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Research brief

Trypanosoma cruzi: Effects of infection on cathepsin D activity in the midgut of *Rhodnius prolixus*

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Abstract

Cathepsin D activity was estimated in midgut homogenates from *Rhodnius prolixus*, uninfected and experimentally infected with *Trypanosoma cruzi*, at different times after blood ingestion. No enzyme activity was found in the anterior midgut and rectum. In the posterior midgut, enzyme activity was found both in lumen and wall. In starved uninfected insects, in lumen and wall, cathepsin D activity was high, decreasing to a constant rate at 1–15 days after feeding. In insects infected with *T. cruzi* cathepsin D activity increased 1 and 3 days after blood meal. We suggest that these changes in cathepsin D activity in *R. prolixus* posterior midgut are due to the establishment of *T. cruzi* infection.

Index Descriptors and Abbreviations: *R. prolixus*, *Rhodnius prolixus*; Triatominae; *T. cruzi*, *Trypanosoma cruzi*; Trypanosomatidae; Cathepsin D (EC 3.4.23.5); LIT, liver infusion tryptose; PBS, phosphate buffered saline; TCA, trichloroacetic acid; BCA, bicinchoninic acid

Chagas disease is one of the most important endemic diseases that occurs in Latin America (Dias, 2000). The etiologic agent of this disease is the flagellate *Trypanosoma cruzi* (Chagas, 1909), and its transmission is related to the blood feeding insect vectors, the triatomines. Most blood sucking Diptera use alkaline proteases (as trypsin and chymotrypsin) to digest blood meal proteins. However, the triatomines produce lysosomal acidic proteases, such as cathepsin enzymes, to digest their diet, as well as lysosomal carboxypeptidase B and aminopeptidase (Garcia and Garcia, 1977; Houseman, 1978; Houseman and Downe, 1980, 1981, 1982). The production of acidic digestive enzymes by this group of insects is probably related to their evolutionary process, that apparently has sap-sucking insects as a common ancestor (Lehane, 1991; Terra et al., 1988).

Previous studies performed by Houseman and Downe (1980, 1981, 1982, 1983) demonstrated that cathepsin B and D are the main proteinases in the *Rhodnius prolixus* midgut. Apparently, the production of these enzymes is controlled by a secretagogue mechanism stimulated by the protein content in the posterior midgut after the blood meal (Garcia and Garcia, 1977). Terra et al. (1988) showed that the major *R. prolixus* proteinase is cathepsin B, with 85% of hydrolytic activity, and cathepsin D a minor one, representing 15% of this activity. Recently, nucleotide sequences of cathepsin L were characterized in *R. prolixus* (Lopez-Ordoñez et al., 2001), and cathepsin B and L in *Triatoma infestans* (Kollien et al., 2004).

Garcia and Azambuja (1991) and Garcia et al. (1995) suggested that *T. cruzi* could interact with the insect midgut digestive enzymes and digestion products, possibly modulating the parasite metabolism and its infectivity. However, no studies on the dynamics of digestive proteases activities in *T. cruzi* infected triatomines have been reported. Thus, the aim of the present work was to investigate if cathepsin D dynamic activity was affected by the presence of the parasite in *R. prolixus* midgut.

The *R. prolixus* used in these experiments originated from a colony maintained under controlled conditions of

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temperature (27 °C ± 2) and humidity (65% ± 5), fed on rabbit blood through an artificial feeding system (Garcia et al., 1989) and the *T. cruzi* clone Dm28c (supplied by Dr. M.A. Souza, Fiocruz, Brazil) was grown in LIT culture medium (Liver Infusion Tryptose, Difco). For the experiments, fifth-instar larvae were randomly chosen after molting, starved for 20 days, and then fed on citrated human blood, using a membrane feeding apparatus (Garcia et al., 1989). Two groups of insects were used: (1) fed only on citrated human inactivated blood supplemented with LIT culture medium and (2) fed on citrated inactivated human blood containing 4×10^6 culture epimastigotes of *T. cruzi*/ml produced by routine serial passages in LIT medium, as described by Garcia and Azambuja (1997).

At different times after infection, the midgut compartments (anterior midgut, posterior midgut, and rectum) of six insects were separately dissected, pooled, and gently ground in phosphate-buffered saline (PBS, pH 7.2), using a small homogenator. Additional PBS was added to final homogenate volumes to 200 µl for the anterior midgut, 200 µl for posterior midgut homogenates, and 50 µl for rectal material. The total number of parasites in each homogenate was determined using a Neubauer hemocytometer. After ingestion of about 1×10^6 parasites/insect, approximately 0.8×10^5 parasites/anterior midgut developed within the initial 5 days, decreasing to 2×10^3 parasites within the following 5 days. No parasites were observed in the anterior midgut of insects after this period. The posterior midgut was colonized by about 3×10^5 parasites at 5 days after infection, remaining at a high level during the following 10 days. In the rectum, the trends of development were very similar to those of the populations in the posterior midgut. At 5 days after infection, the parasite population density was 1×10^4 and maintained about 1×10^5 parasites/insect until day 15 (Fig. 1). The general pattern of infection by the *T. cruzi* Dm28c clone in different gut compartments of *R. prolixus* was similar to that reported previously (Cortez et al., 2002).

