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Degradação de vitelina e hemoglobina no carrapato bovino *Rhipicephalus (Boophilus) microplus*

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"…we all want to change the world.
Don't you know it's gonna be alrigh?…"

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INDICE

ABREVIATURAS

A280 - absorbância a 280 nm

BCIP - 5-bromo-4-cloro-3-indolilfosfato p-toluidina

BmCL1 - "*Boophilus microplus* Cathepsin-L 1"

BSA - bovine serum albumin

BYC - "Boophilus Yolk pro-Cathepsin"

DTT - ditiotreitol

E-64 - L-trans-epoxisuccinil-L-leucilamido(4-guanidino)-butano

Hb - hemoglobina

NBT - nitro blue tetrazolium

PAGE - polyacrylamide gel eletrophoresis

PBS - phosphate buffered saline

RFU - relative fluorescence units

RmLCE - "*Rhipicephalus microplus* Larval Cysteine Endopeptidase"

SDS - sodium dodecyl sulfate

TCA - trichloroacetic acid

TFA - trifluoroacetic acid

THAP - "Tick Heme-binding Aspartic Proteinase"

U - unidade de atividade enzimática

Vg - vitelogenina

Vt - vitelina

RESUMO

O presente trabalho caracterizou alguns aspectos enzimáticos da digestão de vitelina e de hemoglobina no carrapato bovino ***Rhipicephalus (Boophilus) microplus.*** Um novo protocolo foi desenvolvido para a purificação de uma cisteíno endopeptidase de larvas do carrapato (***R. microplus*** Larval Cysteine Endopeptidase; RmLCE). A seqüência parcial de aminoácidos de RmLCE mostrou similaridade com outra cisteíno endopeptidase descrita neste organismo (***Boophilus microplus*** Cathepsin -L1; BmCL1). Vitelina (Vt), preparada a partir de homogenatos de ovos e larvas de ***R. microplus***, foi degradada diferencialmente por RmLCE e VTDCE (Vitellin-Degrading Cysteine Endopeptidase; uma enzima de atividade semelhante purificada de ovos de ***R. microplus***). RmLCE também apresentou capacidade de degradação de hemoglobina em pH 4,0, e a presença de uma atividade cisteíno endopeptidásica acídica em intestino de larvas foi demonstrada. A atividade peptidásica total, presente em homogenatos de ovos, larvas e intestino de partenóginas, sobre substrato sintético em pH 4,0, mostrou-se diferencialmente susceptível a inibidores de cisteíno e aspártico endopeptidases (E-64 e pepstatina A, respectivamente). A ação combinada dos dois inibidores inibiu completamente a atividade peptidásica. Estes inibidores também afetaram a degradação de vitelina e hemoglobina por homogenatos de larvas e intestino de partenóginas. A presença de pepstatina A inibiu cerca de 50 % da atividade sobre hemoglobina em larvas, enquanto E-64 foi capaz de inibir completamente a degradação das proteínas por ambos os homogenatos. Um enzima com atividade aspártico endopeptidásica presente em larvas de ***R. microplus*** foi parcialmente purificada e identificada por Western blot e seqüenciamento N-terminal como ***Boophilus*** Yolk Cathepsin (BYC). Esta enzima foi eluída de uma coluna de gel-filtração em pH ácido associada a uma atividade cisteíno endopeptidásica. A degradação de hemoglobina de forma sinérgica pela aspártico endopeptidase (BYC) e a cisteíno endopeptidase (RmLCE) purificadas de larva indica a existência de uma cascata enzimática responsável pela digestão de hemoglobina. Estes resultados sugerem que RmLCE tem um papel continuo na digestão de vitelina e hemoglobina durante o desenvolvimento de ***R. microplus***.

1 - O carrapato bovino *Rhipicephalus (Boophilus) microplus*

Os carrapatos são ectoparasitas aracnídeos da ordem Acari com hábito hematófago obrigatório, podendo ter como hospedeiros animais selvagens, domésticos e o homem. Com base na extensão das áreas escleróticas em sua cutícula, dois grupos principais são reconhecidos: os "carrapatos duros" (ixodídeos) ou "carrapatos moles" (argasídeos) (Hackman e Filshie, 1982). As diferentes espécies de carrapatos também se distinguem pelo número de hospedeiros necessários para completar seu ciclo de vida ou pela tendência à infestação de determinados grupos de mamíferos. A espécie *Boophilus microplus* (descrita por Canestrini em 1887 e recentemente reclassificada para o gênero *Rhipicephalus*; Murrell e Barker, 2003), é um carrapato ixodídeo que se caracteriza por ser monoxeno, completando o ciclo de vida em um único hospedeiro, quase que exclusivamente bovinos (Fig. I).

O ciclo de vida do carrapato bovino (Fig. II) divide-se em uma fase de vida livre de duração variável, e uma fase parasitária, quando se alimenta ativamente de sangue durante cerca de 21 dias. As mudanças de estágio de larva parasitária, passando por fases de metalarva, ninfa, metaninfa, neógina e partenógina (fêmea adulta parcialmente ingurgitada) até teleógina madura (totalmente ingurgitada) ocorrem durante este período. A queda da fêmea madura ao solo marca o início da fase de vida livre. Cada teleógina é capaz de depositar em média 2.000 ovos durante cerca de 15 dias. Estes ovos irão se desenvolver e eclodir em larvas infestantes após aproximadamente duas semanas (Cordovés, 1997). As larvas de vida livre saem em busca de um hospedeiro 3 a 7 dias após a eclosão e esta fase de procura pode variar significativamente, dependendo de condições ambientais (Davey et al., 1991).

A distribuição geográfica de *R. microplus* inclui as regiões tropical e subtropical entre os paralelos 30° S e 40° N, onde as condições climáticas favorecem seu desenvolvimento (Fig. III). A espécie originou-se no sudeste da Ásia e dispersou pela Austrália, África e América. Na América do Sul, este carrapato é endêmico, exceto em áreas muito áridas ou de altitude muito elevada. Na América do Norte foi erradicado com sucesso nos Estados Unidos (Jongejan e Uilenberg, 2004).

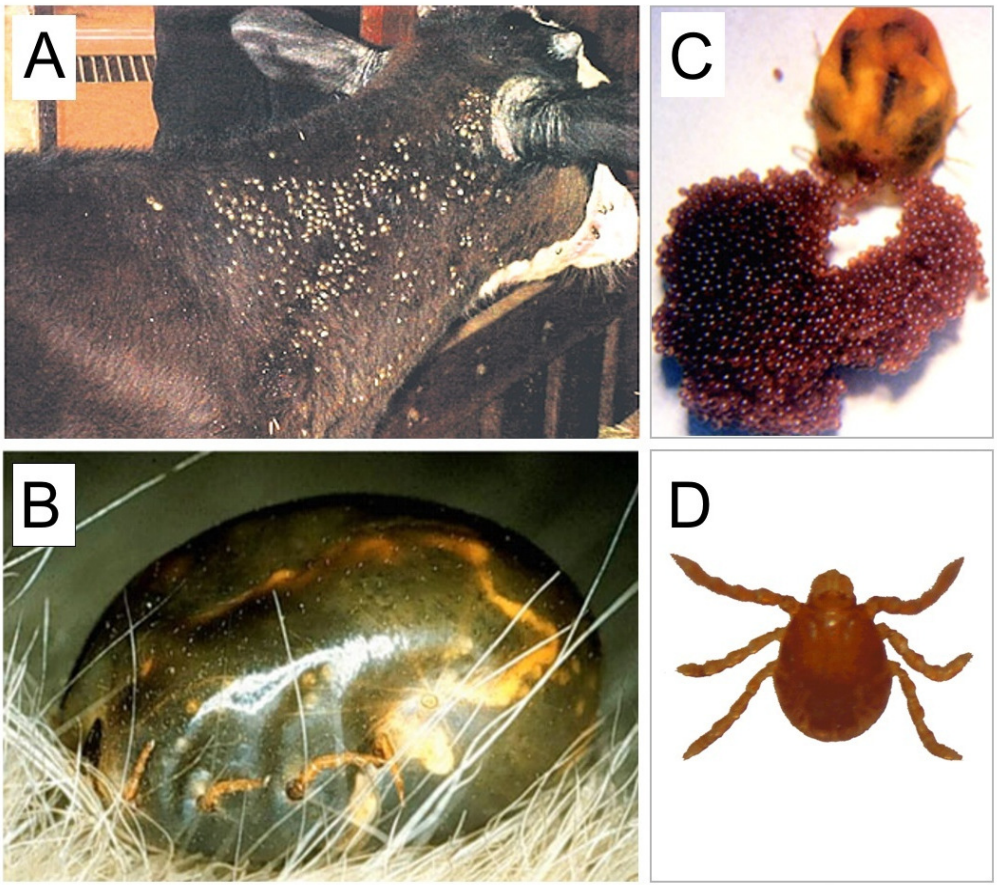

Fig. I - O carrapato bovino *Rhipicephalus (Boophilus) microplus*. (A) Hospedeiro bovino severamente infestado (fonte: www.unesp.br); (B) Fêmea adulta, completamente ingurgitada, ainda fixada no hospedeiro (fonte: www.cnpgc.embrapa.br); (C) Fêmea adulta, após a queda ao solo e postura dos ovos (fonte: www.biotecnologia.com.br); (D) Larva infestante, não-alimentada (foto: José Reck Jr.).

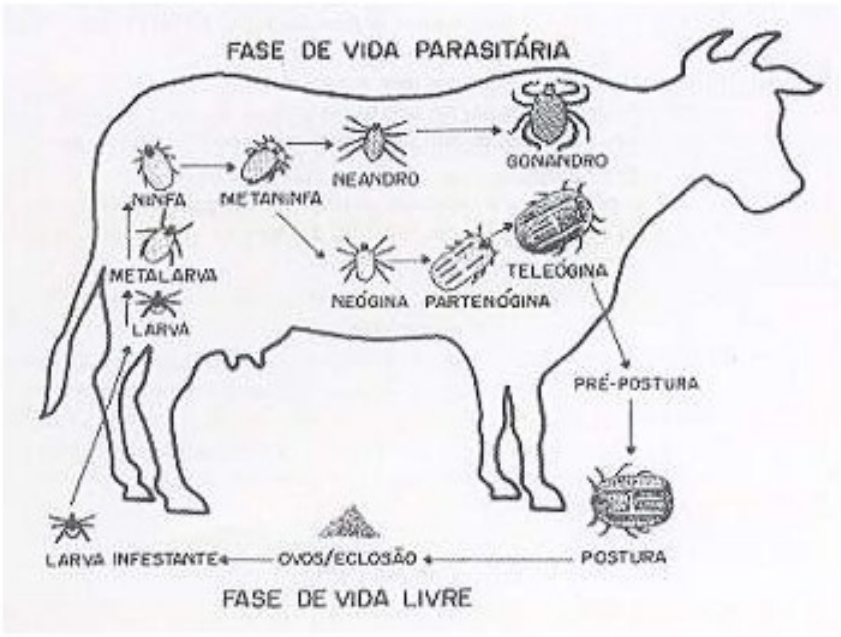

Fig. II - Ciclo de vida de *R. microplus*, incluindo a fase de vida livre no solo e a fase parasitária em hospedeiro bovino (fonte: www.carol.com.br).

