Serine protease activities in *Oxysarcodexia thornax* (Walker) (Diptera: Sarcophagidae) first instar larva

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We report for the first time the expression of multiple protease activities in the first instar larva (L1) of the flesh fly Oxysarcodexia thornax *(Walker). Zymographic analysis of homogenates from freshly obtained L1 revealed a complex proteolytic profile ranging from 21.5 to 136 kDa. Although some activities were detected at pH 3.5 and 5.5, the optimum pH for most of the proteolytic activities was between pH 7.5 and 9.5. Seven of 10 proteases were completely inactivated by phenyl-methyl sulfonyl-fluoride, suggesting that main proteases expressed by L1 belong to serine proteases class. Complete inactivation of all enzymatic activities was obtained using N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (100 µM), a specific inhibitor of chymotrypsin-like serine proteases.*

Keywords: *Oxysarcodexia thornax* - Sarcophagidae - chymotrypsin-like serine proteases

The genus *Oxysarcodexia* belongs to the Sarcophagidae family (flesh flies) whose major biological feature is the ovo-larvipary (Pape 1996). Females may deposit first instar larvae directly onto the vertebrate host at predisposing sites (causing secondary myiasis), carcasses, decomposing organic matter, and dung (Panu et al. 2000). Genus *Oxysarcodexia* is widely distributed in the Neotropical regions (Lopes 1946), and in Brazil, *O. thornax* (Walker) is found in both urban and rural areas of several states. This species is abundant in the state of Rio de Janeiro, occurring preferentially in the summer season, with the population peak between January and February (Oliveira et al. 2002).

In biological systems, proteases may carry out (i) a regulatory function by activation or inactivation of specific proteins via selective proteolysis, and (ii) a non-specific proteolytic function involved in general protein hydrolysis. Such proteolytic mechanisms are highly regulated and are involved in a variety of physiologic processes, such as programmed cell death, stress responses (heat shock and anoxia), skeletal muscle atrophy, cell-cell recognition, signal transduction and learning, morphogenesis, and photoreceptor light adaptation (Mykles 1998). Different classes of proteases such as cysteine proteases, metalloproteases and serino proteases have been described both in adults and larvae of several species of the Diptera (Han et al. 1997, Rosenfeld & Vanderberg 1998, Cho et al. 1999, Noriega et al. 2002, Vierstraete et al. 2003, Okuda et al. 2005, Fazito do Vale et al. 2007, Pires et al. 2007, Rodrigues et al. 2007).

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Despite the existence of information about morphology, taxonomy and ecology of this genus, as far as we know, no data has been published on the biochemical traits of *O. thornax*. In the present study, the proteolytic profile of *O. thornax* first instar larvae was investigated using zymographic analysis. *O. thornax* adults were collected as previously described (Oliveira et al. 2002) and females were kept in a 50 ml plastic vial for 24 h at room temperature. Following death of the females, abundant live first instar larvae (L1) were released and immediately collected. Larvae were washed twice with PBS pH 7.2, and disrupted in four cycles of freeze and thawing in lysis buffer containing 10% glycerol, 0.6% Triton X-100, 100 mM Tris-HCl pH 6.8 and 150mM NaCl. The resulting extract from 10 L1 was centrifuged at 14000 *g*/ 40 min at 4ºC to remove insoluble material, and proteins were resolved as previously described (Cuervo et al. 2006). Thirty micrograms of protein were fractioned on 12% SDS-PAGE co-polymerized with 0.1% porcine gelatin. After electrophoresis, gels were washed twice for 30 min at 4ºC in 100 mM sodium acetate buffer pH 3.5, 5.5, 7.5 containing 2.5 % Triton X-100 and 100 mM Tris-HCl buffer pH 9.5 plus 2.5% Triton X-100. Protease activities were detected by incubating the gels in the reaction buffer containing 100 mM sodium acetate (at pH 3.5, 5.5, 7.5), or 100 mM Tris-HCl buffer (pH 9.5) at 37ºC for 1, 3, 6 and 18 h. Bands of gelatin degradation were visualized by staining the gels with 0.2% Coomassie blue R-250, 50% trichloroacetic acid, and destaining them with 10% acetic acid. The apparent molecular mass of proteases was calculated by comparison with the mobility of a commercial molecular mass standard. Proteases were further characterized regarding sensitivity to specific inhibitors. Larvae extracts were pre-incubated (before electrophoresis) for 30 min at 37ºC with one of the following protease inhibitors: 10 µM *trans*-epoxysuccinyl L-leucylamido-(4-guanidino) butane (E-64); 1 mM phenyl-methyl sulfonyl-fluoride (PMSF); 100 µM Nα-Tosyl-

