A heme-binding aspartic proteinase from the eggs of the hard tick *Boophilus microplus* 

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Running title: a heme-binding aspartic proteinase
Abbreviations used:

Abz – ortho-aminobenzoyl

t-Boc – tert-butyloxycarbonyl

MCA – 4-methylcoumarine amine

Cbz – carboxybenzoxo

EDDNp – ethylenediamine dinitrophenyl

SDS – lauryl sulfate, sodium salt

PAGE – polyacrylamide gel electrophoresis

THAP – Tick Heme-binding Aspartic Proteinase

VT – Vitellin

Tris – tris(hydroxymethyl)aminomethane

DEAE – diethylaminoethyl

SP – sulfopropyl

PVDF – polyvinylidene difluoride

TBS – tris buffered saline

IDA – iminodiacetic acid

Hb – hemoglobin

KGP – Lysine-gingipain
An aspartic proteinase that binds heme with a 1:1 stoichiometry was isolated and cloned from the eggs of the cattle tick *Boophilus microplus*. This proteinase, herein named THAP (Tick Heme-binding Aspartic Proteinase) showed pepstatin-sensitive hydrolytic activity against several peptide and protein substrates. Although hemoglobin was a good substrate for THAP, low proteolytic activity was observed against globin devoid of the heme prosthetic group. Hydrolysis of globin by THAP increased as increasing amounts of heme were added to globin, with maximum activation at a heme-to-globin 1:1 ratio. Further additions of heme to the reaction medium inhibited proteolysis, back to a level similar to that observed against globin alone. Addition of heme did not change THAP activity towards a synthetic peptide or against ribonuclease, a non-hemeprotein substrate. The major storage protein of tick eggs, vitellin (VT), the probable physiological substrate of THAP, is a hemeprotein. Hydrolysis of VT by THAP was also inhibited by addition of heme to the incubation media. Taken together, our results suggest that THAP uses heme bound to VT as a docking site to increase specificity and regulate VT degradation according to heme availability.
Introduction:

Vitellin (VT), the major storage protein from eggs of arthropods, is stored in structures called yolk spheres and constitutes the amino-acid source that supports embryo growth (1, 2, 3). Different kinds of proteolytic enzymes have been implicated in VT degradation, ranging from neutral serine proteinases (4) to acidic cysteine proteinases such as Cathepsins B and L (5,6,7,8). Two aspartic proteinases have also been related to VT degradation. In the blood-sucking insect Rhodnius prolixus, a cathepsin D-like enzyme activity was identified (9). In the tick B. microplus, an aspartic proteinase precursor, BYC (Boophilus Yolk Cathepsin) has been proposed to play a role in VT degradation during embryogenesis (10).

Heme is essential to all living beings as the prosthetic group of essential molecules such as cytochromes and hemoglobins. Almost all organisms are able to synthesize their own heme (11), with the exception of some pathogenic bacteria and protozoa that feed on vertebrate blood. Recently it was shown that Boophilus microplus also acquires its heme by the degrading hemoglobin from the ingested blood (12), being the first multicellular organism shown to be unable to synthesize the protoporphyrin ring. In relation to the reproduction of ticks, an important outcome of this finding is that the egg must provide all the necessary heme to build up a new organism. It is noteworthy that the most distinguishable feature of VTs from ticks is that they are all hemeproteins (13). Thus, VT is probably the source of heme required for embryo growth. Therefore, besides the need for amino acids for synthesis of new proteins, VT degradation should also be regulated according to the embryo’s need for heme.

In this work, we describe the purification, cloning and characterization of a novel aspartic proteinase from the eggs of the hard tick Boophilus microplus (herein named THAP, for Tick Heme-binding Aspartic Proteinase) that is capable of binding heme. We present evidence that THAP heme-binding site functions as a docking-site that recognizes heme on the surface of protein substrates, increasing its specificity towards hemeproteins and providing a possible mechanism of regulating tick VT degradation. This mechanism would represent a novel way of regulating the activity of proteinases.
Experimental Procedures:

- **Animals:**
  Ticks were obtained from a colony maintained at the Faculdade de Veterinária at the Universidade Federal do Rio Grande do Sul, Brazil. *Boophilus microplus* of the Porto Alegre strain, free of *Babesia spp.*, were reared on calves obtained from a tick free area. Engorged adult females were kept in Petri dishes at 28 °C and 80 % relative humidity until completion of oviposition.