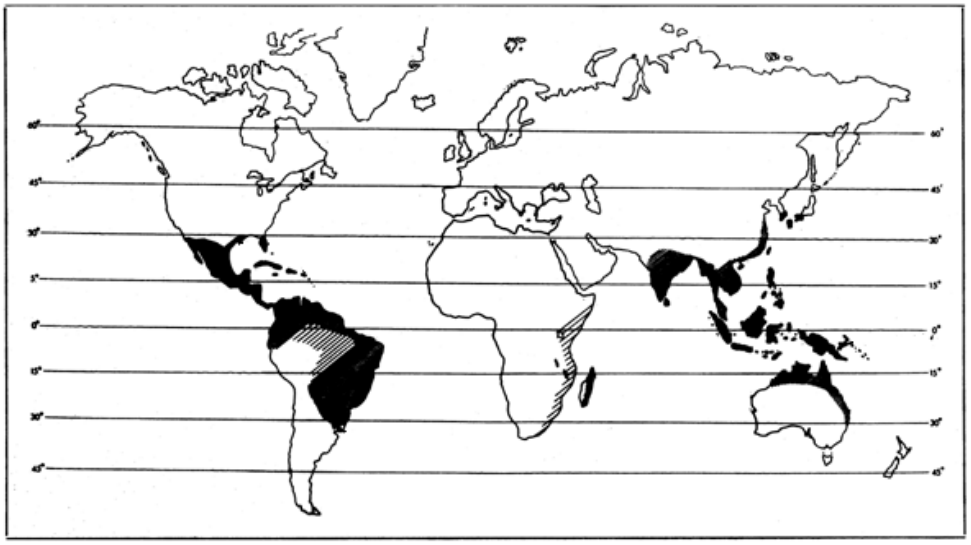

Fig. III - Distribuição geográfica mundial de *R. microplus*, entre os paralelos 30° S e 40° N - áreas marcadas em preto e sombreado (fonte: www.fao.org).

1.1 - Importância econômica e para a saúde animal

A infestação de rebanhos bovinos por *R. microplus* é fonte de grande preocupação na economia pecuária. Diversos estudos, principalmente na década de 80, foram dedicados a estimativas do prejuízo causado pelo parasitismo neste setor econômico. Na América do Sul, calcula-se que cerca de 1 bilhão de dólares são gastos anualmente, incluindo o prejuízo com os danos diretos e indiretos ao rebanho e à produção, e investimentos em estratégias de controle (Da Silva Vaz Jr., 2000).

Entre os danos diretos causados ao rebanho pelo parasitismo estão: perda da qualidade do couro, acentuada queda na produção de leite e carne e a redução da natalidade. Estes efeitos são conseqüência de reações cutâneas inflamatórias causadas pela fixação do carrapato e da espoliação do hospedeiro pela retirada de grandes volumes de sangue (Jongejan e Uilenberg, 2004). Em casos mais graves, a espoliação causada pelo carrapato pode até levar à morte do hospedeiro. Repetidas infestações podem levar o bovino à aquisição de resistência parcial contra o parasita, resultando na redução do engurgitamento e viabilidade reprodutiva dos carrapatos (Brossard e Wikel, 2004).

Através de seu hábito hematófago, os carrapatos atuam como vetores de agentes causadores de diversas doenças. Nenhum outro grupo de artrópodes vetores é capaz de transmitir uma variedade tão grande de patógenos, entre protozoários, bactérias e vírus (Jongejan e Uilenberg, 2004). O complexo de enfermidades conhecido como tristeza parasitária bovina, que inclui a anaplasmose e a babesiose bovinas, é causado pela bactéria da ordem Rickettsiales *Anaplasma marginale* e pelos protozoários do gênero *Babesia* (principalmente *B. bovis* e *B. bigemina*). Estes parasitas intraeritrocíticos são transmitidos aos bovinos por *R. microplus*, e são também responsáveis por perda de produtividade, além de freqüentemente causarem aborto e óbito (Kocan et al., 2004; Bock et al., 2004).

1.2 - Estratégias de controle

Muitos estudos têm sido feitos no intuito de desenvolver métodos alternativos para o controle das infestações por *R. microplus*. Isto ocorre porque os métodos mais difundidos baseiam-se no uso de acaricidas químicos, derivados de arsênico, ciclodienos, formamidinas, organoclorados, organofosfatados e piretróides; estas técnicas apresentam uma série de limitações relacionadas à contaminação ambiental e dos produtos pecuários,

além do desenvolvimento de populações de carrapatos resistentes (George et al., 2004). Muitos destes princípios ativos já não são mais usados, tal a disseminação da resistência.

Entre as alternativas em estudo para complementar ou substituir os acaricidas químicos inclui-se o controle biológico, que utiliza organismos naturalmente hostis ao carrapato (Willadsen, 2006b). Muitos destes predadores de carrapatos são generalistas, o que dificulta seu manejo como métodos de controle. Os fungos deuteromicetes *Metharrhizium anisopliae* e *Beauveria bassiana*, dos quais já existem cepas comercialmente disponíveis, são considerados bons agentes controladores devido à sua capacidade de penetrar na cutícula dos artrópodes e à sua virulência relativamente específica. O controle biológico de carrapatos por fungos, no entanto, ainda é de difícil aplicação e alto custo (Samish et al. , 2004).

O controle imunológico também é um campo de pesquisa em franca expansão. Inicialmente, as respostas imunológicas do hospedeiro submetido a repetidas infestações foram estudadas. As observações decorrentes destes trabalhos logo denotaram a importância de mais estudos sobre a indução artificial de imunidade a antígenos do parasita, através da vacinação (Willadsen, 2006a). Duas vacinas foram disponibilizadas comercialmente, a australiana TickGARD e a cubana Gavac, sendo que a proteção conferida por estas vacinas varia entre diferentes cepas de carrapato (Willadsen, 2004). Ambas foram desenvolvidas com base no antígeno Bm86 identificado através de uma série de testes de fracionamento de homogenatos de carrapatos, imunização e desafio, sem nenhuma referência à função bioquímica ou imunológica do material imunizante. Após a descoberta de sua capacidade imunoprotetora, a Bm86 foi identificada como sendo uma proteína de membrana presente em células epiteliais intestinais de fêmeas de ***R. microplus.*** Proteínas imunogênicas presentes no intestino dos carrapatos, como a Bm86, são exemplos típicos dos chamados “antígenos ocultos”, capazes de induzir uma resposta imune quando inoculados artificialmente, mas que não participam das interações imunológicas naturais entre parasita e hospedeiro, evitando os mecanismos adaptativos que possam reduzir a eficácia da vacina (Willadsen e Kemp, 1988). Outros dois antígenos intestinais identificados foram o Bm91 e BMA7, que apresentaram um potencial de proteção, mas ainda não são utilizados comercialmente (Da Silva Vaz Jr. et al, 2000).

Com o intuito de identificar possíveis alvos para o controle imunológico, com base no papel fisiológico das moléculas, foram realizados muitos estudos sobre componentes salivares atuantes na hematofagia como imunomoduladores e anti-hemostáticos (Ribeiro e Francischetti, 2003; Maritz-Olivier et al., 2007). Além da importância destas moléculas bioativas no sucesso da fixação e da ingestão de sangue pelo carrapato, elas também auxiliam ativamente na transmissão de patógenos (Nuttall e Labuda, 2004). Já está demonstrado que a imunização contra o vetor pode conferir ao hospedeiro proteção contra os patógenos transmitidos (Kocan, 1995; Labuda et al., 2006).

Entre outras linhas de pesquisa que buscam identificar moléculas de importância fisiológica como alvos para uma vacina, as enzimas proteolíticas e seus inibidores têm sido descritos como potenciais antígenos (Willadsen, 2004), destacando-se trabalhos sobre a embriogênese dos carrapatos. A estratégia de reprodução destes artrópodes envolve a produção de grande quantidade de ovos, que são ricos em vitelo (Diehl et al., 1982). Entre os componentes do vitelo estão a vitelina e as proteases responsáveis pela sua degradação. Duas destas enzimas já foram testadas como antígenos em experimentos de vacinação. A imunização de bovinos com uma aspártico endopeptidase de ovos de carrapato, (***Boophilus*** Yolk Cathepsin; BYC) conferiu uma proteção parcial entre 14 % e 36 % com a proteína nativa (Da Silva Vaz et al., 1998) e de 25 % com a proteína recombinante (Leal et al. 2006). Resultado semelhante, uma proteção de 21%, foi obtido quando outra protease de ovo (Vitellin-Degrading Cysteine Endopeptidase; VTDCE) foi utilizada como antígeno (Seixas et al., 2008).

2 - Fisiologia de *R. microplus*: aspectos reprodutivos e a digestão de proteínas

2.1 - Enzimas proteolíticas e digestão proteica em invertebrados

As endopeptidases, enzimas que catalizam a quebra ligações peptídicas entre aminoácidos não terminais em uma cadeia polipeptídica, são classificadas de acordo com o mecanismo catalítico. As endopeptidases são agrupadas em quatro diferentes sub-sub-classes, cada uma com resíduos de aminoácidos característicos em seu sítio ativo (Tabela I). As serino endopeptidases (E.C. 3.4.21._) apresentam um resíduo de serina no centro da sua tríade catalítica. As metalo endopeptidases (E.C. 3.4.24_) possuem um íon metálico, geralmente zinco, participando do mecanismo catalítico. As aspártico endopeptidases (E.C.

3.4.23._) e as cisteíno endopeptidases (E.C. 3.4.22._) caracterizam-se por apresentarem resíduos de ácido aspártico e cisteína, respectivamente, na composição de seu sítio catalítico (Neurath, 1989; Rawlings e Barrett, 1993). Muitas peptidases das sub-sub-classes aspártico e cisteíno endopeptidases são enzimas acídicas, com pH ótimo abaixo de 6,0. Entre as enzimas responsáveis pela proteólise ácida dentro de lisossomos em células animais, por exemplo, as mais ubíquas são as cisteíno endopeptidases (catepsina B, L e H) e a aspártico endopeptidase catepsina D (Bohley e Seglen, 1992).

Tabela I – Classes de endopeptidases (adaptado de Neurath, 1989).

Classe	Endopeptidases representativas	Resíduos característicos do sítio catalítico
Serino	quimiotripsina, tripsina	Asp, Ser, His
Metalo	carboxipeptidase	Glu (Zn)
Aspártico	pepsina, catepsina D	Asp, Asp
Cisteíno	papaína, catepsina B	Cys, His, Asp

A digestão de proteínas no intestino é um exemplo da ação muitas peptidases de diferentes sub-sub-classes, como parte de um processo fisiológico coordenado. Ao contrário do que ocorre em vertebrados, onde as serino endopeptidases da família da tripsina são as principais enzimas atuantes na digestão de proteínas, em invertebrados este processo é resultado principalmente da ação de enzimas do tipo aspártico e cisteíno endopeptidases, descritas em diversos organismos como platelmintos, nematódeos e artrópodes (Delcroix et al., 2006).

2.2 - Proteólise e o desenvolvimento embrionário do carrapato

Animais ovíparos, por não apresentarem aporte externo de nutrientes durante a fase embrionária, dependem de substâncias de reserva de origem materna para completarem seu desenvolvimento. Em carrapatos, estas reservas são compostas principalmente por vitelina (Vt), que é uma lipoglicofosfoproteína de alta massa molecular, possuindo múltiplas subunidades (Canal et al. 1995). A Vt de carrapatos é carreadora de ferro, contendo derivados de heme provenientes da digestão de hemoglobina do sangue do hospedeiro (Logullo et al., 2002). A vitelina é sintetizada pelas fêmeas na forma de vitelogenina (Vg). A Vg é internalizada pelo oócito em desenvolvimento, via endocitose mediada por receptor, processada em endossomos e então armazenadas como Vt em estruturas especializadas,

denominadas grânulos de vitelo (Fagotto, 1995). Os grânulos de vitelo são considerados lisossomos modificados, onde a proteólise ácida ocorre de forma estritamente regulada. A regulação ocorre por mecanismos de latência enzimática, ocorrendo ativação das enzimas por proteólise em pH ácido, e principalmente pela modulação do pH em si, sendo que a acidificação gradual dos grânulos, ao longo do desenvolvimento embrionário, dispara e controla a degradação da vitelina (Fagotto, 1995).

