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Cathepsin L of *Triatoma brasiliensis* (Reduviidae, Triatominae): Sequence characterization, expression pattern and zymography

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ABSTRACT

Triatoma brasiliensis is considered one of the main vectors of Chagas disease commonly found in semi-arid areas of northeastern Brazil. These insects use proteases, such as carboxypeptidase B, aminopeptidases and different cathepsins for blood digestion. In the present study, two genes encoding cathepsin L from the midgut of *T. brasiliensis* were identified and characterized. Mature *T. brasiliensis* cathepsin L-like proteinases (TBCATL-1, TBCATL-2) showed a high level of identity to the cathepsin L-like proteinases of other insects, with highest similarity to *Rhodnius prolixus*. Both cathepsin L transcripts were highly abundant in the posterior midgut region, the main region of the blood digestion. Determination of the pH in the whole intestine of unfed *T. brasiliensis* revealed alkaline conditions in the anterior midgut region (stomach) and acidic conditions in the posterior midgut region (small intestine). Gelatine in-gel zymography showed the activity of at least four distinct proteinases in the small intestine and the cysteine proteinase inhibitors transepoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and cathepsin B inhibitor and *N*-(L-3-*trans*-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) were employed to characterize enzymatic activity. E-64 fully inhibited cysteine proteinase activity, whereas in the samples treated with CA-074 residual proteinase activity was detectable. Thus, proteolytic activity could at least partially be ascribed to cathepsin L. Western blot analysis using specific anti cathepsin L antibodies confirmed the presence of cathepsin L in the lumen of the small intestine of the insects.

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1. Introduction

The heteroxenous flagellate *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) is the causative agent of American Trypanosomiasis, a disease with a strong socioeconomic impact in Latin America (Chagas, 1909; Dias, 2006; Garcia et al., 2007). This tropical parasitic infection is highly abundant in South and Central America, where 5–10 million people are infected and approximately 25 million people are living in risk areas (WHO, 2002, 2010; Garcia et al., 2007). Chagas disease is usually transmitted by the feces of triatomines, which contains metacyclic *T. cruzi* form, but transplantation of organs, blood transfusion and oral infection are alternative transmission routes (Beard et al., 2001; CDC, 2002, 2006; Dias, 2006; Coura and Borges-Pereira, 2010).

Though *Triatoma infestans*, formerly the major *T. cruzi* vector, has been eradicated from Brazil, in the northeastern semi-arid areas of the country *Triatoma brasiliensis* has became one of the main Chagas disease vectors. This triatomine is regularly infected with *T. cruzi* and widely distributed, occurring in six Brazilian states (Guarneri et al., 2000; Costa et al., 2002, 2003; Vitta et al., 2007). *T. brasiliensis* is a native species able to colonize different ecotopes such as households, sylvatic and peridomicilar environments and re-colonizes areas previously controlled by insecticides (Costa et al., 2002, 2003). The potential of these insects to be naturally infected by *T. cruzi* and its large distribution shows the importance for the transmission of the disease in some localities of Brazil.

After infecting the vector, *T. cruzi* must interact with the hostile environment of the insects' digestive tract, in which enzymes and digestion products are some of the factors that might modulate the parasite distribution and its development to infective metacyclic forms (Garcia et al., 1995, 2007, 2011; Azambuja et al., 2005; Araújo et al., 2007, 2008). In order to understand the survival of *T. cruzi* in the hostile environment of the midgut, an investigation of the enzymes involved in the digestion process of the vector, such as cysteine proteinases (EC 3.4.22), hydrolases with a cysteine residue in their active site, is indicated. Cysteine proteinases of triatomines, cathepsin B and L (Tryselius and Hultmark, 1997; Matsumoto et al., 1997; Kuipers and Jongsma, 2004) belong to the papain superfamily and the group of C1 peptidases (Rawlings and Barrett, 1993; Johnson and Jiang, 2005).

Primarily these enzymes are lysosomal peptidases, in mammals generally endopeptidases, though cathepsins C and X are exopeptidases (Turk et al., 2001). Furthermore, cathepsins are

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involved in several pathological processes, such as osteoporosis, neurological disorders, prohormone processing, auto-immune diseases and they also play an important role in apoptosis (Chapman et al., 1997; Tepel et al., 2000; Leist and Jäättelä, 2001; Cimerman et al., 2001; Hou et al., 2002; Brömme et al., 2004). Insect cathepsins are homologous to mammalian cathepsins and the majority of these cysteine proteinases is present in lysosomes, but can also be found in extracellular spaces. Besides their participation in the digestion process (Matsumoto et al., 1997), cathepsins are also involved in intracellular protein degradation, embryogenesis and metamorphosis of insects (Yamamoto and Takahashi, 1993; Shiba et al., 2001; Uchida et al., 2001; Liu et al., 2006).

Triatomine digestion has been studied for many years and several proteinases have been identified and characterized by their specific enzymatic activity (Houseman, 1978; Houseman and Downe, 1980, 1981, 1982; Billingsley and Downe, 1985; Borges et al., 2006). More recent studies have demonstrated the presence of genes encoding cathepsin B and cathepsin B and L in the midgut of *Rhodnius prolixus* and *Triatoma infestans*, respectively (Lopez-Ordoñez et al., 2001; Kollien et al., 2004). Apparently cathepsin L-like enzymes are the main cysteine proteinases, a crucial factor in Hemiptera digestion (Terra and Ferreira, 2005). But there is still a gap between the biochemical and molecular biological findings. Because the digestive tract of triatomines is an interface between the insect and its environment, it is essential to understand its physiology as well as the interaction with *T. cruzi* at all levels. In the present study we report the identification of two novel genes encoding cathepsin L in the midgut of *T. brasiliensis* (*tbcatL-1* and *tbcatL-2*). In addition to the reported cDNA sequences, the expression patterns in different regions of the *T. brasiliensis* digestive tract were analyzed. Finally, we supplemented the molecular biology results with cathepsin in-gel activity assays and immunoblotting experiments.

2. Material and methods

2.1. Reagents

Unless specifically stated, all reagents were obtained from Sigma–Aldrich, St. Louis, MS, USA.

2.2. Insect origin, maintenance and feeding

Adults and fifth instar nymphs of *T. brasiliensis* maintained at 26 ± 1 °C and 60–70% relative humidity, were kindly provided by Prof. Dr. Jurberg (Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos, FIOCRUZ, Rio de Janeiro, Brazil). The insects were reared in plastic beakers, covered with smooth gauze and fed on rabbit blood through latex membranes 2 weeks after molting (Garcia et al., 1989; Mello et al., 1996). Only fully engorged insects were used for further experiments.

2.3. Tissue preparation

For sequence identification and RT-PCR, the salivary glands, anterior midgut (stomach), posterior midgut (small intestine) and fat body of always ten unfed fifth instar nymphs, fifth instar nymphs at 3, 5, 10, and 15 days after feeding (daf) and the same tissues from adult insects at 5 daf including the gonads were dissected. The respective tissues were frozen, pooled in liquid nitrogen and stored at −80 °C.