L-lysine chloromethyl ketone hydrochloride (TLCK); 100 μM N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK); 10 μM pepstatin-A, and 10 mM 1,10-phenanthroline. Samples were then resolved as described above. Assays were performed in triplicate, with similar results obtained in at least three separate larvae extracts pools.

Time course assays of unfed L1 extracts were performed by incubating the gels in the reaction buffer pH 7.5 for 1, 3, 6 and 18 h (Fig. 1). It was observed that protease activities are time-dependent, i.e., both the intensity and number of proteolytic bands progressively increased over time: after 1 h of reaction, only two bands near to 66 kDa and 136 kDa were visualized; with 3 h of reaction, some bands below 45 kDa were also observed; a complete and well resolved profile of proteolytic activities between 21-136 kDa was revealed after 6 h of reaction; incubation of the gel for 18 h resulted in the loss of resolution of the activity bands, and no additional enzymatic activity was observed. These results indicated that L1 exhibit multiple and highly active proteases, which are able to rapidly hydrolyze the substrate gelatin. Six hours of reaction were then used routinely for all subsequent enzymatic assays.

To better characterize the protease profile of *O. thornax* L1, the proteolytic activities were evaluated both for sensitivity to inhibitors and pH dependence. The effects observed with a set of protease inhibitors are shown in Fig. 2. Inhibition of 7/10 proteolytic bands by PMSF indicated that main proteases expressed by *O. thornax* belong to serine protease class. To evaluate whether such enzymes were trypsin- or chymotrypsin-like serine proteases, specific inhibitors were used. Proteolytic profile was resistant to inhibition by 100 μM TLCK, a known inhibitor of trypsin, while 100 μM TPCK, a specific inhibitor of chymotrypsin, completely suppressed all activity bands. These results show that enzymatic activities detected in L1 are serine proteases of the chymotrypsin type. Similarly, the major proteases detected in larvae of other Diptera species also belong to the serine protease class (Tabouret et al. 2003, Fazito do Vale et al. 2007, Pires et al. 2007). Regarding pH dependence, some serine protease enzymes were active at pH 5.5, while most presented an optimal activity between pH 7.5-9.5 (Fig. 3). One proteolytic band migrating at 136 kDa was active over a wide pH range (Fig. 3). Accordingly, serine proteases activities from several insects present similar alkaline pH dependence (Bowles et al. 1988, Terra & Ferreira 1994, Casu et al. 1996, Tabouret et al. 2003). Detection of enzymes with optimum pH in the alkaline range is not surprising given that most of the proteolytic activities present in the larvae of several fly species correspond to digestive serine proteases, which are predominant in the midgut, a highly alkaline environment (Terra & Ferreira 1994, Casu et al. 1996, Muharsini et al. 2000, Tabouret et al. 2003, Fazito do Vale et al. 2007). Furthermore, proteases with broad pH optima have been described in other insect species (Bown et al. 1998, Noriega et al. 2002).

Very little is known about the occurrence and functions of proteases in flesh fly larvae. As far as we know, biochemical analysis of *O. thornax* proteases has not been reported. In the present study, we successfully applied zymography analysis for the characterization of serine proteases from *O. thornax*. The presence of multiple proteolytic activities in unfed L1 obtained under laboratory conditions suggests that this stage of *O. thornax* constitutively expresses highly active serine proteases. Although the role of serine proteases in digestion processes of most of the insects is unquestionable, it is possible that such enzymes may also exert other roles in the preimaginal stages of *O. thornax*. Such a possibility may be supported by the observation that serine proteases are responsible for tissue remodeling during *Sarcophaga peregrine* metamorphosis (Nakajima et al. 1997). In addition, other protease classes,