Além da Vt, são incorporadas ao vitelo enzimas proteolíticas que compõem a maquinaria de degradação das substâncias de reserva. Em ovos de ***R. microplus***, já foram descritas três enzimas envolvidas na degradação de vitelina. A primeira delas, ***Boophilus*** Yolk Cathepsin (BYC), é uma aspártico endopeptidase que possui atividade proteolítica sobre vitelina e é ativada por autoproteólise em pH 3,5 (Logullo et al, 1998). A segunda, denominada THAP (Tick Heme-binding Aspartic Proteinase), pertence a essa mesma classe de peptidases e também possui atividade ótima em pH 3,5 (Sorgine et al, 2000). Uma terceira atividade proteolítica em ovos de ***R. microplus*** foi caracterizada, recebendo o nome de VTDCE (Vitellin Degrading Cysteine Endopeptidase). Esta cisteíno endopeptidase apresenta atividade ótima em pH 4,0. Em extratos de ovos, a enzima, que demonstrou atividade proteolítica sobre Vt, apresenta-se associada a este substrato (Seixas et al, 2003). Em uma análise comparativa, a VTDCE mostrou ser a enzima com maior atividade na degradação de Vt (Seixas et al., 2008).

A utilização das fontes energéticas pelo embrião de ***R. microplus***, durante seu desenvolvimento inicial envolve consumo de oxigênio e redução no conteúdo de lipídeos e carboidratos. Cerca de 15 % da vitelina é consumida nos primeiros 4 dias de embriogênese, sendo que o conteúdo de proteína total presente no ovo embrionado não varia. Até o momento da eclosão, mais 20 % da Vt é consumida, principalmente nas fases mais tardias, quando também começa a aumentar o conteúdo de guanina, produto de excreção do nitrogênio proveniente do catabolismo de aminoácidos. Assim, a degradação de Vt sustenta inicialmente a síntese de novas proteínas no embrião, passando a ser fonte de energia, através de gliconeogênese, na fase final de desenvolvimento (Campos et al., 2006).

Após a eclosão dos ovos, as larvas passam por uma fase crítica denominada fase de encontro, quando podem passar de dias até meses antes de iniciar a alimentação hematófaga. Neste período, a vitelina remanescente é digerida e o conteúdo protéico total

decresce proporcionalmente, indicando uma continuidade no processo de geração de energia por gliconeogênese (Estrela et al., 2007). A atividade de digestão de vitelina na fase larval em ***R. microplus*** é atribuída a uma cisteíno endopeptidase acídica (***Rhipicephalus microplus*** Larval Cysteine Endopeptidase; RmLCE) diferente da enzima descrita no ovo (Estrela et al., 2007).

2.3 - Proteólise e a hematofagia

A partir da fixação das larvas no hospedeiro, com o início da fase parasitária, uma nova fonte nutricional passa a suprir as necessidades metabólicas do carrapato. Proteínas sangüíneas do hospedeiro, principalmente hemoglobina (Hb), começam a ser digeridas no intestino. Diferente do que ocorre em outros artrópodes hematófagos, onde a hemoglobina é digerida extracelularmente, em ***R. microplus*** a digestão de hemoglobina ocorre por uma via intracelular, que inclui a endocitose da proteína por células especializadas no lúmen e a sua degradação em populações específicas de vesículas (Lara et al., 2005). A grande quantidade de heme livre gerada dentro da célula neste processo é detoxificada através da agregação em organelas especializadas chamadas hemossomos (Lara et al., 2003). Este é um mecanismo especialmente importante em ***R. microplus***, onde o heme ingerido é extensivamente reutilizado devido a ausência de uma rota biosintética (Braz, et al., 1999). Outro aspecto relevante da digestão de hemoglobina por carrapatos é a geração de peptídeos com atividade antimicrobiana, componentes importantes da imunidade inata destes parasitas (Fogaça et al.,1999).

Os vacúolos digestivos onde a hemoglobina é degradada nas células intestinais do carrapato são de natureza acídica, assim como os grânulos onde a vitelina é digerida no embrião. Paralelamente, as mesmas classes de peptidases são implicadas na digestão de hemoglobina e vitelina. Aspártico e cisteíno endopeptidases foram descritas como os principais componentes enzimáticos em intestino de fêmeas ingurgitadas de ***R. microplus***, e a atividade proteolítica somente foi observada em pH abaixo de 6,0 (Mendiola et al., 1996). Existem evidências recentes de que a digestão de hemoglobina em carrapato (***Ixodes ricinus***) ocorre pela ação coordenada de peptidases dessas duas classes, através de um mecanismo de ação em cascata onde aspártico endopeptidases são responsáveis pela degradação inicial de hemoglobina e cisteíno endopeptidases atuam na digestão dos

peptídeos resultantes. Além disso, cisteíno endopeptidases podem também estar envolvidas na trans-ativação, por proteólise, das aspártico endopeptidases iniciadoras (Sojka et al. , 2007).

Embora diversas enzimas com capacidade de hidrolisar Vt e Hb tenham sido identificadas em ***R. microplus***, o papel que cada uma delas desempenha na digestão dessas proteínas ainda não está esclarecido.

OBJETIVOS

A melhor compreensão dos mecanismos bioquímicos empregados no desenvolvimento e nutrição de ***Rhipicephalus microplus*** contribui para o conhecimento na área, facilitando a identificação de moléculas que possam servir como alvo para o desenvolvimento de uma vacina contra infestações pelo carrapato bovino. Neste contexto, este trabalho visa investigar as enzimas responsáveis pela proteólise das principais fontes nutricionais em diferentes fases do ciclo de vida do carrapato ***R. microplus***.

Entre os objetivos específicos deste estudo estão:

- purificar e caracterizar uma cisteíno endopeptidase de larvas de ***R. microplus***. (RmLCE);
- elucidar seu papel na degradação de vitelina em larvas não-alimentadas;
- investigar a atuação de RmLCE na digestão de hemoglobina;
- determinar a participação de cisteíno endopeptidases e aspártico endopeptidases na digestão de vitelina e hemoglobina em larvas e fêmeas jovens;
- estudar possíveis interações enzimáticas entre cisteíno endopeptidases e aspártico endopeptidases na digestão de Vt e Hb em ***R. microplus.***

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Vitellin- and hemoglobin-digesting enzymes in *Rhipicephalus (Boophilus) microplus* larvae and females

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Abstract

The aim of the present study was to address the involvement of *Rhipicephalus microplus* Larval Cysteine Endopeptidase (RmLCE) in protein digestion in *R. microplus* larvae and adult female. An improved purification protocol for native RmLCE was developed. The partial amino acid sequence of the purified enzyme shows it has a high similarity with *Boophilus microplus* Cathepsin-L1 (BmCL1). Stage-specific differences in vitellin (Vt) degradation were observed for RmLCE activity in comparison to Vitellin-Degrading Cysteine Endopeptidase (VTDCE) from tick eggs. Purified RmLCE was also able to degrade host hemoglobin (Hb) at pH 4.0, and an acidic cysteine endopeptidase activity was detected in larval gut. It was shown that cysteine and aspartic endopeptidases are involved in Vt and Hb digestion in *R. microplus* larva and female. Interestingly, the aspartic endopeptidase *Boophilus* Yolk Cathepsin (BYC) was demonstrated to be associated with a cysteine endopeptidase, in larvae. Synergic hemoglobin digestion by BYC and RmLCE indicates the presence of an Hb-degrading enzymatic cascade involving these enzymes. Our results suggest that RmLCE has a continued role in vitellin and hemoglobin digestion during tick development.

Keywords: *Rhipicephalus (Boophilus) microplus*, tick, RmLCE, BYC, vitellin, hemoglobin, aspartic endopeptidase, cysteine endopeptidase.

1 - Introduction

Ticks and tick-born diseases cause severe economic losses to animal production worldwide. These haematophagous ectoparasites affect animal health by direct damage and also by transmitting a variety of pathogens (Jongejan and Uilenberg, 2004). *Rhipicephalus (Boophilus) microplus* is a one-host tick that is a major constraint in cattle breeding in tropical and sub-tropical areas. Chemical acaricides currently in use present many limitations related to the appearance of drug-resistant tick population, environmental contamination, and a potential presence of chemical residues in milk and meat. Understanding the physiology of tick vital processes, such as blood digestion and egg and larval development, can help to develop new and improved control methods (Willadsen, 2006).

The high reproductive rate of *R. microplus* females and the success of egg development rely on an efficient conversion of blood meal components into yolk reserve material to sustain embryo demands. Vitellin (Vt), the major reserve protein present in yolk granules, contains heme derived from host hemoglobin digestion (Logullo et al., 2002; Braz et al., 1999). This phosphorylated lipoglycoprotein is the main source of amino acids and energy to support the development of tick embryos and unfed larvae. Yolk material also comprises the enzymatic machinery for Vt degradation. In ticks, maternally derived enzymes are synthesized in both ovarian and extra-ovarian tissues, and internalized by growing oocytes in yolk granules, together with Vt (Fagotto, 1995).

Three *R. microplus* egg proteases, with vitellin degrading capacity and a putative role in yolk mobilization, have been described. Two are aspartic endopeptidases: Tick Heme-binding Aspartic Proteinase (THAP; Sorgine et al., 2000) and *Boophilus* Yolk pro-Cathepsin (BYC; Logullo et al., 1998). BYC is one of the most abundant proteins in egg extracts. While it was also detected in recently hatched larvae, the role of this enzyme in later stages of the tick life cycle has not been investigated.

The third, and most active, Vt-degrading protease in *R. microplus* eggs is a Cathepsin-L like protease (Vitellin-Degrading Cysteine Endopeptidase; VTDCE; Seixas et al., 2003). Indeed, cysteine endopeptidase activities have been implicated in yolk degradation in eggs of another tick (*Ornithodoros moubata*; Fagotto, 1990a), and many insect species, including *Drosophila melanogaster* (Medina et al., 1988), *Aedes aegypti* (Cho et al., 1999),

Bombyx mori (Kageyama & Takahashi, 1990) and *Blatella germanica* (Liu et al., 1996; Yin et al., 2001).

In *R. microplus* larva, a cathepsin-L like cysteine endopeptidase (BmCL1) was cloned from a cDNA library and the recombinant protein displayed vitellin-degrading activity (Renard et al., 2000). A native cysteine endopeptidase (*R. microplus* Larval Cysteine Endopeptidase; RmLCE) was partially purified and characterized (Estrela, et al., 2007), and was also implicated in Vt-digestion during the larval stage.

Intestinal protein digestion in several invertebrates is mainly a result of the combined action of cysteine endopeptidases of the papain family (cathepsin-L like) and aspartic endopeptidases (cathepsin-D like) enzymes (Delcroix et al., 2006). Aspartic and cysteine endopeptidases have often been shown to participate in host hemoglobin digestion in haematophagous parasites (Nisbet & Billingsley, 2000; Sajid & McKerrow, 2002). Mendiola et al. (1996) detected an acid peptidase activity, due to aspartic and cysteine endopeptidases, as the major enzymatic components in the *R. microplus* female gut. Importantly, it is suggested that a network comprising proteases of both classes takes part in tick digestion (Sojka et al., 2007), as occurs in helminths (Williamson et al., 2004; Delcroix et al., 2006), to effect complete breakdown of hemoglobin.

Interfering with digestive enzymes responsible for degrading major protein substrates during tick development can be a suitable strategy to control tick populations, by decreasing tick fecundity. The present work aimed to characterize Vt and Hb-degrading protease activities at different stages of the *R. microplus* life cycle. The purification of a digestive enzyme from tick larvae (RmLCE) is described and the association of larval aspartic and cysteine endopeptidase activities, related to vitellin and hemoglobin digestion, was investigated.

2 - Material and methods

2.1 - Biological material

The Porto Alegre strain of ***Rhipicephalus (Boophilus) microplus*** was reared in Hereford calves maintained in insulated stables. Eggs were obtained by maintaining fully engorged female, spontaneously detached from the host, in Petri dishes at 28 ºC, 80 % relative humidity for oviposition. For larvae production, the eggs were collected and placed in glass tubes closed with a cotton plug under the same conditions until hatching. Larvae were separated at the 12th day after hatching and stored at –70 °C until use.

Egg and larva homogenates (0.1 g/mL) were prepared in sodium phosphate 10 mM, pH 7.0 or pH 6.0, respectively. The preparations were centrifuged (6,500 g for 10 min), the supernatant was filtered through a sequence of pre-filter AP20, 0.45 m and 0.22 m pore filters (Millipore, Bedford, MA, USA), and stored at –20 °C.