2.4. Determination of intestinal pH

The pH-values of the whole midgut and rectum of unfed fifth instar nymphs were estimated using a universal indicator solution (Merck, Darmstadt, Germany). Guts were entirely submerged in indicator solution and the resulting coloration of the tissue was compared with the supplied color card.

2.5. RNA isolation, reverse transcription and amplification of first cathepsin encoding sequence

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturers' protocols. Nucleic acid concentrations were measured by a Bio Photometer (Eppendorf, Hamburg, Germany). Reverse transcription was carried out as described previously (Araújo et al., 2006). Degenerate cathepsin forward and reverse primers, Cat-Deg-F 5′-TGYGGNWSNT-GYTGGGCNTT-3′ and Cat-Def-R 5′-CCCCANSWRTTYTTNAYDATCC A-3′, were designed according to the highly conserved cathepsin L regions, CGSCWSF and WLVKNSWG, respectively (Fig. 2). For the first strand amplification, cDNA from the small intestine at 5 daf was used. The cycling parameters in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) were carried out as described previously and differed only in the annealing temperatures of 51.5 °C (Araújo et al., 2006). Gene amplification products of the predicted size, approximately 500 bp, were cloned into pGEM T-Easy vector (Promega, Madison, WI, USA), following the manufactures' instructions and sequenced at least twice from both directions (Plataforma Genômica – Sequenciamento de DNA/PDTIS-FIOCRUZ/IOC).

2.6. Rapid amplification of cDNA ends (RACE)

5′- and 3′-RACE procedures were carried out using commercial kits (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA from the small intestine of fifth instar nymphs at 5 daf was used for both methods. For the 5′-ends RACE amplification of the *tbcatL-1* and *tbcatL-2* cDNA, the GSP1 primers Cat1-R 5′-AGCTTTTTCATCTCCT-3′ and Cat2-R 5′-TGATGATTCAG-TATCTA-3′ were used for the first strand synthesis. For the subsequent PCR amplifications, the GSP2 primers Cat3-R 5′-GCTTCATA GGGGTATGATGATTC-3′ and Cat4-R 5′-CTAACATATTGGAACGCTT-TATCC-3′ with a forward abridged anchor primer were used. A second PCR was carried out using the GSP3 primers Cat5-R 5′-GTCCACCTTCACAGCCATTGT-3′ and Cat6-R 5′-CCATATTCCTTGGAG CAGTCCATT-3′ with a nested abridged universal amplification forward primer (Invitrogen). For the amplification of the 3′-ends of the cDNA, the first strand was synthesized using the supplied adapter primer. For the subsequent amplification, the supplied nested abridged universal amplification primer and the Cat1-F 5′-GGTAGACTGCTCCACTAGTTAT-3′ and Cat2-F 5′-AATGGACTGCTCCA AGGAATAT-3′ forward primers were used. The resulting products of 600 and 550 bp, respectively, were cloned and sequenced as described above.

2.7. Sequences and identity analyses

Identity analysis of the cDNA sequences with sequences in GenBank was performed using the blastx utility, version 2.2.12 (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequences were aligned using ClustalW v. 1.83 and slight corrections were made subsequently. Predicted signal peptide cleavage sites were calculated using SignalP v. 3.0 (Bendtsen et al., 2004). Isoelectric points and molecular weights were determined with the Compute pI/MW tool (http://www.expasy.org/tools). Phylogenetic analysis of mature cathepsin L amino acid sequences was carried out by the neighbor-joining (NJ) method with pairwise deletion and amino acid *p*-distance correction using MEGA v. 4.0 (Tamura et al., 2007). As outgroups the cathepsin L amino acid sequences of the crustaceans *Lepeophtheirus salmonis* and *Metapenaeus ensis*

(GenBank accession nos. EF490928 and AY126712) were included into the analysis.

2.8. Reverse transcription polymerase chain reaction (RT-PCR)

To exclude genomic DNA contamination, each RNA sample was incubated with RNase free DNase (Promega) for 30 min at 37 °C. For the following cDNA synthesis always 1.0 μg of total RNA isolated from the respective tissue and the oligo-dT_{18}VN primer were used. To verify that no gDNA remained, the gene encoding *T. brasiliensis* defensin 1 (*def1*), which contains an intron of 107 bp, was initially amplified as an internal control (Araújo et al., 2006; Waniek et al., 2009a). For the subsequent PCR amplification of the target gene fragments the specific primers pairs Cat1-RT-F (5′-GGTAGACTGCTC CACTAGTTAT-3′)/Cat1-RT-R (5′-TTTAGAGTAAAATTGAAATGATCC AT-3′) and Cat2-RT-F (5′-AATGGACTGCTCCAAGGAATAT-3′)/Cat2-RT-R (5′-TTCTGAGTAGAAATGGAATGATTC-3′) at the same conditions as described above but with an annealing temperature of 54 °C and 35 cycles were used. Both amplifications resulted in PCR products of 289 bp. The experiment was optimized to exclude signal saturation and carried out three times under the same conditions using technical replicates. Always 5 μl of the respective amplification product was separated on a 2% agarose gel and documented with an EDAS 290 gel documentation system (Kodak, Rochester, NY, USA). Band intensity was analyzed with use of the ImageJ program (version 1.41). Means and standard deviations of the different samples were calculated. Student's *t*-Test was carried out to evaluate significant differences of means at different days after feeding, between *tbcatL-1* and *tbcatL-2* and in different regions of the intestine. For an internal control and standardization the gene encoding β-actin of *T. brasiliensis* was amplified using specific primers RT-Act-F (5′-AGATCATGTTTGAAACGTTCAACACC-3′) and RT-Act-R (5′-TGGTT GTGAAAGAGTAGCCCCT-3′) at the same amplification conditions as described previously (Waniek et al., 2009a). As negative controls PCR reactions without cDNA were carried out.

