SOME HYDROLASE ACTIVITIES FROM THE TICK *HYALOMMA LUSITANICUM* KOCH, 1844 (IXODOIDEA: IXODIDA)

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Summary:

In this work has been made a detection and preliminary characterization of some hydrolases in whole extracts from unfed adult males and females of *Hyalomma lusitanicum*, one of the vectors for *Theileria annulata* that causes Mediterranean theileriosis in cattle. We have elected as targets, proteases as enzymes implicated in the nutritional processes of ticks, esterases that are usually implicated in resistance to organophosphates and phosphatises often implicated in protein phosphorilation and control of ticks salivary gland. The biological role and physiological significance are discussed in terms of the possibility of use these enzymes as possible in future anti-tick vaccination or acaricide resistance.

KEY WORDS : *Hyalomma lusitanicum*, ticks, hydrolases, preliminary characterization.

Résumé : SOME HYDROLASE ACTIVITIES FROM THE TICK *HYALOMMA LUSITANICUM* KOCH, 1844 (IXODOIDEA: IXODIDA)

Ce travail a porté sur la détection et la caractérisation préliminaire de diverses hydrolases extraites de mâles et de femelles adultes et non gorgées de Hyalomma lusitanicum, *l'un des vecteurs de* Theileria annulata, *agent responsable de la theilériose méditerranéenne des bovins. Nous avons ciblé les recherches sur les protéases (retrouvées dans le processus de digestion), les estérases (souvent impliquées dans la résistance aux organophosphorés) et les phosphatases (phosphorilation des protéines et contrôle de l'activité des glandes salivaires) des tiques. Le rôle biologique et l'importance physiologique de ces enzymes sont discutés en termes de possibilité d'utilisation dans la vaccination et la résistance des tiques aux acaricides.*

MOTS CLÉS : Hyalomma lusitanicum, *tique, hydrolase, caractérisation préliminaire.*

Ticks are a hazard to animal and human health through direct effects as well as through the transmission of human pathogens included viruses, bacteria and protozoon parasites. In this sense, *Hyalomma lusitanicum* is one of the ticks implicated in the transmission of the blood parasite protozoon *Theileria annulata* that causes the Mediterranean theileriosis in cattle, which produces economic losses of considerable importance (Viseras & García-Fernández, 1999; Habela *et al.*, 1999).

There are few studies that describe the properties of hydrolytic enzymes as proteases, phosphatases and esterases in ixodid ticks, though they are basic for important physiological processes. Acetylcholinesterase (AChE) catalyses the hydrolysis of the neurotransmitter, acetylcholine and in arthropods, AChE is a target of pesticides. Detoxifying enzymes and esterases in particular, have been associated with organophosphate resistance in *Boophilus microplus* and Villarino *et al.* (2002, 2003) and Li *et al.* (2005) claimed that resistance to carbaryl was likely conferred by insensitive acetylcholinesterase. Proteases have prominent role in the proteolytic cascade for the degradation of the host blood-meal (Mendiola *et al.*, 1996, 2001; Boldbaatar *et al.*, 2006), and acid phosphatases are also involved in digestion (Coons *et al.*, 1986; Gough & Kemp, 1995). Acid phosphatase participates in salivary gland secretion control (Numes *et al.*, 2006) and two protein phosphatases appear to play a principal role in modulating this process (Qureshi *et al.*, 1994).

Saliva in feeding ticks is rich in several biochemical components including various enzymes (Sauer *et al.*, 1995). Immunogenic and pathogenic proteins enter in the mammalian host during feeding via the tick salivary gland (Kaufman, 1989). Currently, tick control depends almost entirely on the use of acaricides. This approach is, however, associated with a number of disadvantages such as development of resistance as well as chemical pollution. Among alternative measures, host vaccination against ticks seems the most promising, so there is a clear need for better awareness of these enzymes. In this work we contribute to the knowledge of the basic physiological significance of these hydrolases in the tick *Hyalomma lusitanicum*.