- **Eggs and Hemolymph:**
  Eggs laid less than 24 h beforehand were collected and homogenized in a Potter-Elvehjem tissue grinder in 20 mM Tris-HCl buffer, pH 7.4 with 0.05 mg/mL of soybean trypsin inhibitor, 0.05 mg/mL leupeptin and 1 mM benzamidine (approximately 1g of eggs/2 mL). Egg homogenate was centrifuged at 100,000 x g for 60 min at 4 °C. The floating lipids and the pellet were discarded, and the crude egg extract supernatant was used for protein isolation.

- **Electrophoresis:**
  Polyacrylamide gels (12 or 10 %) were run in the presence of SDS (14) at a constant current of 20 mA. Gels were stained with Coomassie Blue G according to the method of Neuhoff et al. (15) and destained with deionized water. Molecular masses of polypeptides were determined using the following protein standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (94 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.5 kDa).

- **THAP purification**
  Crude egg extract was applied into a column of DEAE-Toyopearl 650M (20 x 3
equilibrated with 10 mM Tris-HCl, pH 8.4 and eluted with two linear 200 mL NaCl gradient steps (0-100 mM, 100-400 mM). The fractions containing THAP (identified by SDS-PAGE), were dialyzed against 20 mM sodium phosphate pH 6.0 overnight, and applied to a SP-Toyopearl 650 M (column (10 x 1.5 cm) equilibrated with the same buffer and eluted with a 200 mL NaCl gradient (0-210 mM). Fractions containing THAP were applied to an iminodiacetic acid sepharose (IDA) column previously loaded with CuCl₂ and equilibrated with 50 mM phosphate buffer pH 6.0 with 0.2 M NaCl. The column was eluted with a 150 mL continuous glycine gradient (0-300 mM). The fractions containing THAP were concentrated in a Speed-Vac system (Savant SVC 100) and the degree of protein purity was evaluated by SDS-PAGE (12%). Protein concentration was determined by the method of Lowry et al. (16) using bovine serum albumin as standard.

- VT purification:

VT was purified based on the protocol described by Roosell & Coons (13) with some modifications. Egg homogenate was applied to the same DEAE-Toyopearl 650M column used for THAP isolation. The fractions containing VT (identified by SDS-PAGE and by light absorption at 400 nm) were applied to a Sephacryl S-200 gel filtration column (90 x 1.5 cm). The fractions containing VT were concentrated in a Speed-Vac system (Savant SVC 100) and the degree of protein purity was evaluated by SDS-PAGE (10%).

-Amino-acid sequences:

THAP (50 µg) was subjected to SDS-PAGE (12 %) and transferred to a PVDF
membrane (17). After staining with Coomassie Blue for 5 minutes polypeptide bands were destained with 40% methanol, cut, and applied to the protein sequencer. The sequence was obtained by automatic Edman degradation using a liquid phase sequencer (Porton PI 2090). Phenylthiohydantoin amino acids were identified in a Hewlett-Packard HPLC system (mod. 1090) with an amino-quant column (200 mm x 2.1 mm).

-cDNA cloning and analysis:

A forward degenerated primer based on the obtained NH$_2$-terminal sequence (QLGWHDP) was synthesized and used together with a Not I-(dT)$_{18}$ primer (Amersham Pharmacia Biotech) to amplify THAP cDNA from total ovary RNA by RT-PCR. The PCR product was then gel purified and cloned into a pT7Blue-3 vector using the Perfectly Blunt$^\text{TM}$ Cloning kit (Novagen) according to manufacturer’s instructions. DNA sequencing was performed using the dideoxy method at the Molecular Genetics Instrumentation Facility of the University of Georgia.

The obtained sequences were subjected to similarities search in nonredundant sequence data banks (NCBI BlastP search). Similarities were determined using the BESTFIT program from the GCG Software Package (Version 8, University of Wisconsin). Multiple sequence analysis was carried out using Clustal W software (18) and the search for known protein motifs in a sequence was made using Blocks+ database software (19).
-Polyclonal antibodies:

Rabbits were inoculated subcutaneously with THAP (0.5 mg in 0.25 ml of water) emulsified with an equal volume of Freund’s complete adjuvant. After 1 month, animals were boosted with 0.5 mg of THAP in water. Sera were obtained from blood collected at least 1 month after the second injection.