2.9. In-gel zymography

From fifth instar nymphs in different nutrition conditions [unfed, 3, 5, 10 and 15 daf], at least 10 small intestines were dissected and pooled in sample buffer [10 μl/gut, 50 mM Tris–HCl (pH 6.8)]. Stomachs of unfed insects were prepared similarly in parallel. For preparation of the midgut content, the guts were slightly pricked, centrifuged for 10 min at 16,000*g* at 4 °C and the supernatant was transferred to a new tube. Equivalent amounts of the prepared protein samples derived from the gut content and homogenized midguts (10 μl), from which the content was removed, were mixed with the same amount of non-denaturing loading dye. The samples were separated on a 15% polyacrylamide gel containing 0.3% gelatine at a constant voltage of 120 V for 2.5 h at 4 °C. After electrophoresis, the proteins were renaturated by incubation of the gels in 2.5% Triton X-100 for 30 min and Milli-Q water (Millipore, Billerica, MA, USA) for 10 min at room temperature. The gels were then incubated in the respective activation buffer for 24 h at 26 °C. Finally, the gels were stained using coomassie blue staining solution and then destained in 30% v/v ethanol, 7.5% v/v acetic acid to reveal bands of clearing which indicate proteolytic activity. Each experiment was carried out in triplicate, using three independent biological samples. The band intensity was quantified as described above. The optimal pH was determined using activation buffers [25 mM citrate, 50 mM disodium-phosphate, 1.0 mM EDTA, 2 mM potassium-phosphate, 5.0 mM dithiothreitol (DTT)] ranging in pH from 3.5 to 6.0. For determination of proteolytic activity, samples were incubated for 30 min at room temperature and at 4 °C with 20 μM cysteine proteinase inhibitor transepoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 2 μM cathepsin B inhibitor *N*-(L-3-*trans*-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) and with the same amount of diluents lacking the inhibitors, prior to electrophoresis.

2.10. Western blot analysis

Western blot analysis of spatial and temporal cathepsin L distribution was carried out as described previously (Waniek et al., 2009b). The small intestine content was obtained as described above. For each lane 100 μg of total protein from the small intestine content of unfed fifth instar nymphs and at different days after the feeding were used. Monoclonal anti-insect cathepsin L (*Helicoverpa armigera*) antibody (R & D Systems, Minneapolis, MN, USA) diluted 1:1000 in TBST was used as primary antibody (Johnson and Jiang, 2005).

3. Results

3.1. Intestinal pH values

After dipping the whole intestinal tracts of unfed *T. brasiliensis* fifth instar nymphs into the pH indicator, the presence of two regions with differing pH-values became visible (Fig. 1). The anterior part of the midgut (stomach) was greenish, indicating a slightly alkaline or neutral pH (~7.0) environment, whereas the posterior part (small intestine) was reddish, indicating an acid milieu (pH ~5.0). The transition between these midgut regions was abrupt (Fig. 1).

3.2. Characteristics of T. brasiliensis cathepsin L (tbcatL-1, tbcatL-2) sequences

After PCR with degenerate oligonucleotides, 5′- and 3′-RACE and alignment of the nucleotide sequences, two 1112 and 1093 bp cathepsin L-like proteinase encoding cDNAs (*tbcatL-1* and *tbcatL-2*) were obtained (NCBI accession nos. EU643472 and JN099751). Both sequences contained open reading frames of 990 bp, encoding 330 amino acid residues (Fig. 2), 61 and 48 bp of putative 5′-non-coding region and 13 and 35 bp of putative 3′-non-coding region between the stop codon (TAA) and the polyadenylation signal (AATAAA), respectively.

The predicted TBCATL-1 and TBCATL-2 precursors had a molecular weight of 36.8 and 37.1 kDa, respectively. Both deduced enzyme precursors contained a putative signal peptide cleavage site (pre-region) between positions 16 and 17 in the amino acid sequence, a pro-region of 97 amino acid residues and a predicted mature protein of 217 amino acid residues, resulting in a theoretical

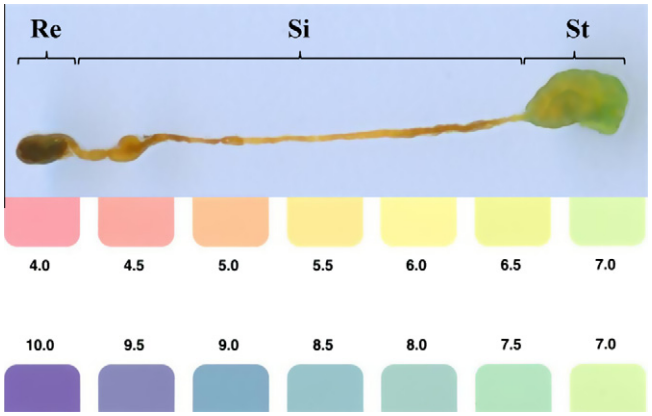

Fig. 1. Physiological pH in the *T. brasiliensis* intestinal tract. After dissection, the gut was submerged in universal indicator solution. Color change was observed under a binocular loupe and compared with the supplied chart.