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MATERIAL, METHODS AND RESULTS

465 unfed adult ticks (males and females) were collected from vegetation in spring (May-June) and each one was examined and classified by binocular lens (Koch, 1844). Males (190) were separated from females (275) and processed individually. Specimens were immersed in 70° alcohol for 10 min and rehydrated for 30 seconds in Mili-Q water and dried in a filter paper. Each ixodid was cut and introduced with PBS-saline (10 mM PBS pH 7.2, 146 mM NaCl) in a Potter-Elvejem on an ice bath and homogenized. Extracts were separated in eppendorf tubes and centrifuged for 1 min at 500 rpm to sedimentate cuticle fragments and tissue rests. Supernatants were collected and centrifuged in new eppendorffs at 14,000 rpm for 5 min, filtered in a 0.22 µm filter (Millipore). Protein concentration was determined by the method of Bradford (Bradford, 1976). The final protein concentration of whole-body homogenates were 1mg/ml for females and 0.635 mg/ml for males.

Proteolytic activity

Proteolytic activity was determined by spectrophotometric and electrophoretic methods in both separated samples from males and females. Gel electrophoresis using 10 % SDS-PAGE minigels copolymerized with 0.15 % gelatine as general substrate was employed. Samples were diluted 1:1 in glycerol (30 %) and separated electrophoretically at 90 V. The gels were then washed in 2.5 % Triton X-100 for 1h to remove SDS, incubated in 0.1 M citrate buffer (pH 3 and 5), or 0.1 M PBS (pH 7) or 0.1 M Glycine-NaOH buffer (pH 9), in the presence or absence of 2 mM Ca Cl_2 for 24 h at 22° C and 37° C. Gels were stained with Coomasie Brilliant Blue and finally proteases are detected as clear bands in the blue gel. Samples were also assayed for proteolytic activity in eppendorff tubes using 5 mg/ml azoalbumin as general substrate with the same buffers. Incubations were realized for 24 h at 22° C and 37° C, and after incubation, proteins were precipitated with 6 % TCA and tubes were centrifuged at 1,000 g. The absorbance of the supernatant was read at 420 nm. All assays were carried out in parallel with their respective controls. Throughout the literature, there are numerous studies that describe protease activity in the midgut and salivary glands of ixodid ticks. Recently, an asparaginyl endopeptidase (Abdul Alim *et al.*, 2007), a serine protease (Miyoshi *et al.*, 2007) and an aspartic protease (Boldbaatar *et al.*, 2006) have been characterized from the tick *Haemaphysalis longicornis*. These and other studies suggest that proteases play an important role in digestion of blood components. By using electrophoresis in polyacrilamide gel copolymerized with gelatine, Mulenga *et al.* (1999) have detected cysteine proteases in midguts of both fed and unfed *H. longicornis* and Hernández-Alvarez *et al.* (2000) have detected neutral protease activity in the intestine of *B. microplus*. We have not found proteolytic activity in unfed *Hyalomma lusitanicum* male and females, under the same experimental conditions. Perhaps, this enzymatic activity in whole-body homogenates was too small to be detected; it is also possible that some enzymes are little or nothing expressed in unfed ticks. Multiple proteolytic activities have been detected at acidic pH in midguts of adult engorged females of *Boophilus microplus* (Mendiola *et al.*, 1996) and a tendency to increased values of aspartic-proteinase activity per milligram of total weight was observed during the development of adult female ticks (Mendiola *et al.*, 2001). Other studies indicate that expression of some peptidases is induced or upregulated by a blood-feeding process (Mulenga *et al.*, 2003; Hatta *et al.*, 2006; Motobu *et al.*, 2007).

Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was measured with a modification of the Ellman assay (Ellman *et al.*, 1961). A microplate method was developed, using acetylthiocholine iodide (ATCI) as substrate, and samples (males and females) were processed by separate. The increases in OD were converted to units per litre. One unit was the equivalent to 1 nmol of substrate hydrolyzed per minute per mg of protein and results are expressed as the mean ± standard error of five independent determinations. In the inhibition assays, samples (20 µg protein) plus 5 µl of each inhibitor were first incubated at 22° C for 10 min, before measuring the increase in OD. The final concentrations and inhibitors used were: 1×10^{-8} M, 1×10^{-7} M, 1×10^{-6} M, 1×10^{-5} M, 1×10^{-4} M, 1×10^{-3} M of 1,5-bis(4-allyl(dimethyammonium phenyl)pentan 3 one dibromide) (BW2845C51) and tetraisopropyl pyrophosphoramide (iso-OMPA). Acetylcholinesterase activity was demonstrated in both male (9.92 ± 0.02 U) and female (33.24 ± 0.03 U) samples. This activity was 3.3 times higher in females than in males. Results have demonstrated a complete inhibition by BW 284C51, a specific and classical inhibitor of acetylcholinesterase, at all concentrations assayed in females but not in males homogenates (Fig. 1a). By opposite when it was assayed iso-OMPA, a pseudocholinesterase inhibitor, enzymatic activity was completely inhibited at 1×10^{-7} M in males, while in females were necessary concentrations of 1×10^{-3} M to obtain the same inhibition (Fig. 1b). Previous studies demonstrated that AchE activity from synganglion of both *Amblyomma americanus* and *B. microplus* male and female was similar (Pruett & Pound, 2006), but the relationship between brain AChE activity and total cholinesterase activity is unknown. At present we do not have information about cholinesterase distribution in ixodid ticks.

