it is an important pest of horses, cattle and dogs. Besides, *A. cajennense* is the main vector of Brazilian spotted fever caused by *Rickettsia rickettsii*. Although it is of economic importance and a vector of human and veterinary diseases, only a few studies on this species are available. Conventional tick control methods have been based mainly on the use of acaricides. However, novel control methods, such as vaccination and biological control, are needed. Although an anti-tick vaccine may be the most promising control method, its development still depends on the identification and characterization of one or more protective tick antigens. α-Fucosidases (EC 3.2.1.51) are glycosyl-hydrolases (GH, family 29) that catalyze the hydrolysis of glycosidic linkages (α-1,2, α-1,3, α-1,4 and α-1,6) present in glycoconjugates (carbohydrates, glycoproteins, glycolipids and glycosaminoglycans) between a fucose and other molecules. The importance of the digestive system as a target of control methods has been already demonstrated and a few studies on carbohydrate processing in ticks are available. **Objectives:** We aimed to characterize carbohydrate digestion in *A. cajennense* and isolate the digestive α-fucosidase. In order to do that, we determined α-amylase, trehalase, α-fucosidase, β-glucosaminidase, chitinase, α-glucosidase, and α-galactosidase activities in the gut of *A. cajennense*. **Methods:** Ticks were fed on rabbits and engorged females were dissected for gut isolation. Guts were homogenized in MilliQ water. In order to characterize carbohydrate digestion in tick, we determined α-amylase (starch), trehalase (trehalose), α-fucosidase (4-methylumbelliferyl-α-L-fucopyranoside), β-glucosaminidase (4-methylumbelliferyl-β-N'-acetylglucosamine), chitinase (4-methylumbelliferyl-β-N',N'',N'''-triacetylchitotrioside), α-glucosidase (4-methylumbelliferyl-α-glucoside), and α-galactosidase (4-methylumbelliferyl-α-D-galactoside) activities. In order to purify digestive α-fucosidase from *A. cajennense*, the midgut was homogenized in acetate buffer pH, 3.5, and centrifuged at 4°C for 30 min at 13,500 rpm. The soluble fraction was desalted using a Hitrap desalting column eluted with MilliQ water and applied to a HiTrap S column in 50 mM citrate-phosphate buffer, pH 5.0. Proteins were eluted in a NaCl gradient (0-1 M) in the same buffer. α-Fucosidase active fractions were pooled and applied to a Resource S column. Protein elution used a linear gradient of 0-0.6 M NaCl in the same buffers used in the previous chromatographic step. **Results and Discussion:** The following enzyme absolute and specific activities were measured: α-amylase 880 mU/gut, (8.3 mU/mg); trehalase: 230 mU/gut (25 mU/mg); chitinase 478 mU/gut (23 mU/mg); α-glucosidase 68.5 mU/gut (2 mU/mg); α-galactosidase 6.9 mU/gut (0.5 mU/mg); β-glucosaminidase 4900 mU/gut (341 mU/mg); and α-fucosidase 416 mU/gut (35 mU/mg). α-Fucosidase activity was purified to homogeneity and demonstrated by a 12% polyacrylamide gel electrophoresis. Main digestive carbohydrase activities were identified in samples of *A. cajennense* gut, and we could identify β-glucosaminidase, α-amylase and chitinase as the most active enzymes.

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2.20 Effect of leech salivary gland compounds on hemostasis and cell survival

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Introduction: Salivary molecules of hematophagous animals have been identified and characterized as new targets for the development of therapeutic agents against several diseases. Salivary glands (SG) from leeches are enriched with molecules that display diverse functions acting against the host's hemostatic, inflammatory and immune responses. **Objectives:** To identify and characterize proteins able to degrade fibrinogen/fibrin, to inhibit factor Xa and also to induce tumor cell death. **Methods:** SG from *Haementeria vizottoi* (Vizotto, 1967) leeches were removed by dissection, stored frozen at – 70 °C and sonicated at 4 °C in 10 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$. After that, the salivary complex extract (SCE) was centrifuged at 12,000 *g* for 3 min at 4 °C and the supernatant filtered. SCE was loaded on a Superdex G-75 column equilibrated with 20 mM Tris-HCl buffer pH 8.0. Fractions were evaluated for their ability to inhibit FXa on chromogenic substrate S-2765, and to modify the human plasma recalcification time. Fibrino(geno)lytic activity was measured by zymography assays on fibrin-agarose-plates and also by thrombin time assays (TT). Cell viability of melanoma (SKmell-28) cells was analyzed by the MTT method **Results and Discussion:** Regarding the chromatographic profile (SGE loaded on Superdex G-75), one protein peak (R1) was able to degrade fibrinogen and fibrin. The molecular mass of the protein responsible for this activity was around 70 kDa detected by zymography. The other isolated peak (R2) showed FXa inhibitory activity and caused an increase in the recalcification time assays. The protein band responsible for this activity was revealed by SDS-PAGE and it is about 10 kDa. SKMell-28 cell viability was significantly altered after 48 h treatment with SGE. Previous LC/MS experiments (proteomic analysis) confirmed the presence in the SGE of two major proteins with molecular masses of 42,115 and 56,145 Da. The *H. vizottoi* SGE contains fibrino(geno)lytic and FXa inhibitor components able to promote blood incoagulability during animal feeding (contributing to the animal's survival and possible development of new therapeutic agents). The identification of the compounds responsible for the cell death effects is now in progress in our laboratory.