-Western blot:

For Western-blot analysis, samples were first separated by SDS-PAGE (10%) and then transferred to nitrocellulose (20). Nitrocellulose sheets were then blocked by incubation with 5% (w/v) non-fat dry milk, 0.01% Tween 20 in 20 mM Tris pH 7.4, 0.15 M NaCl (TBS), and incubated with the antiserum diluted in the same solution. Goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:30,000 (v/v) was used as secondary antibody and developed with 0.033% (w/v) nitroblue tetrazolium and 0.016% (w/v) 5-bromo 4-chloro 3-indolyl phosphate in 100 mM Tris-HCl pH 9.6, 0.15 M NaCl and 5 mM MgCl₂.

-Proteolytic activity:

Unless otherwise stated, 200 µg of each protein substrate (bovine hemoglobin, apohemoglobin, ribonuclease A or purified VT) was incubated in acetate buffer 0.15 M pH 3.5 at 37 °C, in 800 µL. The reaction was started by addition of 10 µg of THAP. At different time intervals, aliquots of 150 µL were taken and immediately added to tubes containing 500 µL of 0.5 M phosphate buffer pH 7.3. Proteinase inhibitors or hemin were
added as indicated in figure legends. Samples were then added to 200 µL of a 0.03 % solution of fluorescamine in acetone in order to titrate newly formed NH₂-terminal ends and the fluorescence (excitation at 375 nm and analysis at 480 nm) was measured using a Hitachi F-4500 spectrofluorimeter (21). A sample collected immediately after THAP addition was used as a blank of the reaction.

Hydrolysis of synthetic peptide substrates by THAP was also measured. THAP (10 µg) was incubated in 100 µL of 0.2 M sodium acetate buffer pH 3.5 at 37 °C, with different concentrations of fluorogenic or chromogenic substrates: 300 µM for –peptidyl-MCA fluorogenic substrates (CHROMOGENIX AB) and 4 mM for Abz-peptidyl-EDDnp fluorogenic substrates (22). The fluorogenic reactions were monitored during one hour with a F-MAX fluorimeter (Molecular Devices Inc.) using a 320 nm excitation filter and a 420 nm emission filter for the EDDnp substrates and a 370 nm excitation filter and a 460 nm emission filter for the MCA substrates. All experiments were repeated at least twice.

-Hemoglobin heme extraction:

Apoantoglobulin was obtained through extraction of the heme group from bovine hemoglobin by the acetone-HCl method (23).

-Dot blot assay of THAP binding to protein substrates:

VT, Hb, Apo Hb or Ribonuclease A (2 µg) were first blotted onto a nitrocellulose membrane, which was then blocked by overnight incubation with 5% (w/v) non-fat dry milk, 0.01% Tween 20 in TBS. After that, the membrane was incubated for 90 min. with a
0.5 mg / ml THAP plus 10^{-5} M pepstatin in TBS. Hb, apoHb, ribonuclease or heme was added to this last incubation as indicated in the legend. After the incubation, the membranes were probed with the anti-THAP rabbit serum diluted in the blocking solution. Goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:30,000 (v/v), was used as secondary antibody. Membranes were developed with nitroblue tetrazolium (0.033%, w/v) and 5-bromo 4-chloro 3-indolyl phosphate (0.016%, w/v) in Tris-HCl 100 mM pH 9.6, 0.15 M NaCl and 5 mM MgCl₂.

-Binding studies:

Binding of heme to THAP was monitored by measuring light absorption at 411 nm while progressively adding a solution of 0.1 mM hemin in 0.1 M NaOH (24). The absorbance was plotted against the amount of hemin added and the hemin necessary to fully saturate the heme-binding capacity of the protein was determined from the break in the curve. The same assay was also used to titrate heme-binding sites of ApoHb.