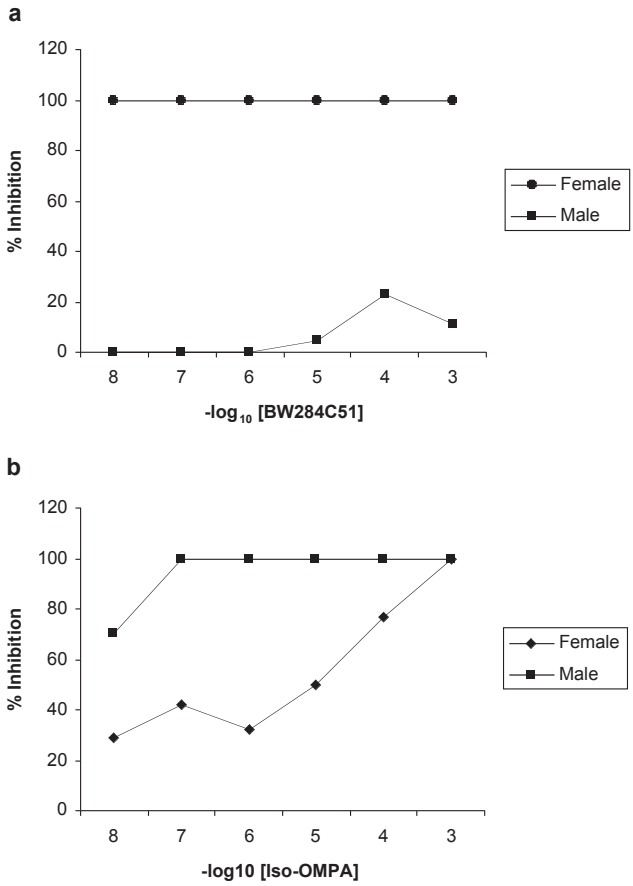

Fig. 1. – 1a. Inhibition of ChE from *Hyalomma lusitanicum* male and female cytosolyc fractions by BW283C52. ChE activity was assayed using ACTI as substrate. All the assays were performed in quintuplicate and standard deviations were very small (< 10 %) and have been omitted. 1b. Inhibition of ChE from *Hyalomma lusitanicum* male and female cytosolyc fractions by iso-OMPA. ChE activity was assayed using ACTI as substrate. All the assays were performed in quintuplicate and standard deviations were very small (< 10 %) and have been omitted.

Phosphatase activity

Quantification of the phosphatase activity was performed by Moulay & Robert-Gero (1995) method using 20 mM p-nitrophenyl phosphate (pNPP) (Sigma, St. Louis, Mo, US) as substrate and the reaction product was measured at 405 nm, using an Ultrospec III Spectrophotometer (Pharmacia, LKB) . Previous assays were realized to verify that the reaction rate was a linear function of assay time and protein concentration in the assay mixture. The pH optimum of AP activity was determined using 50 mM sodium acetate buffer (pH 3.5-6.5) and 50 mM Tris-HCl (pH 7.0-7.5). One unit of specific activity was defined as 1 µmol of p-nitrophenol hydrolyzed per hour per mg of protein under assay conditions (Giménez-Pardo *et al.*, 2003). Phosphatase activity was also assayed in the presence of several phosphatase inhibitors to their optimum pH values. Inhibitors and their final concentrations were: 1 mM of