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2.21 Molecular characterization of four nucleotide pyrophosphatase/phosphodiesterases of *Schistosoma mansoni*: cloning, expression and purification

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Introduction: NPPs are ubiquitous membrane-associated or secreted ecto-enzymes that have a role in regulating extracellular nucleotide metabolism and require divalent cations and alkaline pH. They hydrolyze pyrophosphate or phosphodiester bonds in a variety of extracellular compounds including nucleotides, (lyso) phospholipids and choline phosphate esters, releasing nucleoside 5'-monophosphate. Extracellular nucleotides, and in particular ATP and adenosine, elicit a broad range of responses in biological processes. Catalysis by NPPs could affect multiple physiological processes as diverse as platelet aggregation, apoptosis, cell proliferation, differentiation and motility. **Objectives:** The objective of this work was to identify NPP proteins in the *S. mansoni* genome, characterize them by bioinformatic tools and clone them for *E. coli* expression. **Methods:** We searched for proteins with the NPP domain in the *S. mansoni* genome "GeneDB." Next, Blast and PSI-Blast searches against the NCBI non-redundant protein sequence database were used to identify orthologues of SmNPP. For phylogenetic analysis, alignments of protein sequences were performed using the ClustalX 1.83 software. The tree was constructed using Clustal, and the TreeView program was used to visualize the tree. The signal peptide prediction was performed using the SignalP 3.0 server, and transmembrane helices were analyzed by TMHMM, version 2.0. Molecular weight (MW) and isoelectric point (pI) were calculated with the Compute pI/Mw tool. The *smnpp* genes were amplified by RT-PCR from adult worm RNA, and then fragments with the NPP domain, but without putative signal peptides and putative transmembrane domains, were cloned into an *E. coli* expression vector, pAE. The proteins were expressed as inclusion bodies, which were solubilized with 8 M urea, purified under denaturing conditions by nickel affinity chromatography and dialyzed against PBS buffer. **Results and Discussion:** Four genes with NPP domain were found in the *S. mansoni* genome "GeneDB," and their linear sequence was submitted to an initial characterization by bioinformatic tools. The phylogenetic analyses and the multiple alignment of the SmNPPs showed that three of the four were more similar to human NPP-5, while the other was more similar to human NPP-6, so they were named SmNPP-5a, SmNPP-5b, SmNPP-5c and SmNPP-6. It was observed that SmNPP-5a and SmNPP-6 show a putative N-terminal signal peptide and a putative C-terminal transmembrane domain; SmNPP-5b has two putative transmembrane domains, one N- and the other C-terminal; and SmNPP-5c shows only a putative N-terminal signal peptide. All SmNPPs showed six metal-binding conserved sites and in the position of the catalytic center, an amino acid whose side chain is polar but electrically neutral at neutral pH. The recombinant proteins showed good expression levels, with similar predicted molecular weight. Unfortunately, as proteins precipitated during dialysis, we did not detect enzymatic activity. Therefore, we now plan to improve the refolding process to obtain soluble - and possible enzymatically active - proteins.