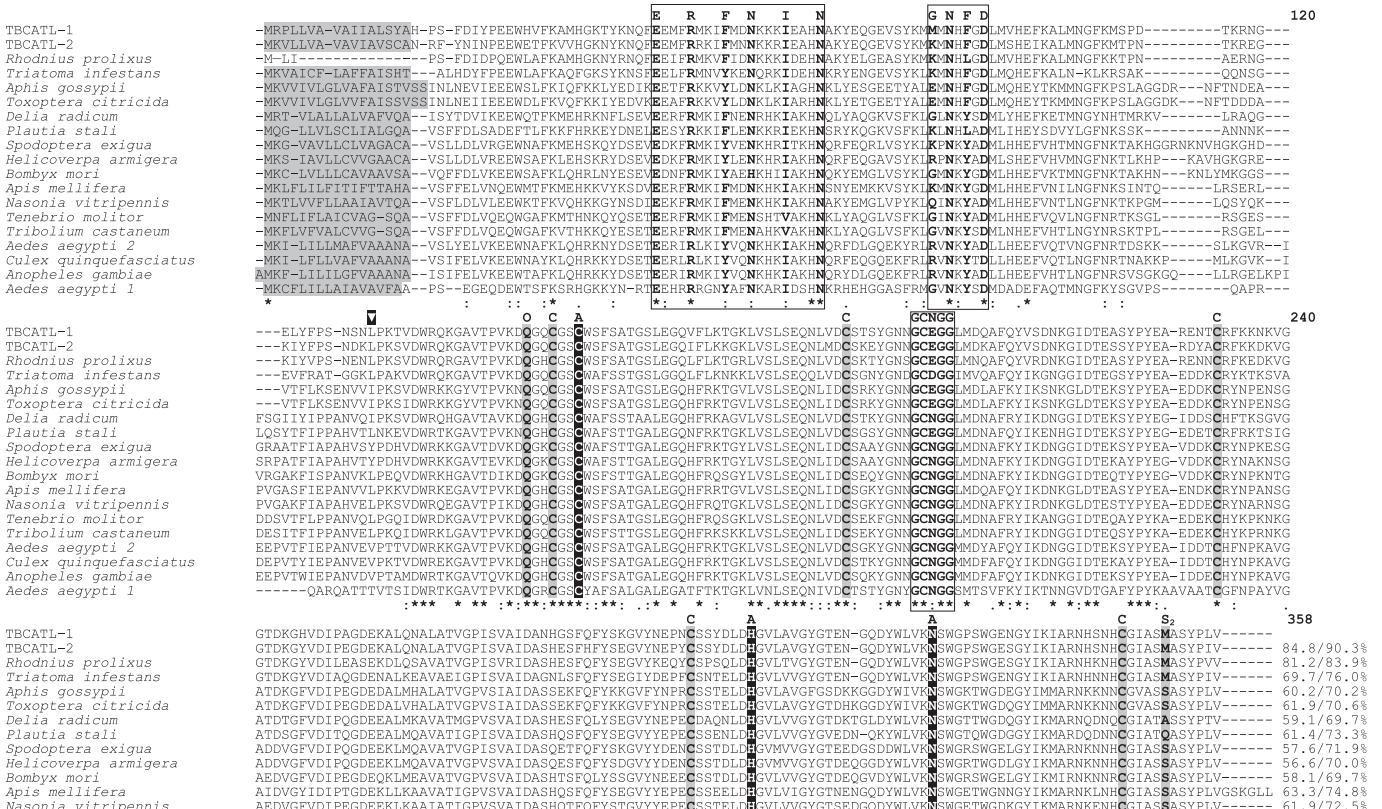

Fig. 2. Comparison of the deduced TBCATL-1 and TBCATL-2 amino acid sequences with various insect cathepsin L-like cysteine proteinases. The amino acid sequences were aligned using the ClustalW (v. 1.83) multiple sequence alignment program. Identical and analogous amino acid residues between TBCATL-1, TBCATL-2 and the other cathepsin L-like cysteine proteinases are indicated beneath the alignment sequences by asterisks and dots, respectively. Putative amino acid residues of the catalytic triad (Cys [C], His [H] and Asn [N]) are black boxed and indicated by A above the alignment; highly conserved regions containing the three cathepsin L-typical consensus sequences are boxed, bold and marked by ERFNIN, GNFD and GCNGG above the sequences. Conserved disulfide bridges forming Cys residues, the oxyanion hole forming Gln (Q) residues and amino acid residues involved in determining enzyme substrate specificity are gray shaded and indicated by C, O and S_2 above the alignment, respectively. After each sequence the percent similarities of the TBCATL-1 precursor (first number) and TBCATL-1 mature enzyme (second number) to those of the other insects are shown. The NCBI GenBank accession nos. for the analyzed sequences are: *Aedes aegypti* 1 and 2 (XP_001661463, XP_001655999), *Anopheles gambiae* (EDO63348), *Aphis gossypii* (CAD33266), *Apis mellifera* (XP_625135), *Bombyx mori* (AAR87763), *Culex quinquefasciatus* (XP_001848344), *Delia radicum* (AAL16954), *Helicoverpa armigera* (AAQ75437), *Nasonia vitripennis* (XP_001602523), *Plautia stali* (BAF94152), *Rhodnius prolixus* (AAL34984), *Spodoptera exigua* (ABK90824), *Tenebrio molitor* (AAO48766), *Tribolium castaneum* (XP_970644), *Toxoptera citricida* (AAU84922) and *Triatoma infestans* (AY36326).

molecular weights of 23.4 and 23.7 kDa, respectively (Fig. 2). The active triad was formed by Cys25, His164 and Asn184 in both mature proteins (Fig. 2). Six cysteine residues forming three disulfide bridges were located at positions 22, 56, 65, 98, 157 and 206 in the mature enzymes. The two motifs, ERFNIN and GNDF, characteristic for cathepsin L-like cysteine proteinases, were found in the preproregion at positions 43–62 and 75–81 of the cathepsin L precursor, respectively. The second motif was modified to MNFD in TBCATL-1and KNFD in TBCATL-2, respectively. The structurally important motif GCNGG was located at position 64–68 in both mature proteins, modified to GCEGG within the amino acid sequence of both mature enzymes (Fig. 2).

Mature TBCATL-1 had an identity of 90.3% to TBCATL-2. When compared with homologous genes available in the GenBank database (blastx using nr database), TBCATL-1 had between 64.7% and 75.7% identity with precursors of cathepsin L like cysteine proteinases from other insects, 76.0% to CatL of *T. infestans* and 83.9% to cathepsin L of *R. prolixus* (Fig. 2).

In the dendrogram of putative mature cathepsin L sequences of different arthropods, both outgroup crustacean cathepsin L amino acid sequences were separated from those of the insects (Fig. 3). All triatomine sequences clustered together in a branch with *Aedes aegypti* cathepsin L 1 and these four taxa were distinctly separated from all other insect cathepsin L mature amino acid sequences with high bootstrap support. *R. prolixus* cathepsin L closely grouped with TBCATL-1 and TBCATL-2 with good bootstrap support, whereas the *T. infestans* cathepsin was more distant to the other three triatomine cathepsin L sequences (Fig. 3).

3.3. Expression patterns of tbcatL-1 and tbcatL-2

Reverse transcription PCR relative to *β-actin* was used to analyze the steady state levels of *tbcatL-1* and *tbcatL-2* in different tissues of unfed fifth instar nymphs and insects at different times after feeding (Fig. 4). In negative controls, lacking cDNA and carried out for each RT-PCR, no amplification products were visible (data not shown). The highest *tbcatL-1* and *tbcatL-2* mRNA abundance was observed in the small intestine (up to 4.6-fold in comparison to stomach at 15 daf) with significant variations of both genes between 3 and 5 daf ($P < 0.01$, 0.05) and *tbcatL-2* between 10 and 15 daf ($P < 0.05$) (Fig. 4A and B). Transcript abundance of *tbcatL-1* and *tbcatL-2* in the stomach was constitutive and generally

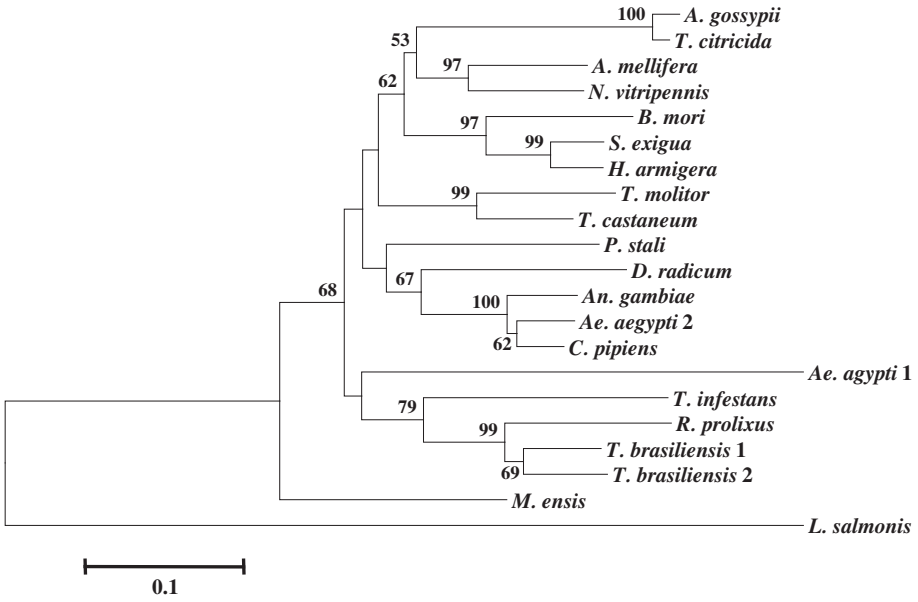

Fig. 3. Neighbor-joining dendrogram of cathepsin L-like cysteine proteinases of different insects. The cathepsin L sequences of the crustaceans *Lepeophtheirus salmonis* and *Metapenaeus ensis* were used as outgroup. Branch labels show the percentages of bootstrap proportions (1000 replicates) supporting that branch.