Larvae and partially engorged female guts were dissected under a stereomicroscope (Stemi DRC, Zeiss, Oberkochen, Germany) and suitable surgical instruments. Female guts were obtained from young females forcibly removed from bovines at 20th day after infestation. After removing the cuticule, the internal organs were submerged in chilled (ice bath) pH 7.0, 10 mM sodium phosphate – 150 mM sodium chloride buffer (PBS). Guts were removed and washed three times in the same buffer, in order to remove the luminal content, homogenized in sodium phosphate 10 mM, pH 6.0, using pestle and mortar, and centrifuged (6,500 g for 10 min). The supernatant was stored at –20 °C.

2.2 - Vitellin and VTDCE preparation

Egg and larval vitellin were purified from egg and larva homogenates, respectively, through a two-step chromatographic protocol in FPLC system (Pharmacia, Uppsala, Sweden). Homogenates were prepared as described above, applied onto a HiTrap™ Q HP (1.6 x 2.5 cm) column (GE Healthcare) equilibrated sodium phosphate 10 mM (pH 7.0 or pH 6.0 for egg and larval preparations, respectively) and eluted with NaCl gradient (0 – 0.8 M). Vt-containing fractions were pooled and applied onto a gel-filtration Superdex™ 75 HR 10/30 (10 x 300 mm) column (GE Healthcare) equilibrated in the same buffer. Vitellin-

degrading Cysteine Endopeptidase from ***R. microplus*** eggs (VTDCE) was purified as previously described (Seixas et al., 2003).

2.3 - Protein quantification

Protein content of preparations was assessed by the the bicinchonic acid (BCA) method reported by Smith et al. (1985), using bovine serum albumin (BSA; Pierce, Rockford, IL, USA) solution as standard.

2.4 - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Zymogram

For eletrophoretic separation, samples were applied onto a gel containing 5 % (w/v) polyacrylamide (stacking gel) and resolving gel (10 %, 12 % or 14 % polyacrylamide, w/v) and run at 100 V for approximately 2 h in a Bio-Rad Mini-Protean Cell II unit (Bio-Rad, Hercules, CA, USA). The gel was then stained with Coomassie brilliant blue solution or Silver Stain Plus kit (Bio Rad), according to manufacturer instructions. Proteolytic activity was assessed by zymogram. Protein substrate, hemoglobin (Hb; 0.1 %) was co-polimerized in 12 % (w/v) polyacrilamide gel. After SDS-PAGE run, gels were treated for 3 h in Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for SDS removing, exhaustively washed with distilled water, and then incubated in conditions suitable for enzyme activity (sodium acetate 10 mM, pH 4.0 for 18 h at 37 °C), before staining with Coomassie brilliant blue.

2.5 - Immunoblotting analysis

For Western blot, proteins in polyacrylamide gels were electrically transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) using a semi-dry system (GE Healthcare). Also, proteins (0.1 g) were directly applied upon nitrocellulose membrane (Dot blot). Immunoblottings were performed treating protein-containing membranes as follows: I) overnight blocking with 5 % (w/v) skim milk solution in PBS at 4 °C; II) incubation with polyclonal anti-BYC rabbit antiserum (1:250; Logullo et al., 1998) or anti-cysteine protease (anti-BmCL1) rabbit antiserum (1:100; Renard et al., 2002) for 2 h at room temperature; as a negative control, membranes were instead incubated with pre-immune rabbit sera; III) washing with PBS three times for 10 min; IV) incubation with alkaline phosphatase-goat anti-rabbit IgG conjugated (Sigma-Aldrich), for 1 h at room

temperature; and V) staining with chromogenic substrate for alkaline phosphatase NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt; USB Corporation, Cleveland, OH, USA).

2.6 - Peptidase assays

Degradation of natural substrates, hemoglobin (Hb) and vitellin (Vt), was assessed labeling N-terminal amino acids with fluorescamine. Fluorescamine is a heterocyclic dione witch reacts with primary amines producing a fluorescent product, allowing the quantification of protease activity (Udenfriend et al., 1972). After incubation at 37 °C, reaction mixtures (50 L) were neutralized with 100 L sodium phosphate 100 mM (pH 7.0) and then incubated for 10 min with 50 L fluorescamine 0.03% (w/v in acetone). The fluorescence generated was measured at 370 nm_{ex}.–460 nm_{em}. in a $M2^{e}$ Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Proteolysis was also verified analyzing reaction products by SDS-PAGE, as described above.

Peptidase activity upon a synthetic substrate for cathepsin-D (Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp; Pimenta, et al., 2001) was analysed by fluorimetry at 320 nm_{ex}.–430 nm_{em}. Samples (10 L) were incubated for 30 min at 37 °C in sodium citrate (68 mM)/sodium phosphate (64 mM) buffer, pH 3.5, in the presence or absence of specific aspartic protease inhibitor (pepstatin A; 2 M) and/or cysteine endopeptidase inhibitor (L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane; E-64; 10 M), prior substrate addition (final concentration 20 M). Fluorescence increase was monitored at 11 s intervals for 1 h. One activity unit (U) is defined as the relative fluorescence units (RFU) released per second. Similarly, cysteine endopeptidase activity was tested upon the fluorogenic substrate N–Cbz–Phe–Arg–MCA in sodium citrate (42 mM)/sodium phosphate (116 mM) buffer (pH 5.5), and DTT 10 mM solution (as described in Estrela et al. 2007). Pepsin and papain were used as positive controls for cathepsin D – like (aspartic endopeptidase) and cathepsin L – like (cysteine endopeptidase) activities, respectively. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.7- Enzyme purification and gel-filtration analysis of enzyme association

RmLCE was purified from 1 to 12-days-old larvae, in FPLC system (GE Healthcare, Uppsala, Sweden). Homogenates were acidified with citric acid 1 M to pH 4.0 and incubated at 37 °C for 15 h (autolysis). Preparation was then centrifuged (6,500 g for 10 min) and the supernatant was applied (0.5 mL/min) onto a Superdex™ 75 HR 10/30 (10 x 300 mm) gel-filtration column, equilibrated in sodium phosphate 10 mM (pH 6.0). Fractions containing cysteine endopeptidase activity were pooled and processed in an ion-exchange chromatography (1.0 mL/min) in Mono Q™ 5/50 GL (5 x 50 mm) column, equilibrated in the same buffer. Retained proteins were eluted with NaCl gradient (0 - 0.8 M).

Aspartic endopeptidase was partially purified from larvae homogenates using a two-step chromatographic protocol in FPLC system. The material was first processed in an ion-exchange chromatography (2.0 mL/min) in HiTrap™ Q HP (1.6 x 2.5 cm) column, equilibrated in start buffer (sodium phosphate 10 mM, pH 6.0). Retained proteins were eluted with a non-linear NaCl gradient (0 - 0.8 M) in the same buffer. Fractions containing peptidase activity upon Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp at pH 3.5 in the presence of 10 M E-64, were pooled and applied (0.5 mL/min) onto a Superdex™ 75 HR 10/30 (10 x 300 mm) gel-filtration column equilibrated in start buffer. The protein content of the column eluates was monitored by absorbance at 280 nm.

Gel-filtration chromatographies were performed to study the relationship between aspartic and cysteine protease activities in partially purified preparations. A fraction (0.5 mg) from aspartic endopeptidase partial purification protocol, containing both aspartic and cysteine endopeptidase activities, was applied onto Superdex™ 75 HR 10/30 (10 x 300 mm) column equilibrated with sodium acetate 10 mM, pH 4.0, in the presence or absence of 1 M NaCl. Protein content of eluted fractions were monitored by absorbance reading at 280 nm and analyzed by SDS-PAGE and dot-blot. Enzymatic activity was tested as described above.

2.8 - Amino acid sequence analysis

For amino acid sequencing by Edman degradation, sample was subjected to SDS-PAGE on 12% (w/v) polyacrylamide gel and then electroblotted to a PVDF membrane

(Bio-Rad, USA). Proteins visualized with Coomassie brilliant blue were excised for sequencing. The N-terminal amino acid sequence determination was performed on a protein sequencer (PPSQ-23A Protein Sequencer, Shimadzu, Kyoto, Japan).

For mass spectrometry analysis, purified sample (5 g) was desalted with TCA precipitation protocol, by adding equal volume of TCA 60 % (v/v) and incubating for 2 h at 4 °C. After centrifugation (6,500 g for 10 min) the pellet was washed with cold acetone for 1 h at – 20 °C, and then dissolved in 20 L of NH_4HCO_3 4 M, Urea 8 M. The proteins were reduced by addition of 5 µl 45 mM DTT and alkylated by addition of 5 µl 100 mM iodoacetamide (both 30 min at room temperature). Distilled water (130 L) and sequencing-grade modified porcine trypsin (Promega Corporation, Madison, WI, USA) 2 % (w/w) were added. Reaction was incubated at 37 °C for 24 h. Tryptic peptides solution was processed in a C 18 reverse phase mini-column equilibrated in TFA 0,046 % (v/v), eluted with acetonitrile 80 % (v/v) and concentrated in a vacuum centrifuge for analysis on a Finnigan LCQ ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data were analyzed by database searching using the Sequest algorithm.

3 - Results

3.1 - RmLCE purification

A new protocol was established to purify the cysteine endopeptidase activity first described in Estrela et al., 2007 (RmLCE). A homogeneous preparation of the enzyme was obtained after treatment of larva homogenates by autolysis at 37 °C, pH 4.0, followed by two chromatographic separations at pH 6.0; gel-filtration in Superdex 75 and Mono Q ion-exchange chromatography (Fig. 1 A and B, and Table 1). The autolysis procedure resulted in an almost 3-fold increase in total activity (Table 1). A visible reduction in the brownish color of the preparation indicated that the bulk of Vt was removed. Fig. 1A shows the gel-filtration fractionation of autolyzed larva homogenates. Cysteine endopeptidase containing-fractions eluted with a low total protein content (high specific activity), although the activity peak was partially overlapped by an abundant, non-enzymatic protein peak. Active fractions were pooled and applied onto an ion-exchange column (Fig. 1B). Elution with a

NaCl gradient resulted in a fraction with high specific activity, containing 38% of the total cysteine endopeptidase activity with a purification factor of 3,794 fold (Table 1).

The homogeneity of the purified enzyme was demonstrated by SDS-PAGE (Fig. 1C, left panel). A single protein of approximately 30 kDa was detected by silver staining. A similar eletrophoretic migration was observed in an Hb-degrading zymogram of a partially purified RmLCE preparation (Fig. 1C, center panel). This sample also showed a 30 kDa protein recognized by anti-cysteine protease (BmCL1) rabbit antiserum (Fig. 1C, right panel). Four peptides derived from purified RmLCE, analyzed by mass spectrometry (Fig. 1D), showed 100 % identity with the deduced amino acid sequence of a previously described cysteine endopeptidase cloned from a ***R. microplus*** larvae cDNA library (Renard et al., 2000).

3.2 - Vitellin digestion by cysteine endopeptidases in ***R. microplus*** egg and larva

The protease activity of RmLCE upon egg and larval vitellin was compared to that of a vitellin-degrading cysteine endopeptidase from ***R. microplus*** eggs (VTDCE). Fig. 2A shows that, though both enzymes are able to degrade Vt from egg and larva, egg Vt is more rapidly degraded by the egg enzyme (VTDCE; Fig. 2A, left panel, gray bars), fluorescence of RmLCE reaction products after 60 min representing only 31 % of that released by egg Vt incubated with VTDCE. After 120 min (Fig. 2A, left panel, black bars), this difference decreased and RmLCE presented almost 90 % of the Vt-degrading activity of VTDCE. The scenario was reversed when analyzing larval Vt degradation. RmLCE was more active upon this substrate, showing higher fluorescence release (34 % and 22 % at 60 and 120 min, respectively) than VTDCE (Fig.2A, right panel).