Quenching of intrinsic tryptophan fluorescence of THAP upon binding of hemin was used to determine the Kd of THAP-heme interaction. Protein fluorescence spectra were recorded using an ISS GREG-200 (Urbana Il) spectrofluorimeter in which the excitation wavelength was 275 nm and the emission was measured between 250 and 450 nm. Binding was determined by gradually adding small volumes of a 0.01 mM hemin solution to 1 ml of THAP solution (40 µg/ml) in 50 mM sodium phosphate pH 7.2 until maximum quenching of protein fluorescence was attained. A control was performed using N-acetyl-l-tryptophanamide instead of THAP in order to verify whether the observed
quenching could be due to absorption by the heme molecule of light produced from fluorescence of the indole group. No significant effect on the fluorescence of N-acetyl-L-tryptophanamide was observed at these concentrations of heme.

Kd was determined according to Vincent & Muller-Eberhard (25) by plotting the molar ratio of bound hemin per mole of THAP against concentration of added hemin. The fraction of bound hemin was calculated from (Fo-F)/Fo, where Fo is the fluorescence of protein in the absence of ligand and F is the fluorescence after ligand addition. The value of Kd was obtained by nonlinear regression analysis of the obtained data (25).

-Binding to hemin-agarose:

An assay for screening heme-binding proteins using a hemin-agarose gel was performed as described by TsuiTsui & Mueller (26). 40 µg of protein from the DEAE-Toyopearl peak containing THAP were added to 500 µL of 50 mM phosphate buffer pH 7.0, 0.15 M NaCl with or without 10 nmoles of hemin (as indicated in figure legend) and incubated for 30 min with 10 µL of hemin-agarose (Sigma Chemical Co.). After the incubation the gel was washed five times with 0.5 ml of the same buffer, heated with SDS (40 µl of SDS sample buffer) and the bound polypeptides were analyzed by 12% SDS-PAGE.
Results:

*B. microplus* egg extract was fractionated on a DEAE-Toyopearl column (Figure 1A) and the peaks were tested for the capacity to bind to a hemin-agarose gel in order to identify putative heme-binding proteins. Two polypeptides from the first peak of the DEAE column bound to hemin agarose (Figure 2). Starting from the peak from the DEAE column, a preparative isolation was carried out by chromatography on a SP-Toyopearl column (Figure 1B) followed by another chromatographic step on a IDA-Cu⁺² column (Figure 1C), leading to co-isolation of the 37 and 32 kDa polypeptides (Figure 1D). The polypeptides were then blotted onto a PVDF membrane and sequenced separately by automated Edman degradation (Figure 3A). After the 19th amino acid, the sequence of the 37 kDa polypeptide became identical to that of the 32 kDa one, indicating that both of them are in fact different proteolytic states of the same protein (Figure 3B). Gel filtration chromatography on a Superose 12 (Pharmacia Biotech) column showed a single peak of approximately 35 kDa (data not shown), excluding the possibility that these two polypeptides were subunits of an oligomeric protein.

The amino-terminal sequence shown in figure 3B was used to design a primer (5’CARYTNGGNTGGCAYGAYCC 3’) that was used together with a *Not*I-dT(18) primer to clone THAP from total ovary RNA. When translated, the resultant clone presents an amino-terminal sequence identical to the 50 amino-acid sequence obtained by overlapping the two segments deduced from the Edman procedure. The cDNA encodes a protein of 353 amino acids (38313 Da) with a pI of 7.16 (Figure 3C). Proteins showing the closest identities with THAP were cathepsin Ds from several organisms (40-44% identity) and an aspartic proteinase of the mosquito *Aedes aegypti* (45% identity), and accordingly we consider that this enzyme can be characterized as cathepsin D-like. Selected sequences used in this analysis are aligned with THAP in figure 4. When subjected to search for known structural motifs, THAP presented several blocks characteristic of aspartic proteinases such as the active site regions and the characteristic cysteines at conserved positions (Figures 3C and 4). We have previously described another aspartic proteinase, also isolated from *Boophilus* eggs called BYC (10). However, none of BYC sequences were identified in THAP cDNA, confirming that
BYC and THAP are in fact different proteins.