lower, about half as much in comparison to the small intestine with a significant reduction of the transcript abundance only between 10 and 15 daf of *tbcatL-1* ($P < 0.05$). In the fat body, transcript abundance of both genes increased at 3 daf, remained on a high level at 5 daf and significantly declined at 10 daf ($P < 0.001$, 0.05). In the salivary glands the transcript abundance was elevated at 5 daf and decreased significantly 10 daf ($P < 0.001$). In both fat body and salivary gland tissue, *tbcatL-1* transcripts increased significantly at 15 daf ($P < 0.05$, 0.01) (Fig. 4B). When comparing the transcript abundance of both cathepsin gene isoforms, the only significant difference was evident in the small intestine at 15 daf ($P < 0.05$). When comparing different small intestine sections at

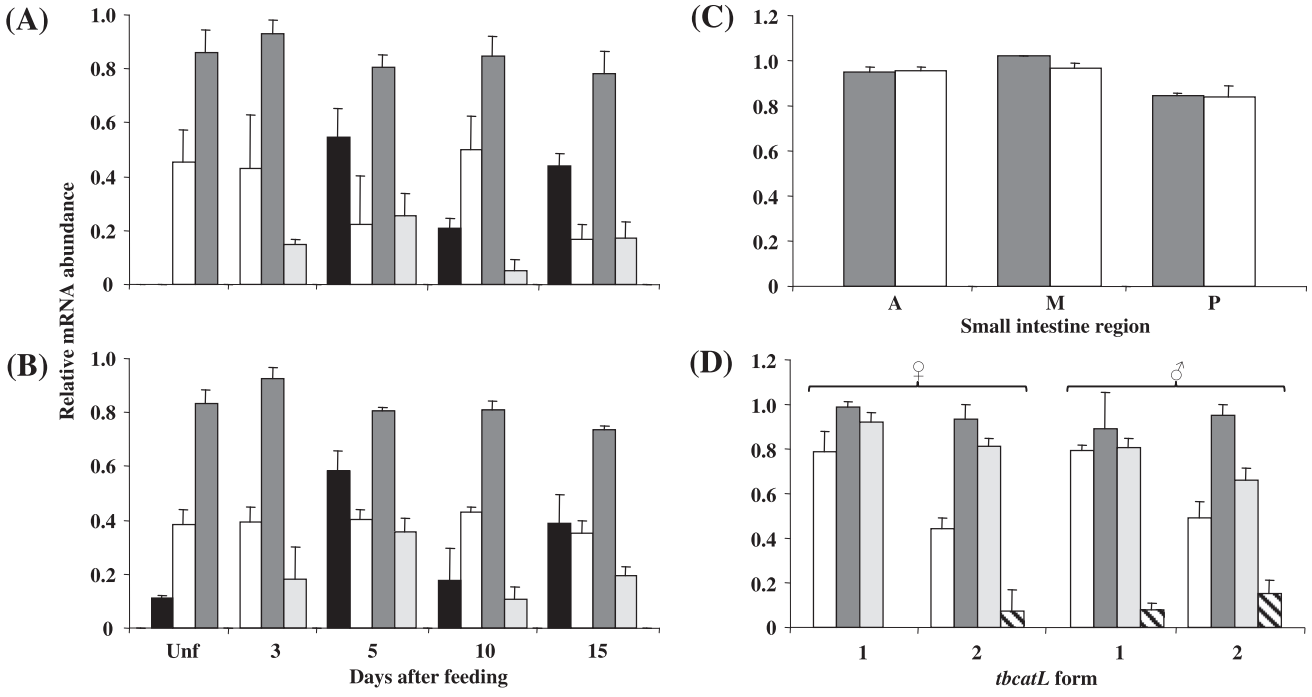

Fig. 4. RT-PCR of *tbcatL-1* (A) and *tbcatL-2* (B) using total RNA from different *T. brasiliensis* tissues of fifth instar nymphs at different times after feeding. The highest transcript abundance of both genes was detected in the small intestine (dark gray column) and lower in the salivary glands (black column), stomach (white column) and fat body (light gray column). (C) *tbcatL-1* (gray column) and *tbcatL-2* (white column) mRNA abundance in the anterior (A), middle (M) and posterior (P) region of the *T. brasiliensis* small intestine at 5 daf. The RT-PCR products were almost identical in the three examined midgut sections. (D) *tbcatL-1* and *tbcatL-2* abundance in different tissues of adult female (♀) and male (♂) insects at 5 daf. The RT-PCR products showed strong signals in the small intestine (dark gray column), fat body (light gray column) and stomach (white column) of both sexes and weak signals of *tbcatL-2* in the female gonads (hatched column) and both cathepsin encoding gene forms in the male gonads (hatched column). RT-PCR product of β-actin (*tbact*) was used as an internal control of each reaction.

5 daf, only slight differences in the transcript abundance of both genes were observed between tissues coming from anterior, middle and posterior region of this midgut section (Fig. 4C).

In adult insects at 5 daf the highest *tbcatL-1* and *tbcatL-2* mRNA concentrations were detected in the small intestine without significant differences between genes and sexes, respectively (Fig. 4D). Slightly lower concentrations were detected for *tbcatL-1* and *tbcatL-2* transcripts in the female and male stomach and fat body (Fig. 4D). The *tbcatL-2* transcript abundances detected in the small intestine tissue of female and male insects, respectively, were always significantly higher in comparison to that of fat body ($P < 0.05$, 0.01). In comparison to other tissues the abundance of both cathepsin L encoding mRNAs in the small intestine was in general significantly higher ($P < 0.05$–0.0001), except when comparing the *tbcatL-1* small intestine concentrations with those of male stomach and fat body of both sexes. Transcript abundances of *tbcatL-1* were significantly higher than *tbcatL-2* in the stomach ($P < 0.01$, 0.05) and fat body ($P < 0.05$). In female fat body both cathepsin L encoding mRNAs were significantly more abundant than in males ($P < 0.05$). *TbcatL-2* transcripts were abundant in the gonads of both sexes whereas *tbcatl-1* was only detectable in the testis, always in a significant lower level than in the other tissues of adult insects (Fig. 4D). When comparing the transcript concentrations in the fat body of fifth instar nymphs at 5 daf with adult insects, in female and male bugs both *tbcatL-1* and *tbcatL-2* were significantly more abundant ($P < 0.0005$, 0.005, 0.001).