SDS-PAGE analysis of purified vitellins (Fig. 2B) showed that egg Vt presented a disperse pattern of high molecular weight (>40 kDa) subunities, while larval Vt is mainly composed of a 90 kDa-polypeptide, also observed in egg Vt. Analyzing the reaction products of egg Vt digestion by VTDCE and RmLCE after 120 min of incubation (Fig. 2B, left panel), and comparing with non-digested egg Vt, the main difference (indicated by *) is that the 90 kDa Vt- polypeptide was poorly degraded by the egg cysteine endopeptidase while it was readily degraded by the larval enzyme. The difference between VTDCE and RmLCE ability to degrade larval Vt was also observed by SDS-PAGE analysis (Fig. 2B,

right panel). After 120 min- incubation with VTDCE, there was a slight decrease in 90 kDa-protein intensity and the appearance of lower molecular weight products. Contrarily, RmLCE exhibited a more rapid degradation of the major larval Vt polypeptide as well as digestion of the lower molecular weight peptides.

3.3 - Digestion of hemoglobin by RmLCE

Purified RmLCE also degraded hemoglobin, as shown in Fig. 3A. Reaction products of Hb degradation, after 90 min of incubation with RmLCE at 37 °C, pH 4.0, were quantified by the fluorescamine method. RmLCE activity upon Hb increased fluorescence release 11-fold when compared to the intact Hb control (Ctrl). The presence of cysteine endopeptidase inhibitor (E-64; 10 M) abolished Hb digestion by RmLCE to the control level. In accordance with a digestive role for RmLCE, larval gut presented cysteine endopeptidase activity at pH 4.0 (Fig. 3B).

3.4 - Peptidase activity in egg, larva and partially engorged female gut

Using a fluorogenic peptide substrate preferred by cathepsin-D (Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp), an acidic peptidase activity was detected in *R. microplus* egg, larvae and female gut (Fig. 4A). Cysteine endopeptidase inhibitor (E-64) had a minor, effect upon the hydrolysis of the synthetic substrate. Egg homogenate retained more than 90 % activity in the presence of E-64, while this inhibitor abolished about 25 % of the larval and gut activity. In turn, aspartic protease inhibitor, pepstatin A, inhibited 50 % of the egg and larval activities and almost 95 % of the gut peptidase activity. Moreover, a combination of both inhibitors, inhibited substrate degradation by all three homogenates (residual activity < 10%).

Larval and young female gut homogenates were able to degrade both vitellin and hemoglobin at pH 4.0, 37 °C (Fig. 4B). Vitellin hydrolysis by larvae was not affected by aspartic endopeptidase inhibitor pepstatin A. In gut homogenates, Vt-degrading activity decreased by 32 % in the presence of this inhibitor. Pepstatin A had a greater effect upon hemoglobin hydrolysis by larvae (52 % inhibition), while Hb-degrading activity in gut decreased only 16 % in the presence of this aspartic endopeptidase inhibitor. Cysteine

endopeptidase inhibitor (E-64) completely abolished Vt and Hb proteolysis by larva and female gut homogenates (Fig. 4B).

3.5 - Aspartic endopeptidase purification from ***R. microplus*** larva

Larva homogenate was fractioned by ion-exchange chromatography, in HiTrap Q column, and aspartic endopeptidase activity eluted between 0.2 M and 0.4 M NaCl (Fig. 5A). The fractions containing highest activity upon synthetic substrate for cathepsin-D (Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp) were further processed on a Superdex 75 column (Fig. 5B). The peak containing highest peptidase activity upon Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp, was mainly composed of a 47 kDa protein recognized by anti-***Boophilus*** Yolk pro-Cathepsin (anti-BYC) rabbit antiserum in Western blot (Fig. 5C). This fraction co-eluted with cysteine endopeptidase activity (data not shown). N-terminal amino acid sequence by Edman degradation of a partially purified larval aspartic protease preparation showed 100 % identity (19 N-terminal residues, positions 15 to 33) with BYC amino acid sequence (Fig. 5D). Also a 19-amino acids peptide presenting 100 % identity with a BYC internal sequence was detected (Fig. 5D).

3.6 - Enzyme association

Further analysis of partially purified aspartic endopeptidase by gel-filtration at pH 4.0 showed that cysteine endopeptidase activity co-eluted with BYC (Fig. 6A). The three eluted protein peaks presented cysteine endopeptidase activity upon the synthetic substrate N-Cbz-Phe-Arg-MCA (Fig. 6A; black bars 1, 2 and 3). SDS-PAGE (Fig. 6B, upper panel) revealed a characteristic pattern of BYC processing, corresponding to a proteolytic activation of BYC by the successive cleavage of two pro-enzyme isoforms (54 kDa and 49 kDa) producing the mature form of BYC (47 kDa). All forms were recognized by anti-BYC (Fig. 6B, lower panel).

The presence of 1 M NaCl disrupted enzyme association (Fig. 6C). This is demonstrated by the cysteine endopeptidase activity peak (1) elution from gel-filtration isolated from the other protein peak (2). SDS-PAGE and immunoblotting analysis of the two fractions (Fig. 6D) revealed the protein peak is mainly composed of a 47 kDa protein and is recognized by anti-BYC rabbit antiserum in a dot blot. The cysteine endopetidase

containing-fraction (Fig. 6C; 1), in turn, was not recognized by anti-BYC antibodies and was recognized only by anti-cysteine protease rabbit antiserum.

3.7 - BYC and RmLCE synergistic activity upon hemoglobin

Hb-degrading activity was analyzed in five different reactions using purified larval cysteine endopeptidase (RmLCE) and partially purified larval BYC (Fig. 7). After 6 h incubation, degradation of Hb by RmLCE and BYC alone resulted in 317 and 222 RFU, respectively. Simultaneous action of these two enzymes increased fluorescence to 615 units. Simulating an enzymatic cascade by pre-incubating Hb with RmLCE for 3 h and then adding cysteine endopeptidase inhibitor (E-64) before incubation with BYC for 3 h, resulted in Hb degrading activity not much greater than the activity of each enzyme alone (473 RFU). However, when the cascade is inverted and Hb is pre-incubated with BYC before inhibition by pepstatin A and RmLCE addition, Hb degradation was greatly increased (1104 RFU).

4 - Discussion

Arthropods with haematophagous habits such as the cattle tick *Rhipicephalus microplus*, pass through a transition phase when the nutrient source shifts from maternal derived- yolk reserves to blood meal. Initially, a differential gastrulation process encloses the yolk material in the embryo gut (Gillott, 1980), where it is stored during development. After attachment, degradation of blood-acquired nutrients occurs in the larval gut. Vitellin, the main protein for tick nutrition during the unfed larval stage, is then gradually replaced by major host proteins, mainly hemoglobin. An important, common feature of yolk- and blood-proteins digestion in ticks is the acid environment where these reactions occur. Vitellin proteolysis depends on acidification of yolk granules (Fagotto, 1995), while hemoglobin digestion is an intracellular process catalyzed by acid hydrolases in digestive cells vacuoles (Lara et al., 2005). Understanding the biochemistry of digestion in ticks is an important issue that must be assessed as an overall process. A vital axis of tick physiology involves the conversion of blood meal to yolk components and digestion of such yolk reserves during starvation stages, linking nutritional and reproductive aspects (Grandjean, 1983).

Here we described a novel protocol to purify an acidic Vt-degrading enzyme (*Rhipicephalus microplus* Larval Cysteine Endopeptidase; RmLCE). Compared to the previous protocol (Estrela et al., 2007), much better purification (3,794 x) was achieved. The enzyme was identified as a 30 kDa-protein with amino acid sequence identity with *Boophilus microplus* Cathepsin-L 1 (Renard et al., 2000). Other characteristic features such as optimal pH (5.0 - 5.5), substrate specificity and molecular weight suggest we have purified the native form of the enzyme specified by the cDNA cloned by Renard et al. (2000). Also, expression of BmCL1 has been described in 5- to 10-day old larvae and in the female gut (Renard et al., 2002).

An investigation of stage-specific vitellin digestion by RmLCE and VTDCE (Fig. 2), revealed a differential pattern of Vt-degradation by the two enzymes. Our results suggest that RmLCE is responsible for digestion of Vt polypeptides not degraded during egg development, completing Vt-digestion initiated by embryo enzymes and providing the energy for unfed larvae development. RmLCE was also able to degrade hemoglobin at acid pH, which is compatible with a continued digestive role in protein digestion during *R. microplus* development. Indeed, this is the first demonstration on the presence of a cysteine endopeptidase activity in larval gut, the site of Vt degradation in unfed larvae and of Hb digestion after the blood ingestion starts.

Thus, in order to have a wider perspective of protein digestion enzymes at different stages of *R. microplus* life cycle, acidic peptidase activities present in eggs, larvae and young female gut were investigated (Fig. 4). Inhibition profile using aspartic and cysteine endopetidase specific inhibitors confirmed the presence of both classes of enzymes in these homogenates, although stage specific differences had been observed. Mendiola et al. (1996) showed some features of protease activity present in fully engorged female gut crude extracts. Using a variety of synthetic peptides and natural protein substrates (hemoglobin, albumin, casein and immunoglobulin G), these authors found that proteolysis occurs exclusively at acid pH (below pH 6.0), and that aspartic and cysteine endopeptidases are the major enzymes responsible for this activity.

Native and recombinant aspartic endopeptidase *Boophilus* Yolk pro-Cathepsin (BYC) have been tested in bovine immunization trials, and a partial efficacy, ranging from 14 % to 36 % of overall protection against *R. microplus* challenges, was observed (Da Silva Vaz Jr

et al., 1998; Leal et al., 2006). The aspartic endopeptidase present in larva homogenates was partially purified and characterized. Analyses of the final preparations confirm identity of the enzyme. In addition to recognition by polyclonal anti-BYC antibodies, the N-terminal sequence of the 47 kDa-protein obtained is coincident with BYC N-terminal sequence after cleavage of a putative signal peptide. Actually, the same chromatographic principles used to purify BYC from tick eggs (Logullo et al., 1998) were applied for larval BYC purification. A sequence of anion-exchange and size-exclusion fractioning was able to isolate the abundant aspartic protease from vitellin (Vt), the major protein present in egg and larva extracts. Applying the same chromatographic protocol to purify BYC from partially engorged female gut resulted in a final preparation containing the 47 kDa protein also recognized by anti-BYC in Western blots (data nor shown). The presence of BYC in newly hatched larvae has already been briefly reported by Logullo et al. (1998). BYC was described as an abundant egg enzyme, activated by auto proteolysis at acid pH and involved in yolk degradation processes (Logullo et al., 1998). The relationship between BYC Vt-degrading activity and cortical acidification during egg development (Abreu et al., 2004) and the particularities of BYC amino acid sequence and structure, likely responsible for low specific activity upon Vt (Nascimento-Silva et al., 2008) were demonstrated. However, detailed studies of BYC in later stages of the tick life cycle and its possible roles in general protein digestion, had not been carried out to date.

Besides a role in yolk degradation in ***R. microplus*** eggs (Logullo et al., 1998; Sorgine et al., 2000; Seixas et al., 2003) and larvae (Estrela et al., 2007), participation of acidic aspartic and/or cysteine endopeptidases in host-proteins digestion is well documented in some ticks, as well as in other parasites (Nisbet & Billingsley, 2000; Sajid & McKerrow, 2002). In this context, acid hydrolysis of Vt and Hb by tick larva and gut homogenates, and the contribution of aspartic and cysteine endopeptidases to these activities, were investigated in ***R. microplus***. The use of specific inhibitors showed the presence of both aspartic and cysteine endopeptidase activity upon Vt and Hb (Fig. 4). Apparently, aspartic protease contribution to vitellin degradation is more prominent in gut. In larvae, this class of peptidase is most active upon hemoglobin. Protein digestion in these stages of the tick life cycle (larvae and young adult female gut) was completely prevented by cysteine endopeptidase inhibitor. Protein degradation by egg extracts was poorly or not detectable,

possibly due to the presence of controlling mechanisms responsible for limited proteolysis in tick eggs (Fagotto, 1995).