When THAP proteolytic activity against hemoglobin was evaluated, it showed an optimal pH of 3.5 (data not shown) and was specifically inhibited by pepstatin, a selective inhibitor of this group of enzymes, but not by inhibitors of other classes of proteinase (Table 1). THAP was also assayed against several different synthetic fluorogenic and chromogenic substrates. Substrates for the main classes of acidic proteinases (cathepsin L, cathepsin B, cathepsin G and cathepsin D) were assayed (27, 28). Only Abz-AIAFFSRQ-EDDnp, a substrate based on the sequence susceptible to enzymes such as pepsin and related aspartic proteinases (28) was hydrolyzed by THAP (Table 2). As expected for a cathepsin D-like enzyme, analysis of the peptide after hydrolysis revealed that cleavage occurred between the two Phe residues (data not shown).

The binding of THAP to hemin-agarose (Figure 2) was a strong indicative of the presence of a heme-binding site in this protein. Titration of THAP with hemin showed that each THAP molecule is capable of binding a single molecule of hemin (Figure 5A) with a Kd of 190 nM (Figure 5B). In order to determine whether the binding of heme would affect THAP activity, we followed Abz-AIAFFSRQ-EDDnp hydrolysis in the presence of heme. Addition of heme in a 1:1 or 2:1 heme-to-THAP ratio did not affect its Km or Vmax, indicating that heme interferes neither in the binding of the substrate to the active site nor in the catalytic efficiency of the enzyme (Figure 6).

A completely different result was obtained when hemoglobin hydrolysis was investigated. ApoHb produced after removal of the heme prosthetic group was a poor substrate for THAP, but the hydrolysis was greatly increased upon hemoglobin
reconstitution by addition of hemin (Figure 7A). Stimulation of the activity was maximal at 1:1 globin-to-heme ratio. This observation could mean that the binding of heme induced a conformational shift in the globin molecule leading to exposure of susceptible peptide bonds. This did not seem to be the case, as increasing the heme-to-globin ratio to 1.2 : 1 resulted in strong inhibition of hemoglobin hydrolysis (Figure 7A). The same experiment using ribonuclease A instead of globin did not show any effect of heme on THAP proteolytic activity (Figure 7B). These data may be explained by THAP using heme (bound to a protein) as a docking site, with the bound heme increasing the affinity of THAP towards the protein substrate. For the same reason, after the saturation of globin heme-binding sites, the addition of free hemin to the reaction medium would competitively inhibit docking of THAP to the substrate by occupying the THAP heme-binding site.

Since VTs from ticks are hemeproteins (13), we carried out experiments to verify whether or not heme would be able to modulate THAP proteolytic activity against VT. Similarly to the result obtained with hemoglobin, hydrolysis of VT by THAP was inhibited by the presence of free heme in the reaction medum (Figure 8). This suggests that free heme can compete with heme bound to VT (13) for THAPs heme-binding site. Further support for this hypothesis is provided by comparing binding of THAP to different substrates (Figure 9). Pepstatin-treated THAP was incubated with a nitrocellulose sheet blotted with Hb, apoHb, VT or ribonuclease A, and binding of THAP was revealed using polyclonal anti-THAP antibodies. THAP was able to bind to hemoglobin and VT, but not to apoHb or to ribonuclease A (Figure 7). Moreover, the binding to VT and Hb was blocked by hemin or Hb added to the incubation media, but not by ribonuclease A.
Discussion:

During turnover of heme-proteins such as hemoglobin and cytochromes the heme group is destroyed by the action of heme oxygenase (29). Although the iron atom may be used again (included in de novo heme synthesis), the porphyrin ring is not recycled to a significant extent in most organisms (29, 30). It has recently been shown that the cattle tick *Boophilus microplus* is not able to synthesize heme (12), relying on the host hemoglobin to obtain the heme necessary for its own development. One consequence of this finding is that during the course of evolution the tick must have developed efficient mechanisms for heme recycling. Here we show that the egg of *Boophilus microplus* has a proteinase capable of binding heme, and we present evidence that this provides a mechanism for modulating its activity toward a physiological substrate (VT, a hemeprotein). To our knowledge, this is the first proteinase shown to be regulated by heme.