3.4. Proteolytic activity of triatomine midguts

To determine the pattern of midgut proteinase activity with respect to pH in fifth instar nymphs of *T. brasiliensis* the wide-ranging proteinase substrate gelatine was used. Gelatinase activity of electrophoretic separated proteins led to a degradation of the gelatine matrix and appeared in colorless, non-stainable areas in the gel. Only fresh midgut content samples showed proteolytic activity, samples stored at −20 °C lost the major part of their activity and could not be visualized by the methodology used in the present study (data not shown). Both, the small intestine content (Fig. 5) and the small intestine tissue samples (data not shown) showed up to four distinct bands of proteolytic degradation, although the activity of the gut content was always more intense. Stomach content of unfed fifth instar nymphs never generated proteolytic activity bands (data not shown).

Content of small intestine at 5 daf produced three broad proteolytic activity bands corresponding to the molecular weights of cysteine proteinases (about 28–35 kDa), showing the maximum intensity at pH 4.5. Therefore further experiments were carried out at this pH value. Also among the other tested conditions proteolytic degradation of gelatine became visible (Fig. 5A). Only at a pH 3.5 and 4.0 an additional band of about 45 kDa was visible in *T. brasiliensis* samples. In small intestine homogenates of *T. infestans* this 45 kDa band remained visible also in all tested pH values in a similar intensity (data not shown). The other activity band detected in the small intestine of *T. infestans* slightly differed in their molecular weight from those of *T. brasiliensis* (Fig. 5B).

Using specific proteinase inhibitors, the analysis revealed that the midgut activity contained cysteine like enzymes in small intestine samples at 5 daf (Fig. 5B). E-64 fully inhibited all proteinase activity bands of *T. brasiliensis* after 30 min incubation at room temperature, while in *T. infestans* a residual activity of the 45 kDa band remained (Fig. 5B). After incubation with the specific cathepsin B inhibitor CA-074, in *T. infestans* 22.9% and in *T. brasiliensis* 72.5% of remaining activity was detected. After incubation with E-64 at 4 °C a residual activity was visible in *T. brasiliensis* small intestine samples, indicating a minor affinity of the inhibitor to the enzyme at low temperatures (data not shown).

Cathepsin activity was detected in unfed insects and at 3, 5 and 10 daf, at 15 daf no activity was observed. Proteolytic activity increased at 3 daf and reached its maximum at 5 daf (Fig. 5C).

3.5. Detection of T. brasiliensis cathepsin L by immunoblotting

To verify the zymography results of intestinal triatomine cathepsins, the midgut content samples were separated by SDS–PAGE and analyzed by immuno blotting using specific antibodies to *Helicoverpa armigera* cathepsin L. *H. armigera* mature cathepsin L amino acid sequence has an identity of 70.0 and 69.6% with that of TBCATL-1 and TBCATL-2, respectively. The antibodies used in the experiment showed affinity – even at the dilution of 1:1000 – to *T. brasiliensis* cathepsin L. Various band signals with a molecular weight ranging from about 30–38 kDa, similar to zymography, were detected (Fig. 6). Since the samples were separated under reducing conditions, the molecular weights differed slightly from those observed in in-gel zymograms.

4. Discussion

The establishment of a *T. cruzi* infection in the intestinal tract of the vector depends on many factors which modulate the parasite-vector interaction (Azambuja et al., 2005; Garcia et al., 2007). The midgut of triatomines is the interface for development and multiplication of parasites and exerts in its physiological and biochemical conditions a great influence on the *T. cruzi* development (Kollien and Schaub, 2000; Garcia et al., 2007, 2011). In some hematophagous insects (e.g. Pediculidae, Culicidae) the midgut is responsible for both storage and digestion of the blood, whereas in Hemiptera these two functions occur in different midgut regions (Lehane, 2005; Waniek, 2009). Dipteran insects use serine proteinases (trypsins and chymotrypsins) as their major luminal proteolytic enzymes in their digestion process, which are active at alkaline pH (Johnston et al., 1991; Chougule et al., 2005), the phylogenetically distant hemipterans possess an rather different digestion, using cysteine and/or aspartic proteinases, which are highly active in acidic conditions (Houseman, 1978; Houseman and Downe, 1980, 1981, 1982; Houseman et al., 1984; Lehane, 1994; Borges et al., 2006). These peculiarities of the triatomine midgut physiology and digestion must be specifically taken into account in the studies of triatomine–trypanosomatid interactions.

So far, triatomine cathepsin L encoding cDNA sequences have been identified and characterized in *R. prolixus* and *T. infestans* (Lopez-Ordoñez et al., 2001; Kollien et al., 2004). In their deduced amino acid sequences triatomine cathepsin L precursors are structurally similar, possess all characteristic motifs and are highly conserved but less as for example triatomine defensins or lysozymes (Kollien et al., 2004; Araújo et al., 2006; Waniek et al., 2009a,b). Triatomine cathepsins are synthesized as pre-proenzymes. In general signal peptides are approximately 20 amino acids long, hydrophobic and cleaved during their passage to the endoplasmatic reticulum (von Heijne, 1983; Turk et al., 2000). Signal peptides of insect cathepsins L are within the usual boundaries and all *Triatoma* cathepsin B and L signal peptides, so far identified, are composed of 16 amino acid residues. Activation peptides are important for the proper folding of the protein and for protection of the cell from potentially negative effects of unregulated proteolytic activity. Mainly at their C-terminus cathepsin L activation peptides are more heterogenous in different species and in their length they exceed those of cathepsins B (Coulombe et al., 1996; Turk et al., 2000). The activation peptide length varied from 94 to 110 amino acids in insect cathepsin L sequences analyzed in the present study. After cleavage, these peptides act as cathepsin L inhibitors, playing an important role in the activity regulation of