In addition to the information about class-specific contribution to proteolytic activity, results in Fig. 4 revealed an interesting pattern of inhibition. The high inhibition of synthetic substrate degradation by the combined action of pepstatin A and E-64 can be due to the sum of distinct activities, although this seems not to be the case for egg and larva homogenates. By the other hand, despite the partial contribution of aspartic endopeptidases to Vt and Hb hydrolysis, the degradation of these proteins is completely prevented by cysteine endopeptidase inhibitor, E-64. These data indicate a possible synergic role of the different enzymes in protein digestion.

Analysis by gel-filtration chromatography of partially purified larval BYC from ***R. microplus*** offers some evidence of association between this Vt- and Hb- degrading aspartic endopeptidase and a cysteine endopeptidase activity, at acid conditions. Co-elution of cysteine protease activity with three BYC-containing protein peaks, likely corresponding to different stages of BYC proteolytic activation, was demonstrated (Fig. 6). This association was disrupted in the presence of 1 M NaCl. Interestingly, the elution of the associated proteins in gel-filtration carried out at low pH does not correspond to the actual size, suggesting this pH favors protein binding to the chromatographic matrix. Probably this is a BYC property, because, even after dissociation by NaCl, BYC (47 kDa) eluted from gel-filtration column after RmLCE (30 kDa).

Many studies demonstrated the presence of digestive-enzymes cascade systems responsible for hemoglobin breakdown in hematophagous endoparasites. A review by Williamson et al. (2003) describes the presence of aspartic, cysteine and metallo endopeptidases in blood-feeding nematodes and suggests these enzymes form a proteolytic cascade for Hb digestion. This hypothesis was further explored in ***Schistosoma mansoni*** (Delcroix et al., 2006) and ***Ancylostoma caninum*** (Williamson et al., 2004). Only recently the presence of a similar enzymatic system was suggested in ixodid ticks. Characterization of cysteine proteases (asparagyl endopeptidases or legumains) in ***Haemaphysalis longicornis*** (HlLgm; Abdul Alim et al., 2007) and in ***Ixodes ricinus*** (IrAE; Sojka et al., 2007) indicates the participation of these enzymes in an Hb-degrading network in tick gut. A functional activity scan of the peptidase complement in gut tissue extracts, followed by

genetic screens of gut-derived cDNA, was conducted to elucidate this hypothesis in *I. ricinus* (Sojka et al., 2008). Some of these studies, particularly in nematodes, pointed an aspartic protease as responsible for the first step in Hb proteolysis. In ticks, however, the cascade is often described as semi-ordered and the sequence of reactions is not well established. The clear importance of cysteine endopeptidases in these cases may involve their ability to do trans-processing proteolysis. Indeed, in addition to Hb direct hydrolysis, it is possible that IrAE contributes to activation of other cysteine and aspartic proteases (Sojka et al., 2007). Also, many yolk-degrading enzymes in ticks suffer proteolytic processing prior to activation (Fagotto, 1990b; Logullo et al., 1998)

A direct analysis of hemoglobin digestion using purified cysteine endopeptidase (RmLCE) and aspartic endopeptidase (BYC) from *R. microplus* larvae, provided more evidence that these two protease classes work together in protein digestion in cattle tick. Prior incubation of Hb with BYC increased RmLCE activity upon this substrate, showing that digestion of Hb occurs initially by the action of BYC and the hydrolysis of resultant peptides is completed by RmLCE. Taken together, the results point to a model of enzymatic network responsible for hemoglobin digestion in *R. microplus* similar to the model described in *S. mansoni* (Delcroix et al. 2006) and proposed to *I. ricinus* (Sojka et al., 2007). Accordingly, aspartic endopeptidases are implicated in initial Hb breakdown and cysteine endopeptidases contribute to activation of these initializing enzymes, as well as in digestion of resultant Hb peptides.

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Figures and Tables

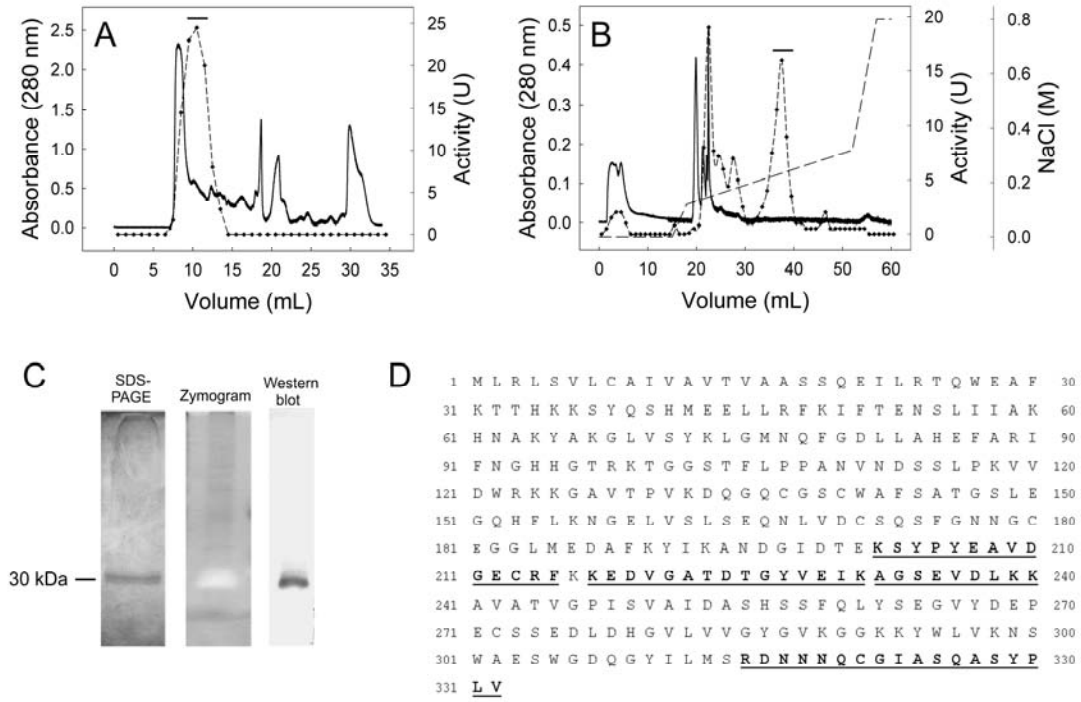

Fig. 1 - RmLCE purification. Elution profile of proteins (solid line) and peptidase activity (short-dash line) from: **(A)** autolysed larvae homogenate applied onto a gel-filtration chromatography (Superdex™ 75 HR 10/30; 10×300 mm) equilibrated in 10 mM sodium phosphate (pH 6.0); and **(B)** selected active fractions from the first step applied onto an ion-exchange column (Mono Q™ 5/50 GL; 5 x 50 mm) in the same buffer. Elution with NaCl gradient (0 – 0.8 M; long-dash line). Peptidase activity was tested upon N-Cbz-Phe-Arg-MCA at 37 °C in sodium citrate (42 mM) /sodium phosphate (116 mM) buffer (pH 5.5), DTT 10 mM. Black bars indicate pooled active fractions. **(C)** Left panel: SDS-PAGE 12% of purified RmLCE (5 g) visualized by silver staining; Center panel: zymogram at pH 4.0 showing Hb-degrading activity of partially purified RmLCE (active fractions from the first purification step, 40 g), stained with Coomassie brilliant blue; Right panel: Western blot analysis of partially purified RmLCE (35 g) probed with anti-BmCL1 antibody (1:100), labeled with anti-rabbit Ig-alkaline phosphatase conjugate and revealed with NBT/BCIP as substrate. Molecular weight, calculated from migration of standard proteins, is indicated on the left. **(D)** Partial sequence analysis of purified RmLCE by mass spectrometry showing RmLCE peptides (underlined, bold) identity with deduced amino acid sequence from *Boophilus microplus* cathepsin-L 1 (*Bmcl*1) gene (accession number AF227957).

Table 1 - Purification process for *R. microplus* Larval Cysteine Endopeptidase (RmLCE)

Purification Step	Volume (mL)	Protein		Activity		Purification (fold)	Yield (%)
		mg/mL	total (mg)	total (U)	specific (U/mg)		
homogenate	7.0	50.80	355.60	11060	31	1	-----
autolysis	7.0	17.50	122.50	30520	249	8	100
Superdex 75	3.0	2.00	6.00	11070	1845	59	85
Mono Q	2.0	0.0142	0.0284	3340	117606	3794	38

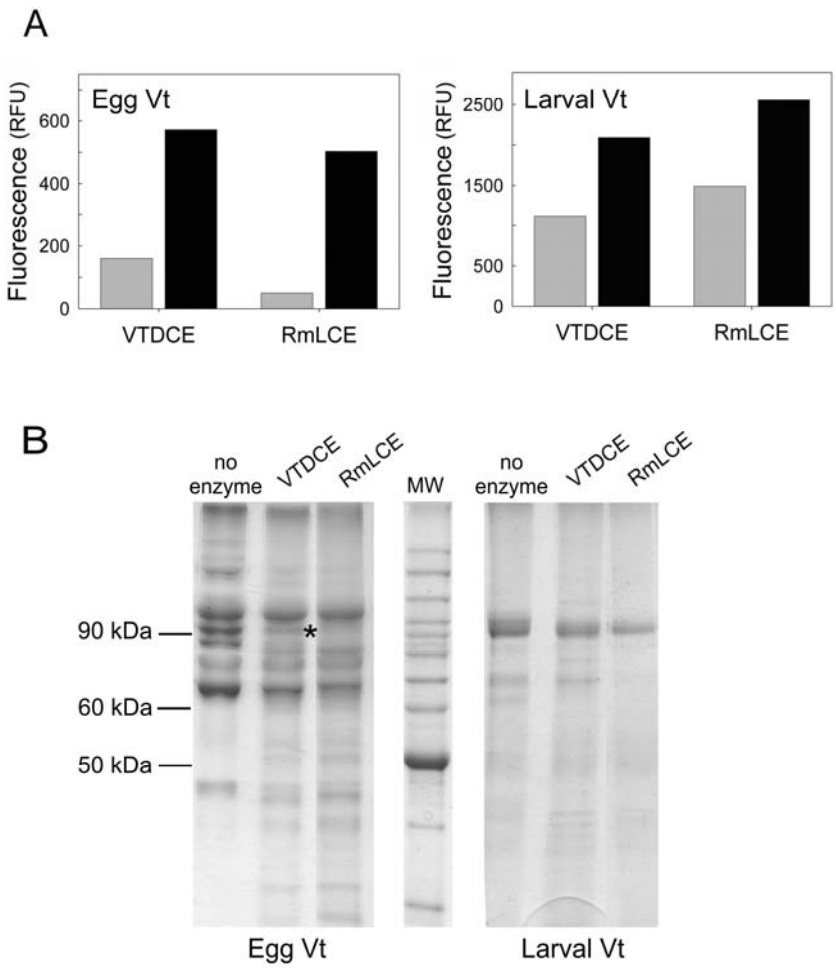

Fig. 2 - Vitellin digestion by egg (VTDCE) and larval (RmLCE) cysteine endopeptidases. **(A)** Vt proteolysis after 60 min (gray bars) and 120 min (black bars) of incubation at 37 °C in sodium citrate (61 mM)/sodium phosphate (77 mM) buffer (pH 4.0), DTT 10 mM. Egg Vt (left panel) or larval Vt (right panel), 1.4 g/ L, was incubated with VTDCE or RmLCE (1 U). Products were quantified by the fluorescamine method; RFU: relative fluorescence units. **(B)** Analysis by SDS-PAGE 10% of egg Vt (28 g/lane; left panel) and larval Vt (28 g/lane; right panel) not degraded (no enzyme) and after a 120 min-incubation at 37 °C, in sodium citrate (61 mM)/sodium phosphate (77 mM) buffer (pH 4.0), DTT 10 mM, with VTDCE or RmLCE (1 U). Relative position of molecular weight standard (MW) is indicated on the left. * A 90-kDa polypeptide degraded by RmLCE.