Several pieces of evidence suggest that THAP interaction with heme relies on a site that is distinct from the active site of the enzyme. Heme binding by THAP did not interfere with its activity against ribonuclease or synthetic substrates (Figure 6 and Figure 7B). Further support for this hypothesis comes from the observations that the active proteinase bound heme (Figure 2 and Figure 5), and that pepstatin-treated THAP also retained the ability to bind to Hb and VT, even though its active site was occupied by the inhibitor (31) (Figure 9). An analogous behavior is exhibited by thrombin, a key proteinase of the blood coagulation pathway that converts soluble fibrinogen to a fibrin gel. Thrombin has been shown to bind fibrinogen through a site unrelated to the enzyme active center (32) and this bind can be blocked independently of the site that catalyzes proteolysis (33).

Analysis of bovine Hb crystalline structure (pdb identifier 2HHD) available at the Protein Data Bank (http://www.rcsb.org/pdb) using RasWin molecular graphics version 2.6 (34) reveals that the edge of the heme ring is exposed to the solvent and that the propionate side chains of the porphyrin project from the protein surface and thus are available to interact with other proteins. Therefore, we postulate that THAP interaction with heme in Hb occurs through the propionate radicals of protoporphyrin IX.

It has recently been shown that pathogenic *Escherichia coli* strain EB1 can secrete a serine proteinase that is able to bind Hb, degrade it and capture the released heme (35). So far, no regulatory role has been
proposed for heme in this case. It has also been demonstrated that the oral anaerobic bacteria *Porphyromonas gingivalis* synthesizes a cysteine proteinase that binds Hb (36). Recently, DeCarlo et. al., (37) have shown that this proteinase known as Lys-gingipain (KGP), binds to Hb molecule through its heme moiety. Like the THAP:heme interaction, the interaction of KGP with heme occurs through a site different from the active site (37).

It is important to note that THAP (like KGP) has no heme-binding motif in its primary sequence. Thus neither of these proteins has a classical heme-pocket and presumably neither of them interacts with the iron which, in any case is not exposed. Instead we are propose that the interaction of THAP (and possibly KGP) with heme occurs through a different mechanism involving the protoporphyrin IX propionyl radicals.

The Kd for heme binding to THAP was 190 nM. *In vivo*, the most likely substrate for THAP is Vitellin, the main egg storage protein. The concentration of Vitellin, in eggs is about 1 mM (data not shown) , 5,000 X higher than the Kd of THAP for heme. Considering that VT is not equally dispersed throughout the egg but instead highly concentrated in the yolk granules (3) the concentration in this compartment may be even higher. It remains to be shown were THAP is localized in relation to VT.

A potentially important consequence of the fact that THAP activity is regulated by binding of heme is related to heme cytotoxicity. Heme is a generator of oxygen free radicals through Fenton-type reactions that lead to damage of many important molecules (38, 39, 40, 41). As a protective measure through the animal kingdom, free heme is not found in cells, being instead always bound to proteins. During oocyte growth, large amounts of VT containing heme are internalized by the oocytes of ticks, and this VT is subsequently degraded during embryogenesis (42). The rate of VT degradation must match that of heme utilization in order to avoid the formation of a potentially dangerous pool of free heme. As the heme content of VT favours THAP proteolytic activity (Figure 8), the
release of an excess of free heme from VT degradation may inhibit THAP binding to VT, thus slowing the pace of VT proteolysis and preventing oxidative damage.

Bugs, mosquitoes, and other hematophagous organisms face the same challenge as *Boophilus* during feeding and each species has found a secure way to avoid the oxidative stress generated by the release of free heme (38, 39, 40, 41). Some of these protective mechanisms have already been identified, including the synthesis of hemozoin in the parasite *Plasmodium falciparum* (43) and in the blood-sucking bug *Rhodnius prolixus* (44).

Regulation of hemeprotein proteolysis (and consequent reduction of heme release) may also prove to be an important adaptation of hematophagous species to this unique way of feeding. This hypothesis is currently under investigation.
References:


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Figure legends:

**Figure 1: Isolation of THAP from crude egg extract.** (A) DEAE-Toyopearl chromatography. (B) SP-Toyopearl chromatography. (C) IDA-Cu$^{+2}$ chromatography. Fractions containing THAP are indicated with a bar. (D) Summary of THAP isolation by SDS-PAGE (12%). Egg: Egg extract; DEAE: DEAE-Toyopearl column; SP: SP-Toyopearl column; IDA: IDA-Cu$^{+2}$ column.