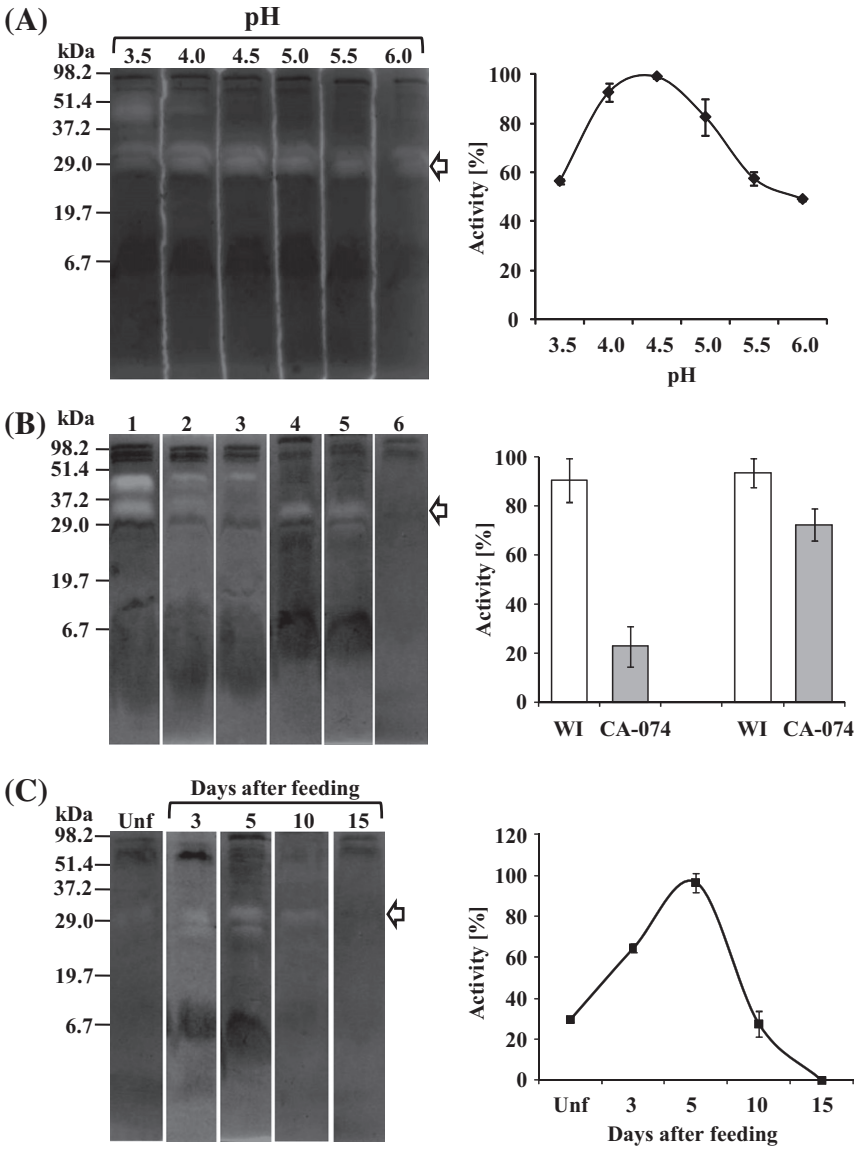

Fig. 5. Cysteine proteinase activity of small intestine content by gelatine in-gel zymography. (A) pH (3.0–6.0) dependence of *T. brasiliensis* intestinal proteolytic activity in samples at 5 daf, (B) Enzymatic inhibition of midgut proteinases: 1 and 3 without inhibitor (WI), 2 and 4 CA-074 (20 μM), 3 and 6 E-64 (2 μM), lanes 1–3 *T. infestans*, lanes 4–6 *T. brasiliensis*, (C) Time course of intestinal proteolytic activities, unfed and different days after feeding. Graphs on the right side reflect the intensity of the respective band. Bands used for quantitation are marked with arrowheads.

these digestive enzymes (Coulombe et al., 1996; Cygler and Mort, 1997). Considering the ERFNIN and GCNGG motifs, important for the globular folding of the N-terminus of the activation peptide (Coulombe et al., 1996), the *T. infestans* cathepsin L sequence (ERYNIN, GCDGG) differed from that of *T. brasiliensis* and *R. prolixus* (ERFNIN, GCEGG). The GNFD motif was more variable, modified to KNFD in TBCATL-2 and *T. infestans* cathepsin L, MNFD in TBCATL-1 and KNLF in the *R. prolixus* cathepsin L amino acid sequence (Lopez-Ordoñez et al., 2001; Kollien et al., 2004). The initial amino acids of the mature enzyme (Leu-Pro), the number of disulfide bridge forming cysteine residues, the active site and S2 residues were identical in all four triatomine cathepsin L sequences. Both mature *T. brasiliensis* cathepsin L amino acid sequences had a closer identity with cathepsin L of *R. prolixus* than that of *T. infestans*. Therefore the sequence of *T. infestans* was separated from the other three triatomine cathepsins in the dendrogram. This result indicates the occurrence of, at least, two cathepsin L subgroups in triatomines. *T. brasiliensis* and *T. infestans* are phylogenetically closer than *T. brasiliensis* and *R. prolixus*, therefore TBCATL-1 and TBCATL-2 should cluster together with the amino acid sequence of *T. infestans*. Since this is not the case, we can conclude that TBCATL-1/-2 and *R. prolixus* cathepsin L encoding genes might be orthologous counterparts, whereas the more distant *T. infestans* cathepsin L belongs to a second triatomine cathepsin L group. If we include different cathepsin B, cathepsin D, carboxy- and amino-peptidase isoforms, so far identified at DNA and protein level, the complexity of the triatomine digestive system becomes clearer.

Expression analyses by RT-PCR and northern blotting have shown high cathepsin L transcript abundance in the posterior intestine (small intestine) of *R. prolixus* whereas in the crop (stomach) cathepsin L mRNA was absent (Lopez-Ordoñez et al., 2001). These authors also have shown high cathepsin L transcript abundance in second instar nymphs, lower in unfed first instar nymphs and in fed first, third and fourth instars nymphs but absent in fifth instars. These findings are surprising as the last nymphal stage is also strongly dependent on blood digestion in view of nutrient

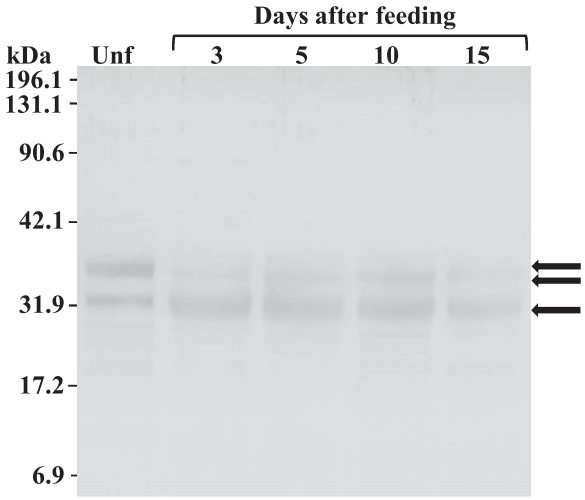

Fig. 6. Western blot analysis of *T. brasiliensis* small intestine content samples at different times after feeding, using anti-*Helicoverpa armigera*-cathepsin L antibodies. Proteins were separated by Tris–HCl–SDS–PAGE. Distinct bands in the range of ~32–40 kDa are marked by arrow heads.

demand for the metamorphosis to adults and because in adult *R. prolixus*, cathepsin L mRNA has been detected by northern blotting. By contrast, in the present study both cathepsin L transcripts were highly abundant in the small intestine of fifth instar nymphs. The differing cathepsin abundance in fifth instar nymphs of *R. prolixus* and *T. brasiliensis* remains puzzling. One possibility for this contradiction might be the differing phylogenetic origin and biology of these two triatomine species and thus divergent gene expression and physiology (Araújo et al., 2009). As we showed in the present study, several cathepsin L isoforms are expressed in the triatomine midgut. It is also possible that other isoforms assume the role of this specific *R. prolixus* cathepsin L, but are not detectable by a highly specific methodology like RT-PCR. However, since we analyzed the cathepsin L transcript abundance in a more detailed way – using more tissues – the cathepsin L expression pattern became clearer. The transcript abundance pattern indicates a major role of the respective enzymes predominantly in the small intestine of fifth instar nymphs and adult insects.