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2.22 N-terminal determination of Mlx-6, a protein isolated from the venom of the snake *Micrurus lemniscatus*

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Introduction: Toxins isolated from venoms of Old World elapids have been extensively studied as sources of presynaptic PLA2s and postsynaptic neurotoxins. These toxins have been shown to be valuable tools for the characterization of the structure and function of muscle cholinergic receptors as well as for identifying subtypes of nicotinic and muscarinic receptors that control specific functions in the brain. Therefore, few investigations have dealt with isolated toxins from venoms of Brazilian snakes of the genus *Micrurus* (coral snakes, family Elapidae) and their effects on the brain. Previously, we reported that the toxin Mlx-6 isolated from the *Micrurus lemniscatus* venom induced a displacement on the H3-QNB binding in hippocampal membrane suggesting an activity on the muscarinic acetylcholine receptors. **Objective:** The aim of this study was to describe the N-terminal of the toxin Mlx-6 isolated from the *Micrurus lemniscatus* venom, and compare the obtained sequence to proteins isolated from the venom of *Micrurus surinamensis*. **Methods:** To isolate Mlx-6, venom of the *M. lemniscatus* snake was loaded onto a C18 RP–HPLC column and eluted with an ACN gradient containing 0.1% TFA. After concentration, the fraction of interest was chromatographed again to avoid eventual contaminants. To determine the N-terminal portion, the obtained fraction was submitted to Edman degradation in a PPSQ-21 Sequencer (Shimadzu). **Results and Discussion:** The 10 amino acids of the N-terminal portion of the Mlx-6 were determined; however, the obtained sequence shows similarity to other proteins isolated from the genus *Micrurus*. Mlx-6 shows similarity to the Three-Finger proteins that act on cholinergic receptors and our protein could be considered a new member of this group not described before. With the aim to understand the functional importance of this toxin, studies have been designed to determine if Mlx-6 acts like an agonist or antagonist on the muscarinic acetylcholine receptors. Therefore, studies using preparations of isolated organs and behavioral studies of learning and memory are in progress in our laboratory.

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2.23 *Phoneutria* from Western Amazonia: antimicrobial peptides

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Introduction: Infectious diseases are among the main causes of death in human beings. The main reason is the resistance of microorganisms to different antibiotics. Owing to this, the search for antimicrobial molecules in Brazilian fauna and flora could be important. In arachnid toxins, mainly from spiders and scorpions, some antimicrobial peptides have been identified. **Objectives:** The objective of this study was to identify antimicrobial peptides in the venom of spiders of the genus *Phoneutria* from the Western Amazonian rainforest. In this region, three species of the genus can be found: *P. fera, P. reidyi* and *P. boliviensis*. Contrary to the non-Amazonian species *P. nigriventer*, the *Phoneutria* spp. from the Amazon have been poorly studied with regard to their venom. **Methods:** The venom was milked by electric stimulation and lyophilized. The venom was reconstituted in 0.05% trifluoroacetic acid (TFA) and the soluble part was submitted to HPLC reversed-phase chromatography on a semi- preparative Jupiter C18 column. Elution was performed with a linear gradient of ACN/TFA 0.05% over 60 min at a flow rate of 1.5 mL. The column effluent was monitored by absorbance at 225 nm. The presence of antibacterial activity was determined by a liquid growth inhibition assay against the Gram-negative bacteria *Escherichia coli SBS363*, Gram-positive bacteria *Micrococcus luteus A270* and yeast *Candida albicans*. **Results and Discussion:** Several fractions inhibited the growth of the bacteria *M. luteus* and *E. coli* and the yeast *C. albicans*. Two groups of antimicrobial molecules were found in the venom of *Phoneutria* spiders: hydrophilic and hydrophobic. The same hydrophilic molecules were observed in the venom of the three spiders in this study, a molecule with 410 Da also found in *P. nigriventer* venom, the nigriventrin neurotoxin. Nigriventin is a non-protein low-molecular-mass neurotoxin, isolated from the hydrophilic fraction of *P. nigriventer* venom. This molecule inhibited the growth of *E. coli*. The fractions with hydrophobic molecules are peptides and were not pure on analysis by mass spectrometry (MALDI-TOF). These molecules should be submitted to new stages of purification for their full characterization. The purification and characterization of these antimicrobial factors are in progress.

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2.24 Is the antifungal rondonin widespread in all Theraphosidae?