**Figure 2: Binding of THAP to hemin-agarose gel.** 30 µg of protein from the first DEAE peak were incubated with the hemin-agarose gel in the absence or presence of hemin. The polypeptides bound were analyzed by 12% SDS-PAGE. DEAE: 30 µg of protein from the first peak of DEAE-column; Hemin-agarose: proteins that bound to the hemin agarose gel; Hemin-agarose + hemin: proteins that bound to the hemin-agarose gel after preincubation with 10 nmoles of hemin.

**Figure 3: Amino-acid sequence of THAP.** (A) The polypeptides present in the THAP preparation were fractionated by 12% SDS-PAGE, blotted onto a PVDF membrane and sequenced by automated Edman degradation. The boxes indicate the identical sequences found in both polypeptides. (B) Overlapping of the sequences of both THAP polypeptides obtained through protein sequencing. Arrow indicates the putative cleavage site. (C) THAP complete sequence. Portion determined by protein sequencing is underlined.

Symbols indicate beginning of sequence deduced from cDNA ( ), catalytic Asp residues ( ), conserved
Cys residues ( ), potential Asn glycosylation site ( ).

**Figure 4: Sequence alignment of THAP with several known aspartic proteinases.**

Alignment was made using Clustal W (V. 1.8). Aedae_ASPP - *Aedes aegypti* lysosomal aspartic protease (accession number M95187); *Schistosome japonicum* aspartic proteinase (L41346); Chicken cathepsin D (Q05744); Mouse renin (MMREN1A); Sheep chymosin (P18276); Pig pepsin A (A90185); Human cathepsin E (P14091). * = identical or conserved residues in all sequences in the alignment, : = indicates conservative substitutions "." = indicates semi-conservative substitutions. The active site region is boxed.

**Figure 5: Titration and affinity of THAP heme-binding sites.** (A) For determination of the number of THAP heme-binding sites, 1 nmol of THAP (35 µg) in 0.15 M NaCl, 20 mM Tris-Cl pH 7.0 was titrated by addition of hemin and measurement of the absorbance at 411 nm (+). Lower curve shows heme added to buffer alone (•). (B) The Kd for THAP:heme interaction was determined by measuring the quenching of THAP intrinsic fluorescence between 250 and 450 nm while exciting at 275 nm. Data shown are typical of three experiments carried out with different THAP preparations.

**Figure 6: Hydrolysis of Abz-AIAFFSRQ-EDDnp by THAP.** THAP (10 µg) was incubated in 100 µl of 0.2 M sodium acetate buffer pH 3.5 at 37 °C for 40 min. with different concentrations of the fluorogenic substrate and the indicated heme : THAP ratios. A nonlinear least-squares fit to the Michaelis-Menten equation is shown by the solid line.
double reciprocal plot is shown in the inset. Error bars indicate S.E.M. for three experiments.

**Figure 7: Effects of heme on hydrolysis of globin and RNaseA by THAP.** 150 µg of each protein substrate were incubated in 800 µl of acetate buffer (0.15 M pH 3.5) at 37 °C, at the indicated heme:protein ratios. Reactions were started by addition of 10 µg of THAP. At the indicated times, aliquots of 150 µl were taken and formation of new NH₂-terminal groups was titrated with fluorescamine as described in Experimental Procedures. A sample collected immediately after THAP addition was used as a reaction blank.

(A) Apohemoglobin; P indicates 10⁻⁵ M pepstatin in the reaction medium. (B) RNaseA. Data shown are typical of three experiments carried out with different THAP preparations.