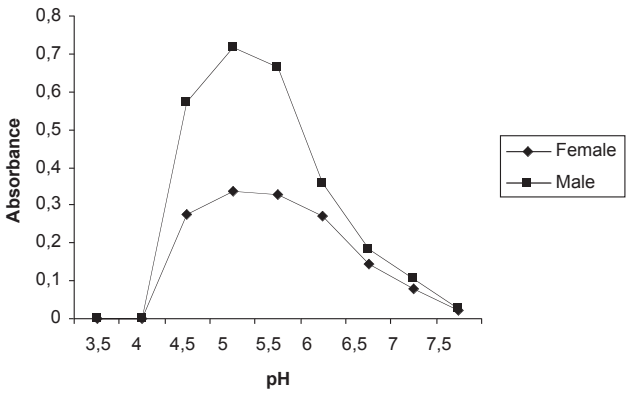

Fig. 2. – Effect of pH on phosphatase activity from cytosolyc fractions of *Hyalomma lusitanicum* males and females. All the assays were performed in quintuplicate and standard deviations were all very small (< 10 %) and have been omitted.

L(+) tartaric acid, 1 mM NaF, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM levamisole, 100 µM sodium orthovanadate, 10 mM Molybdate and the divalent ions at 1 mM: $CuCl_2.H_2O$, $ZnCl_2$, $MgCl_2$, $HgCl_2$, Cl_2Ca and $CoCl_2.6H_2O$. Inhibitors were preincubated with the samples in the reaction buffer at 22° C for 10 min before the addition of the substrate. Phosphatase activity was two fold higher in males than in females, *i.e.* 1.18 ± 0.03 U and 0.53 ± 0.02 U respectively. For both female and male homogenates, the phosphatase activity reached a maximum at pH 5.0 (Fig. 2) and subsequent determinations were conducted at this pH values. It is important to point out that we have found acid phosphatase activity in unfed adult ticks of *H. lusitanicum*. Acid phosphatase activity has been previously detected in tick gut and in the salivary glands of females of *B. microplus*, partially fed or during feeding (Gough & Kemp, 1995, Nunes *et al.*, 2006). Semi-engorged females showed a larger amount of acid phosphatase compared to those at beginning of feeding (Nunes *et al.*, 2006). The effects of several potential inhibitors on phosphatase activity from both males and females extracts are summarized in Figure 3. The enzymatic activity of the females was more sensitive than that of a male to sodium fluoride and tartaric acid, two well recognized acid phosphatase inhibitors, as well as to sodium orthovanadate, a protein phosphatase inhibitor. Surprisingly, the alkaline phosphatase inhibitor levamisole inhibited this enzymatic activity in male and female extracts. Phosphatase activity was also affected by EDTA and divalent cations.

DISCUSSION

Knowledge of these hydrolases about their detection, activity expression at different stages (juvenile and adult stages) and different feeding

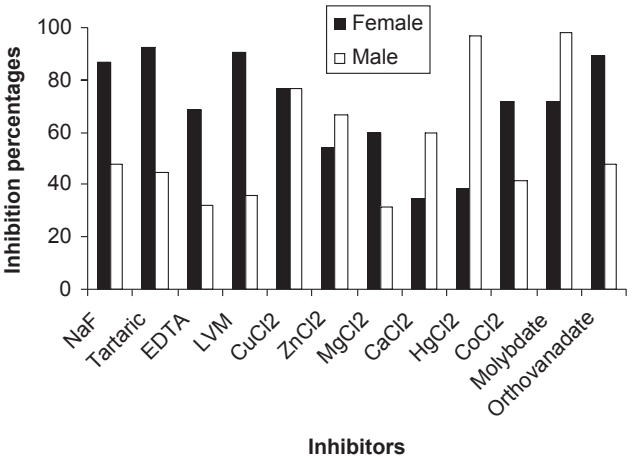

Fig. 3. – The effect of various potencial inhibitors on the acid phosphatase activity of the cytosolyc fractions of adult males and females H. lusitanicum. Assays were performed in quintuplicate and standard deviations were all very small (< 10 %) and have been omitted.

stages (unfed, semi-engorged and fed) and location, could help to clarify some basic biological processes that happens in ixodid ticks. It is not a simple matter to assess the physiological significance of these enzymatic activities and studies like that are essential due to influence that global warming is bound to have on ticks distribution and on ticks role in the transmission of diseases.

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