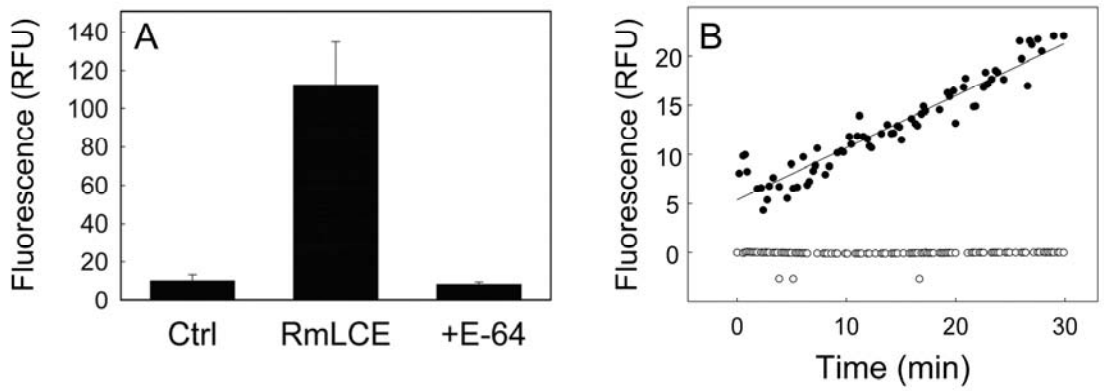

Fig. 3 - Hemoglobin-degrading activity of RmLCE **(A)** Hb (0.5 g/ L) incubated for 18 h at 37 °C, in sodium acetate 10 mM (pH 4.0), DTT 10 mM, without enzyme (Ctrl), with purified cysteine endopeptidase (RmLCE) or with RmLCE in the presence of cysteine endopeptidase inhibitor (E-64; 10 M). Mean ± SD for three replicates. **(B)** Peptidase activity of larval gut homogenates upon N-Cbz-Phe-Arg-MCA at 37 °C in sodium citrate (42 mM)/sodium phosphate (116 mM) buffer (pH 5.5), DTT 10 mM, in the presence (open circles) or absence (solid circles) of cysteine endopetidase inhibitor (E-64; 10 M).

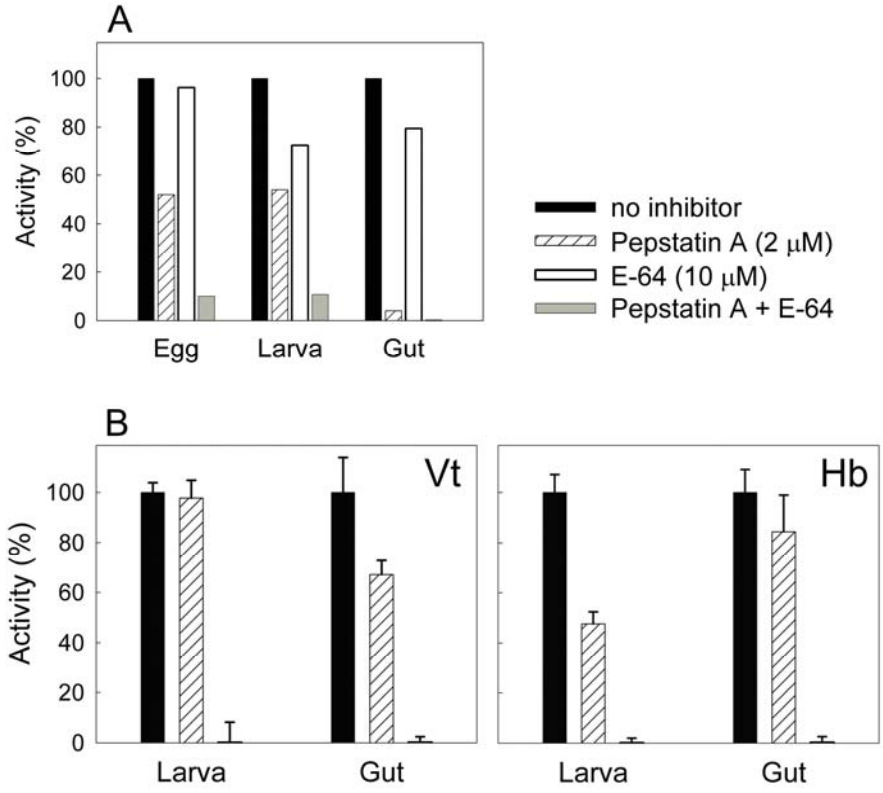

Fig. 4 - Inhibition of peptidase activities in *Rhipicephalus microplus* eggs, larvae and female gut. **(A)** Activity was tested upon Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp. Homogenates (10 L) were incubated for 30 min at 37 °C in sodium citrate (68 mM)/sodium phosphate (64 mM) buffer (pH 3.5), in the absence or presence of inhibitors (pepstatin A 2 M and/or E-64 10 M). Subsequently, the synthetic substrate (20 M) was added and the enzymatic activity determined as described in materials and methods. **(B)** Activity upon vitellin (Vt; left panel) and hemoglobin (Hb; right panel). Protein substrates (1 mg) were incubated with extracts (250 g of protein) for 6 h at 37 °C, in sodium acetate 10 mM (pH 4.0), in the absence or presence of inhibitors (pepstatin A 2 M or E-64 10 M). Reaction products were quantified by the fluorescamine method. Mean ± SD for three replicates.

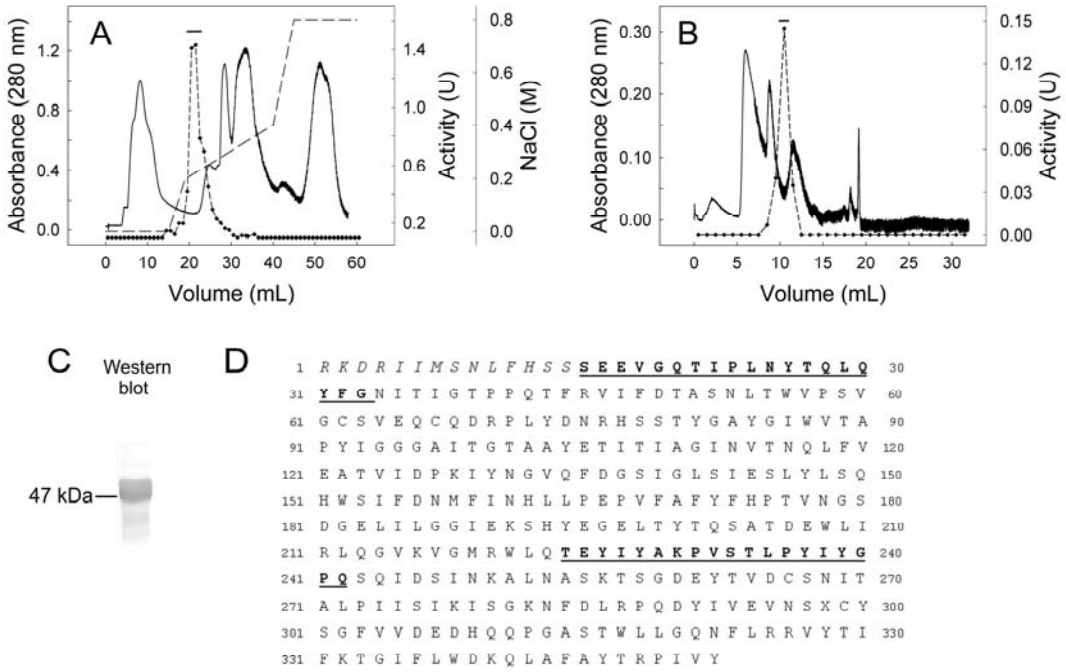

Fig. 5 - Aspartic endopeptidase partial purification. Elution profile of proteins (solid line) and peptidase activity (short-dash line): **(A)** larval extracts applied onto an ion-exchange column (HiTrap™ Q HP; 1.6×2.5 cm) in 10 mM sodium phosphate (pH 6.0). Proteins were eluted with NaCl gradient (0 - 0.8 M; long-dash line); **(B)** selected active fractions from the first step applied onto a gel-filtration column (Superdex™ 75 HR 10/30; 10×300 mm) in the same buffer. The peptidase activity of each fraction was tested upon Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Gln-EDDnp at 37 °C in sodium citrate (68 mM)/sodium phosphate (64 mM) buffer (pH 3.5). Black bars indicate selected active fractions. **(C)** Western blot analysis of partially purified aspartic endopeptidase (50 g) probed with anti-BYC antibody (1:250), labeled with anti-rabbit Ig-alkaline phosphatase conjugate and revealed with NBT/BCIP as substrate. Molecular weight, calculated from migration of standard proteins, is indicated on the left. **(D)** Partial sequence analysis of partially purified aspartic endopeptidase by Edman degradation showing peptides (underlined, bold) identity with amino acid sequence from *Boophilus* Yolk pro-Cathepsin (BYC; accession number AAX76981).

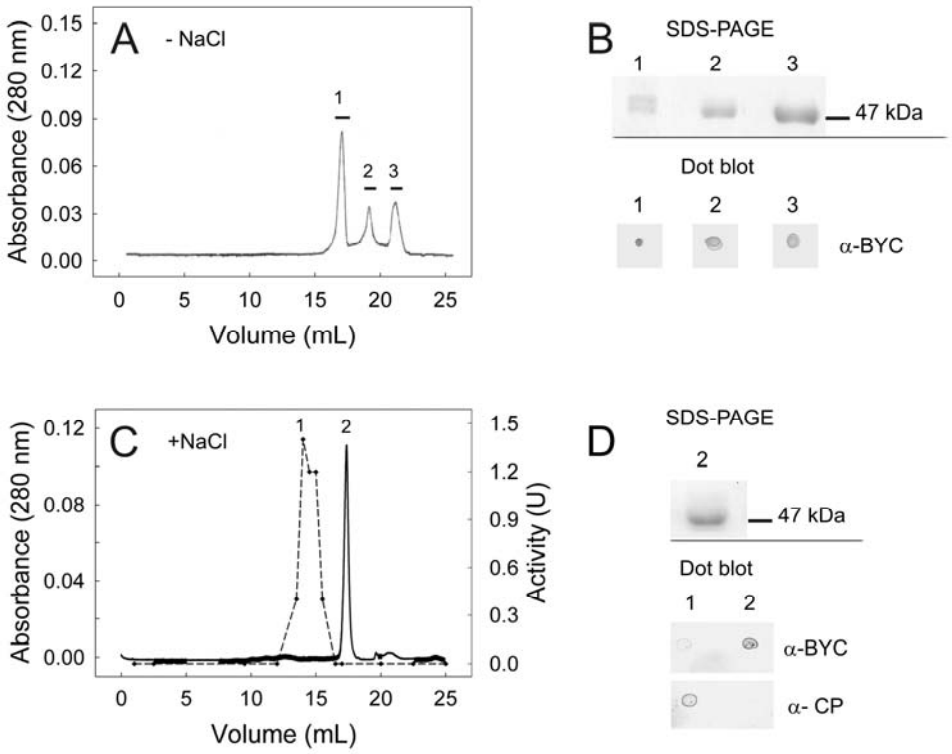

Fig. 6 - Gel-filtration analysis of enzyme association. **(A)** Co-elution of partially purified larval-BYC (1 mg) with cysteine endopeptidase activity in gel-filtration chromatography (Superdex™ 75 HR 10/30; 10×300 mm) equilibrated with sodium acetate 10 mM (pH 4.0). Bars indicate protein peaks (1, 2 and 3) containing cysteine endopeptidase activity upon N–Cbz–Phe–Arg–MCA at 37 °C in sodium citrate (42 mM)/sodium phosphate (116 mM) buffer (pH 5.5), DTT 10 mM. **(B)** SDS-PAGE 12% (upper panel; 20 g/lane) and dot blot (lower panel; 0.1 g/dot) of selected active fractions indicated in (A). SDS-PAGE was stained with Coomassie brilliant blue. Relative position of molecular weight standard is indicated on the right. Immunoblotting was probed with anti-BYC antibody (1:250), labeled with anti-rabbit Ig-alkaline phosphatase conjugate and revealed with NBT/BCIP as substrate. **(C)** Chromatographic separation of partially purified larval-BYC (600 g) from associated cysteine endopeptidase activity by gel-filtration chromatography (Superdex™ 75 HR 10 /30; 10×300 mm) equilibrated with sodium acetate 10 mM (pH 4.0), in the presence of 1 M NaCl. Solid line shows elution profile of proteins and short-dash line shows cysteine endopeptidase activity of fractions tested upon N–Cbz–Phe–Arg–MCA at 37 °C sodium citrate (42 mM)/sodium phosphate (116 mM) buffer (pH 5.5), DTT 10 mM. Bars indicate cysteine endopeptidase activity peak (1) and protein peak (2). **(D)** SDS-PAGE 12% (upper panel; 20 g/lane) and dot blot (lower panel; 0.1 g/lane) of selected fractions indicated in (C). SDS-PAGE was stained with Coomassie brilliant blue. Relative position of molecular weight standard is indicated on the right. Immunoblotting was probed with anti-BYC antibody (1:250) or anti-BmCL1 antibody (1:100) and labeled with anti-rabbit Ig-alkaline phosphatase conjugate and revealed with NBT/BCIP as substrate.