**Figure 8: Effect of heme on hydrolysis of VT by THAP.** 0.5 nmols of VT were incubated in 800 µl of acetate buffer 0.15 M pH 3.5 at 37 °C, in the absence or presence of 22 nmols of hemin. The reaction was started by addition of 10 µg of THAP. At the indicated times, aliquots of 150 µl were taken and formation of new NH₂-terminal groups was titrated with fluorescamine as described in Experimental Procedures. A sample collected immediately after THAP addition was used as a reaction blank. Data shown are typical of three experiments carried out with different THAP preparations.
Figure 9: Dot blot assay of THAP binding to protein substrates. 2 µg of each protein (VT, *Boophilus* vitellin; Hb, hemoglobin; ApoHb, apohemoglobin; RNaseA, ribonuclease A) was dotted onto a nitrocellulose membrane. The membrane was blocked and incubated for 1.5 hours with a 1.4 µM THAP solution (previously inhibited with 10^{-5} M pepstatin) in TBS in the presence of: (1) No addition; (2) 15 nmoles/ml hemin; (3) 5 mg/ml Hb; (4) 5 mg/ml RNaseA. After the incubation, the membrane was probed with anti-THAP rabbit serum and developed using a goat anti-rabbit IgG conjugated to alkaline phosphatase as described in Experimental Procedures. Data shown are typical of two experiments carried out with different THAP preparations.

Table 1: Effect of different proteinase inhibitors on THAP proteolytic activity. Reactions were started by addition of 10 µg of THAP. After 40 minutes, formation of new NH$_2$-terminal groups was titrated with fluorescamine as described in Experimental Procedures. A sample collected immediately after THAP addition was used as a reaction blank. Data shown are mean ± SD (N=3).

Table 2: THAP proteolytic activity towards different synthetic substrates: Fluorogenic substrates for cysteine (24) and aspartic (25) proteinases were used: N-Cbz-F-R-MCA (cathepsin L), N-t-Boc-G-R-R-MCA (cathepsin B), Abz-P-F-F-S-R-EDDnp (cathepsin G) and Abz-A-I-A-F-F-S-R-Q-EDDnp (aspartic proteinases). Reactions were started by addition of THAP (10 µg). The reactions were monitored during one hour.
as described in Experimental Procedures. n.d. not detected.
A

37 kDa: EFAŁQLGWHPXVTEIRGRALGDPI

32 kDa: ALGDPIPIPIILTNYNNMQFYGIITXGXPPQ

B

EFALQLGWHPXVTEIRGRALGDPIPIPIILTNYNNMQFYGIITXGXPPQ

C

EFALQLGWHPVTEIRGRALCDPFP

ILTNYYNNMQFYGIIITIGTPQSFKL

LMDTGSSNFQWPSPINCQDQSMACRDHA

KYDSSKSSSTFTKSGRYIIRYSGGVV

RGITSDNVGVGPATVTQYKFAEMDH

SDGKLFRNAKYDGFIFGLAPSPISQNN

QLPLFDAMVKQGVVRQAVFSILYLSKQ

PSEQNGGEIYFGGINAQRYTGATHYV

PVSQAQHWQVMNINVQGTTLCSVGG

CPTVVDSGTSDLGSAGPSADVEGLNRFVI

GATKTAAGYFEVNCATISSLPDIVFN

LNGKSFPLQGEAYTIRIPLTTGGEOQC

FTR1SESDASGTLNWLILGAVFTQTYY

TVFDRAQNLRVFATA
<table>
<thead>
<tr>
<th>Heme : THAP</th>
<th>Km</th>
<th>Vmax (nmoles/h.mg ptn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>1.1 µM</td>
<td>27</td>
</tr>
<tr>
<td>1:1</td>
<td>1.2 µM</td>
<td>28</td>
</tr>
<tr>
<td>2:1</td>
<td>1.2 µM</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Hb</td>
<td>ApoHb</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>-------</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Relative Activity (% control)</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Pepstatin (10 μM)</td>
<td>6.2 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Leupeptin (1 mM)</td>
<td>108.1 ± 12.6</td>
<td></td>
</tr>
<tr>
<td>L-trans-epoxysuccinil-leucylamide – (4guanidino)-butane (100 μM)</td>
<td>98.7 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>EDTA (2 mM)</td>
<td>104 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Soybean trypsin inhibitor (250 μM)</td>
<td>99.1 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Iodoacetamide (2.5 mM)</td>
<td>112.0 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>Hydrolysis rate</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>--------------------------</td>
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<tr>
<td>N-Cbz-F-R-MCA (24)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>N-t-Boc-G-R-R-MCA (24)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Abz-P-F-F-S-R-EDDnp (24)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Abz-A-I-A-F-F-S-R-Q-EDDnp (25)</td>
<td>27 nmols/h.mg ptn</td>
<td></td>
</tr>
</tbody>
</table>