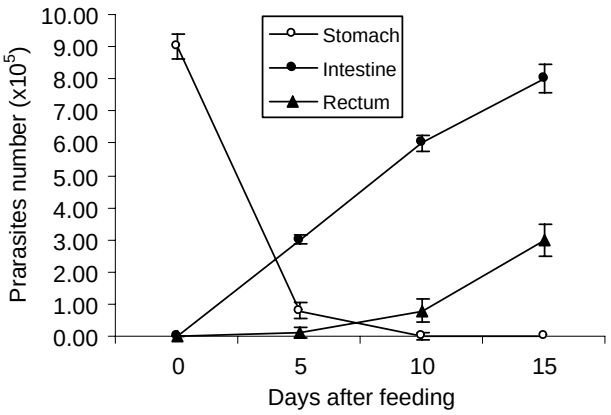

Fig. 1. The course of the infection of *Trypanosoma cruzi* Dm28c clone in the anterior midgut, posterior midgut, and rectum of fifth-instar larvae of *Rhodnius prolixus*. White circles, anterior midgut; black circles, posterior midgut; and black triangle, rectum. Each point represents the mean ± SD of parasites in the gut compartments from six insects per day.

Six insects from the uninfected group and six from *T. cruzi* infected group were dissected before and at 1, 3, 6, 9, and 15 days after feeding/infection. The digestive tract was dissected in a Petri dish after being freed from the Malpighian tubules, rinsed with cold saline and divided into anterior midgut, posterior midgut, and rectum. After disruption of the anterior midgut and rectum walls to collect contents, 100 µl of cold saline was added to each sample, while the contents of posterior midguts were recovered as a fluid leaking from the organ each time it was flushed with 100 µl saline and centrifuged at 5000*g* for 5 min at 4 °C to wash the tissue walls (four times). The supernatants were removed on each centrifugation and correspond to the posterior midgut content (lumen) extracts in a final volume of 100 µl saline. The tissues walls (anterior and posterior midguts and rectum) were homogenized with a pellet pestle and centrifuged for 10 min at 10,000*g* at 4 °C. The supernatants were suspended in a final volume of 100 µl saline and stored at −20 °C until use. To each sample of 10 µl preparation from both experimental groups, 150 µl of bovine hemoglobin (10 mg/ml Sigma) was added and 0.1 M glycine–HCl buffer, pH 2.8, was used to complete a final volume of 620 µl. Three hundred and ten microliters of this mixture was mixed immediately with 300 µl of 20% of trichloroacetic acid (TCA) to measure the time zero of the reaction. After incubation for 4 h at 30 °C the reaction in the remaining mixture (310 µl) was stopped by adding 300 µl of 20% of TCA and the enzyme activity was measured by reading TCA soluble product absorbance at 280 nm, as described by Houseman and Downe (1982). To confirm the specificity of the reaction, the hemoglobin hydrolysis at pH 2.8 was measured in the presence of pepstatin A (Sigma), an aspartic protease inhibitor, in a final concentration of 20 µg in the reaction mixture. For each sample, total protein content was determined by the BCA method (bicinchoninic acid, BCA Protein Assay Reagent, Pierce) using a bovine serum albumin standard. The enzyme units were expressed as absorbance × 100/µg protein.

Cathepsin D activity was present only in the posterior midgut, and not in the anterior midgut and rectum (data not shown). In the present investigation, we showed that in starved insects the highest level of activity was present at day 0, decreasing 1 day after feeding and remaining stable until the 15th day in lumen and wall of uninfected insects (Fig. 2). Cathepsin D activity levels in triatominae midgut was firstly demonstrated by Houseman and Downe (1983), who showed that the protease reached its highest activity 6 days after feeding, returning to levels similar to those prior feeding on the 10th day and to low levels 20 days after the blood meal. However, these authors used total posterior midgut from adults *R. prolixus*, which probably explains the differences found. Terra et al. (1988) evaluated the properties of *R. prolixus* fifth-instar nymph midgut proteases, 7 days after the blood meal. In the anterior midgut they found glycosidases (produced by the insect) and amylases (produced by symbiont bacteria). In the posterior midgut they observed two main proteases activities: a major one,