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Introduction: Antimicrobial peptides have become recognized as important components of the nonspecific host defense or innate immune system in a variety of organisms ranging from plants and insects to animals, including mollusks, crustaceans, amphibians, birds, fish, mammals, and humans. The widespread occurrence of these antimicrobial substances suggests that they play a role in innate immunity against microorganisms and other pathogens. We found an antimicrobial peptide in the plasma of *Acanthoscurria rondoniae* with antifungal activity, and this molecule was characterized by mass spectrometry as a single molecule with 1,236.405 Da. This peptide has been submitted to "de novo" sequencing, elucidating its primary structure: IIIQYEGHKH, which showed similarity with a hemocyanin fragment and called rondonin. These studies have made it increasingly clear that due to the continuous use of antibiotics multi-resistant bacterial strains have developed all over the world, and as expected, antibacterial and antifungal peptides have attracted attention in recent years in order to find new therapeutic agents. **Objectives:** The objective of this study was to determine if rondonin is widespread among the genera of the family Theraphosidae. **Methods:** The hemolymph was collected from *A.gomesiana*, *Vitalius wacketi, Nhandu coloratovilosum*, *Avicularia juruensis* and *Grammostola pulcra*, by cardiac puncture with an apyrogenic syringe. The hemocytes were removed from plasma by centrifugation at 800 x g for 10 min at 4ºC. The plasma collected was mixed with acidified water (TFA – 0.05% trifluoracetic acid) and kept in agitation on ice for 30 min and centrifuged at 16,000 x g. The soluble part was loaded onto classic Sep–Pak C18 cartridges. The Sep-Pak fractions were concentrated in a vacuum centrifuge and reconstituted with 0, 5% and 40% TFA and submitted to a reverse phase chromatography on a semi-preparative Jupiter C18 column. Elution was performed with a linear gradient of 2% to 60 % ACN/0.05% TFA over 60 min at a flow rate of 1.5 mL/min. The presence of antibacterial activity was determined by a liquid growth inhibition assay against *Candida albicans* MDM8. These fractions were submitted to mass spectrometry to verify the presence of the same molecular mass. **Results and Discussion:** According to our results, we found the same peptide only in *A. gomesiana* and *N. coloratovilosum*; it showed the same molecular mass as that of rondonin and the same activity against *C. albicans*. It is possible because they belong to the same subfamily Theraphosinae. Maybe rondonin is a specific character of the subfamily Theraphosinae since it is not present in *Avicularia* which belongs to the subfamily Aviculariinae. We need to analyze the results for *Grammostola* which now belongs to the subfamily Theraphosinae but that has already been placed in a subfamily, Grammostolinae.

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2.25 Collagen-induced arthritis and bee venom treatment affect aminopeptidases in synovia, synovium and plasma of rats

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Introduction: Rheumatoid arthritis affects about 1% of the population worldwide. It is characterized by cellular infiltration in the synovium, progressive erosion of cartilage and bone and pannus formation in the affected joints, as well the immune response to cartilage components and the presence of rheumatoid factors. The destruction of cartilage, inflammatory mechanisms and antigenic processing are related to aminopeptidases, such as neutral (APN), basic (APB) and dipeptidyl-peptidase IV. Moreover, bee venom therapy has been used in oriental medicine to treat inflammatory diseases, such as rheumatoid arthritis, due to its property to decrease pain and inflammation. **Objectives:** To analyze: (1) the involvement of basic (APB), neutral (APN) and dipeptidyl IV (DPPIV) aminopeptidases (APs) in the rat synovia, synovium and plasma in collagen-induced arthritis (CIA); and (2) if changes in the activity levels of these APs could be mitigated after treatment with *Apis mellifera* venom (BV). **Methods:** Male Wistar rats (150-160g) received a single dose of type-II collagen, id, into the base of the tail. After 21 days, animals were treated with 0.25 mg BV/ kg body wt, sc, in the back, every other day. On the 41st day after immunization, plasma and synovial fluid and synovial membrane were collected from the groups: control (A1), A1 treated with BV (A2), arthritic (B1), B1 treated with BV (B2), and injected with collagen which did not develop arthritis (B1R). The synovium was homogenized and ultracentrifuged in order to obtain soluble (SF) and solubilized membrane-bound (MF) fractions. APs were measured fluorometrically and expressed as pmoles of hydrolyzed substrate/min/mg protein [mean±SEM (n)]. **Results and Discussion:** DPPIV in the synovial fluid was increased by BV and/or CIA, since A2 [200±25 (6)], B2 [329±48 (3)] and B1R [271±27 (3)] showed higher DPPIV activity, while B1 [215±25 (3)] showed a tendency for an increase, compared to A1 [103±29 (4)]. No alteration of any activities was found in synovium MF, but in SF APB was higher in B1R [3698±896 (3)] than in other groups [A1=1421±403 (4); A2=928±172 (6); B1=1902±903 (3); B2=1183±455 (3)], while DPPIV was higher in B1 [1086±339 (3)] than in B1R [296±60 (4)]. APN in plasma was higher in B1 [506±13 (3)] than in B2 [386±9 (3)]. Alteration of the AP activities examined and their possible functions during arthritis may depend on their localization. In SF, DPPIV could be causal, while APB might be involved in the mechanisms of resistance. BV decreased plasma APN activity in CIA, but it is still not clear what benefits this effect could have in this disease.

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