Intestinal pH is one important physiological parameter which affects the efficiency of digestive enzymes (Terra et al., 1996). Activity maxima of proteolytic enzymes, evaluated in various studies, emphasize the acid character of the small intestine content in triatomines (Houseman and Downe, 1980, 1981, 1982, 1983). Using a microelectrode, the pH measured in the stomach of *T. brasiliensis* has been between 7.02 and 7.16 (Barros et al., 2009). However, mixing contents of different midgut regions – e.g. anterior and posterior midgut or ecto- and endo-peritrophic (extra cellular membrane layer in Hemiptera) regions – with contrasting pH values will certainly give inaccurate results (Terra and Ferreira, 1994). Thus determination of the pH in the whole midgut might reflect the intestinal conditions more precise. The ingested blood surely contributes to the neutral or rather slightly alkaline environment in the stomach, but also guts of non-fed bugs show a pH within the range of 7.0. It remains unclear whether or not cathepsin L is secreted into the lumen of the stomach because, (i) at the neutral pH value present in this midgut region their activity would be very low and (ii) consequently also low propeptide cleavage and enzyme activation by autocatalysis will occur in this environment. Hence only the small intestine lumen with its acid pH offers proper conditions for reasonable cathepsin activity.

So far, intestinal proteolytic activities of triatomines have been analyzed by photometric assays. By using specific substrates (e.g. BAPNA, BANA, LPNA and Z-Phe-Arg-pNA), the luminal activity of cathepsin B, D and L, carboxypeptidase A and B and an aminopeptidase has been shown (Houseman and Downe, 1981, 1982, 1983; Kollien et al., 2004; Borges et al., 2006). Using a biotinyl affinity assay, several putative cysteine proteinases in the range of 30–35 kDa has been shown in the small intestine of *T. infestans* at 5 daf (Kollien et al., 2004). In the present study the intestinal proteinase activity was visualized using in-gel gelatine zymography. The broadness of the activity bands in context with the variety of cathepsin cDNAs also emphasized the presence of several cysteine-like proteinases in the small intestine of *T. brasiliensis*. The difference between the derived protein mass of the cDNA sequences and the real protein activity band can be explained by post-translational modification of these enzymes. Indeed, both cathepsin B and L amino acid sequences possess predicted glycosylation sites. The major activity of the *R. prolixus* cathepsin B-like proteinases has been shown at a pH of 3.8 and 4.0, respectively (Houseman and Downe, 1981). In the present study, the optimum pH for the cysteine proteinases was determined at 4.5, but also with high activities at 4.0 and 5.0. This wide activity range makes a correlation between maximum proteolytic activity and intestinal pH difficult. The slight pH shift in comparison to previous studies might be explained by the use of another and unspecific substrate as well as different reaction buffer compositions. It can also not be excluded that midgut proteinases of *T. brasiliensis* require less acidic conditions due to their adaptation to different environmental conditions. Because the activity optimum of the *T. brasiliensis* cathepsin L doesn't exactly match the intestinal conditions, we also should take into consideration that the pH value in the small intestine might represent a compromise, important for satisfying activity of a large number of proteolytic enzymes depending on different conditions.

Kollien et al. (2004) have shown a strong inhibition of intestinal gelatinase activities by the unspecific cysteine protease inhibitor E-64 (25.7% residual activity) and lesser inhibition by the specific cathepsin B inhibitor CA-074 (35.8% residual activity) in *T. infestans* at 5 daf and a pH of 5.0. Residual activity values in *T. infestans* at different days after feeding also have emphasized a strong variation of intestinal proteinases which might be based on individual properties. These results have confirmed the presence of both cathepsin B and L in the intestinal lumen of triatomines. In the present work, after 30 min of incubation at room temperature, E-64 almost fully inhibited proteolytic activity in *T. infestans*, whereas CA-074 inhibited the activity up to 75% (Fig. 5B). A higher inhibitor concentration (2 and 20 μM instead of 1 μM) used in the present study was possibly responsible for differing results. In *T. brasiliensis*, E-64 fully inhibited proteolytic activity but in the CA-074 treated samples an activity of 72.5% remains. These results strongly indicate the presence of cathepsin B and L in the small intestine of *T. brasiliensis* but indicate a differing cathepsin B/L activity ratio in comparison to *T. infestans* at 5 daf. The presence of different cathepsin L forms in the *T. brasiliensis* small intestine was also strongly supported by the immunoblotting experiments which showed at least three distinct bands of this cysteine proteinase. In addition to the activity bands with an expected molecular weight, a further band of ca. 45 kDa became visible at low pH in the small intestine samples of *T. brasiliensis*. A strong inhibition by CA-074 and an absence of the respective band in immunoblots points at cathepsin B. It is possible that different cathepsin isoforms, which might be present in the midgut, differ in their post translational modification and thus lead to a divergent enzymatic activity pattern. Both the presence of different cathepsin B encoding genes in the intestine of *T. infestans* and a strong discrepancy between the theoretical and real molecular weight of cathepsin B has been shown previously (Cho et al., 1999; Kollien et al., 2004, GenBank accession no. DQ376250).

In previous studies, highest enzymatic activity in the triatomine midgut has been shown at 5–6 daf. Cathepsin B, D and lysosomal carboxypeptidase A of *R. prolixus* have shown maximum activity

at 6 daf (Houseman and Downe, 1983). In the *T. brasiliensis* small intestine, muramidase activity has reached its highest activity at 5 daf (Waniek et al., 2009b). The results of the present study showed highest proteolytic activity at 5 daf and thus strongly corroborate these previous findings (Fig. 5C). It seems that 5–6 daf is the period with the highest metabolic activity in triatomines. Also in the *T. brasiliensis* small intestine the proteolytic activity increased strongly at 3 daf and reached its peak at 5 daf. It is interesting that at 15 daf proteolytic activity was not detectable by in-gel zymography, whereas in unfed bugs a clear band was visible. This apparent paradox might be explained by loss of water and a subsequent higher protein concentration in the intestinal tract of long-lasting starved (unfed) insects.

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