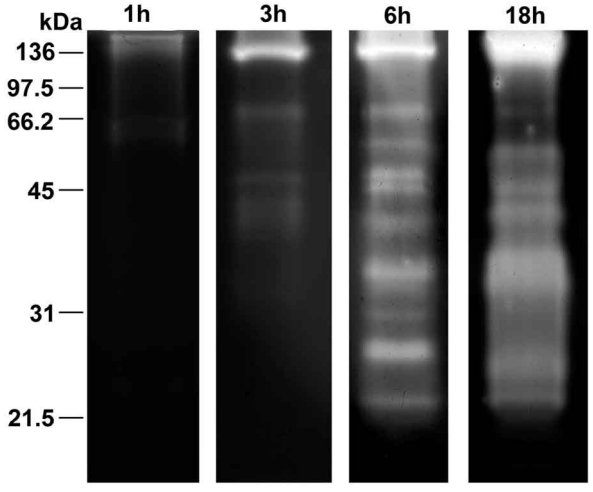

Fig. 1: gelatin-SDS-PAGE showing the time course of proteolytic activities exhibited by *O. thornax* L1 homogenate. Protease activities were detected by incubating gels in reaction buffer pH 7.5 for 1, 3, 6 and 18h. The number on the left indicates the apparent molecular mass of the reactive polypeptide, expressed in kiloDalton (kDa).

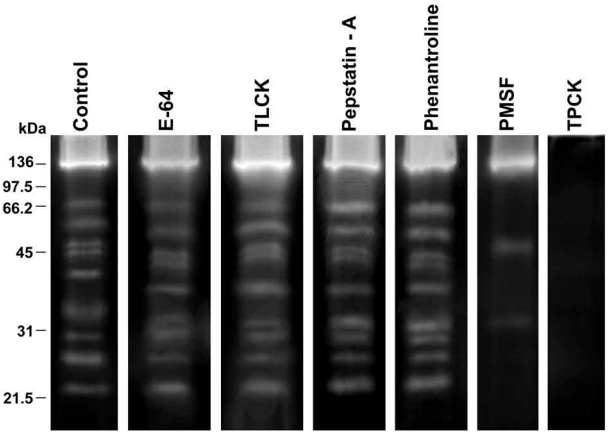

Fig. 2: effect of protease inhibitors on the proteolytic profile of *O. thornax* L1 in gelatin-SDS-PAGE. Proteolytic assays were performed in the absence (control) or presence of each of the following protease inhibitors: 10 μM E-64; 1 mM PMSF; 100 μM TLCK; 100 μM TPCK; 10 μM pepstatin-A (pep-A), and 10 mM 1,10-phenanthroline (1,10-phen). The number on the left indicates the apparent molecular mass of the reactive polypeptide, expressed in kiloDalton (kDa).

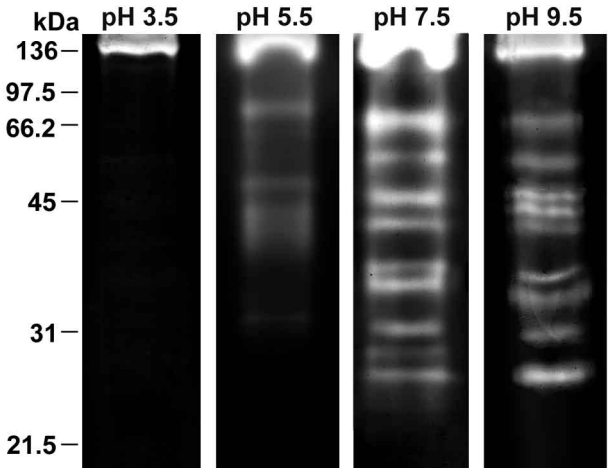

Fig. 3: gelatin-SDS-PAGE showing the influence of pH on the proteolytic pattern of *O. thornax* L1. Enzymatic activities were evaluated by incubation the gels in reaction buffer containing 100 mM sodium acetate at pH 3.5, 5.5, 7.5, and 100 mM Tris-HCl buffer at pH 9.5. The number on the left indicates the apparent molecular mass of the reactive polypeptide, expressed in kiloDalton (kDa).

such as cathepsin B and cathepsin L-like cysteine proteases, have been described to participate in the developmental cycle of *S. peregrine* (Yano et al. 1995, Philip et al. 2007). The identification and biochemical characterization of chymotrypsin-like serine protease in *O. thornax* larva may represent the first steps to a better understanding of the roles played by these enzymes in the physiology of this species.

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