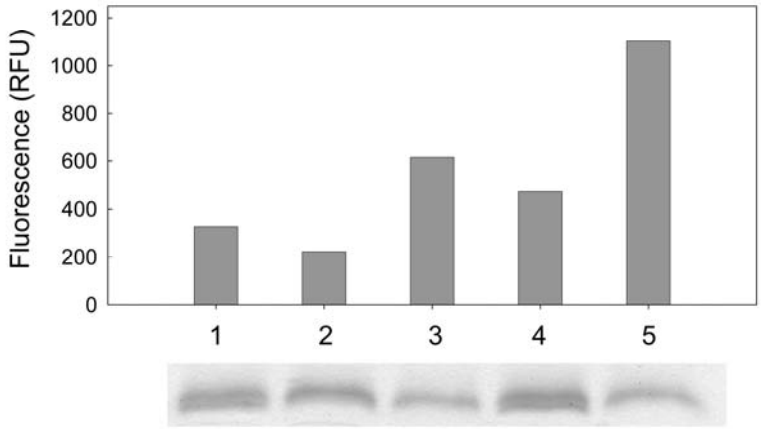

Fig. 7 - Synergistic action of RmLCE and larval BYC upon hemoglobin. Quantification (fluorescamine method) of products generated by Hb incubation at 37 °C in sodium citrate (61 mM)/sodium phosphate (77 mM) buffer (pH 4.0), submitted to the following enzyme treatments: 1) RmLCE alone for 6 h; 2) BYC alone for 6 h; 3) RmLCE and BYC for 6 h; 4) RmLCE for 3 h, addition of E-64 (10 M) and then BYC for 3h; 5) BYC for 3 h, addition of pepstatin A (2 M) and then RmLCE for 3 h. Lower panel shows SDS-PAGE 14% of reaction products (40 g/lane).

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O carrapato bovino ***Rhipicephalus (Boophilus) microplus*** está entre as espécies de carrapato de maior impacto econômico. Apesar da pesquisa intensiva na busca por métodos de controle mais eficientes, o conhecimento sobre a bioquímica e fisiologia deste parasita ainda é escasso e fragmentado, impedindo o avanço neste campo (Willadsen, 2006b). O presente estudo traz novas informações sobre a maquinaria enzimática envolvida na digestão de proteínas em diferentes estágios do ciclo de vida de ***R. microplus***, considerando a importância deste processo na biologia do carrapato.

Neste trabalho, desenvolvemos um novo e eficiente protocolo para a purificação de uma cisteíno endopeptidase de larvas de ***R. microplus*** (***Rhipicephalus microplus*** Larval Cysteine Endopeptidase; RmLCE). A seqüência parcial de aminoácidos de RmLCE purificada mostrou identidade com BmCL1 (***Boophilus microplus*** Cathepsin-L1), uma cisteíno endopeptidase clonada a partir de uma biblioteca de cDNA de larvas (Renard et al., 2000). RmLCE foi reconhecida pelos anticorpos policlonais gerados contra BmCL1 recombinante, que detectaram a expressão de BmCL1 em extratos de larvas e em vesículas intracelulares em intestino de fêmeas parcialmente ingurgitadas (Renard et al., 2002). Algumas propriedades enzimáticas em comum também foram identificadas entre RmLCE e BmCL1 recombinante, que apresenta pH ótimo de 5,5 e atividade sobre vitelina e hemoglobina em pH ácido (Renard et al., 2000). É possível que RmLCE seja a forma nativa da enzima codificada pelo gene ***Bm***cl1.

Nossos resultados indicam que RmLCE atua na digestão de vitelina (Vt) em larvas não-alimentadas. A degradação desta proteína de reserva é importante para a sobrevivência das larvas na fase que antecede o início da alimentação hematófaga. Efetivamente, menos da metade da Vt presente nos ovos é consumida antes da eclosão (Campos et al., 2006). Interessantemente, RmLCE apresenta atividade sobre um polipeptídeo de Vt que não é totalmente degradado no ovo, indicando ser uma atividade estágio-específica e que complementa a ação da enzima da mesma classe encontrada em ovos (VTDCE). Esta especialização parece fazer parte de um mecanismo que evitaria o consumo excessivo de Vt pelo embrião para disponibilizar material nutritivo para a fase larval.

Além disso, mostramos a capacidade de RmLCE de degradar hemoglobina. A demonstração de que a atividade cisteíno endopeptidásica está presente em intestino de

larvas não-alimentadas é compatível com um papel desta enzima na degradação de hemoglobina. Assim, é possível que a enzima seja também responsável pela digestão de hemoglobina durante a fase parasitária. De fato, nossos resultados indicam que a degradação de vitelina em larvas é efetuada principalmente por cisteíno endopeptidases, enquanto a degradação de hemoglobina, tanto em larvas como em intestino de partenóginas, depende da atuação de atividades cisteíno e aspártico endopeptidásicas acídicas. Esta observação está de acordo com dados da literatura que mostram a presença de cisteíno e aspártico endopeptidases em intestino de teleóginas (Mendiola et al. 1996).

Determinamos um protocolo de purificação para uma aspártico endopeptidase de larvas de *R. microplus*, identificada como *Boophilus* Yolk Cathepsin (BYC). Esta enzima foi caracterizada em ovos como responsável pela degradação de vitelina (Logullo et al., 1998). No entanto, BYC apresenta uma atividade específica muito baixa sobre este substrato, degradando-o lentamente (Nascimento-Silva et al., 2008). Nossos resultados mostram que, em larvas, BYC está fisicamente associada a uma cisteíno endopeptidase, atuando na digestão de hemoglobina. Demonstramos que BYC e RmLCE agem de forma ordenada para degradar Hb, em uma cascata enzimática iniciada por BYC. Este tipo de interação enzimática, embora já descrito em outros parasitas hematófagos, como platelmintos (Delcroix et al., 2006) e o carrapato *Ixodes ricinus* (Sojka et al., 2007), ainda não havia sido comprovado em *R. microplus*.

Mais estudos são necessários para entender completamente a natureza e o funcionamento destas interações. A importância fisiológica dos seus componentes, no entanto, é evidente, uma vez que abrange a digestão das principais fontes nutricionais em diferentes estágios do ciclo de vida do carrapato. Enzimas proteolíticas e outras proteínas de intestino são freqüentemente descritas como alvos para o desenvolvimento de uma vacina contra carrapatos, porém, muitos dos experimentos de imunização têm resultado em efeitos insuficientes para garantir a eficácia de uma vacina. Uma abordagem sugerida o para o uso destes antígenos é a sua combinação em vacinas multiantigênicas, potencialmente mais eficazes do que os antígenos aplicados isoladamente (Willadsen, 2006a).

Os resultados apresentados aqui apontam a cisteíno endopeptidase de larvas de *R. microplus* (RmLCE) como uma molécula de grande importância na fisiologia do parasita e,

portanto, um potencial alvo para controle imunológico. Além disso, a demonstração de que esta enzima atua em combinação com outras peptidases, na degradação de vitelina (VTDCE) e hemoglobina (BYC), ambas mostrando uma capacidade protetora parcial quando testadas como antígenos, sugere que RmLCE possa ser utilizada como componente na formulação de uma vacina multiantigênica contra o carrapato bovino.

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ANEXO 1

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A cysteine endopeptidase from tick (*Rhipicephalus* (*Boophilus*) *microplus*) larvae with vitellin digestion activity

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Abstract

The hard tick *Rhipicephalus* (*Boophilus*) *microplus* is a blood-sucking ectoparasite. *R. microplus* free-living stage comprises egg development, hatching, and subsequent larval development until encountering a host. In order to complete the embryological development, this tick relies on yolk reserve substances, mainly vitellin (Vt), which is still present in the larval stage. The present study demonstrates presence and digestion of Vt in unfed *R. microplus* larvae. An increasing proteolytic activity is observed in larval development, as well as a decrease in total protein and in Vt content. Partial purification and characterization of a *R. microplus* larval cysteine endopeptidase (RmLCE) with Vt-degrading activity is also described. RmLCE has optimal activity at 37 °C at pH 5.0, being unstable at pH ≥ 7.5. This enzyme is active upon fluorogenic peptide substrates and is able to degrade Vt, its putative natural substrate. These results indicate that RmLCE has a role in supporting the nutritional needs of unfed *R. microplus* larva through Vt proteolysis, allowing survival until the first blood meal.

Keywords: Cysteine endopeptidase; *Rhipicephalus* (*Boophilus*) *microplus*; RmLCE; Tick larva; Vitellin

1. Introduction

The hard tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) is a hematophagous ectoparasite of great importance to tropical and subtropical livestock because it causes not only direct economic losses by blood-sucking but is also the vector of the protozoan *Babesia* and the rickettsia *Anaplasma* (Jongejan and Uilenberg, 2004).

The free-living stage of the life cycle begins when the fully engorged female drops from host, starts oviposition, and extends through the larval stage until larva encounters a host. Sometimes, unfed larva can survive up to 160 days without a blood meal, depending on temperature, relative humidity, and other conditions (Davey et al., 1991; Corson et al., 2004).

Before hatching, embryos are sustained by a nutritional supply provided by yolk reserve substances, as occurs in oviparous species. The major reserve protein, vitellin (Vt), is a glycolipohaemoprotein produced from a precursor called vitellogenin (Vg). Vg is synthesized by female, transferred to oocytes *via* a receptor-mediated pathway (Friesen and Kaufman, 2004), processed, and then stored in structures named yolk granules (Fagotto, 1990). Maternal derived proteolytic enzymes are also internalized within yolk granules together with Vt. These proteases are responsible for Vt degradation in embryos through a process that is developmentally regulated (Giorgi et al., 1999).

Cysteine endopeptidases have frequently been described as acting in the yolk degradation processes in many species of arthropoda, such as fruit fly, *Drosophila melanogaster* (Medina et al., 1988), cockroach, *Blattella germanica* (Liu et al., 1996; Yin et al., 2001), silkworm, *Bombyx mori* (Kageyama and Takahashi, 1990), and the tick *Ornithodoros moubata* (Fagotto, 1990). In *R. microplus* eggs, vitellin-degrading cysteine endopeptidase (VTDCE) has been found to participate in vitellin degradation and embryo nutrition (Seixas et al., 2003).

Little is known about the processes responsible for larval nutrition during the period of starvation preceding the first blood meal, although some studies have reported the presence of yolk proteins and their derivatives in tick larvae. Chinzei and Yano (1985) demonstrated the presence of Vt in *O. moubata* for

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several months following hatching and a similar temporal organization of Vt has been reported for *R. microplus* (Canal et al., 1995).

The present work confirms presence of Vt in *R. microplus* larval stage and is the first report on Vt digestion during development of unfed larva. Additionally, partial purification and characterization of a novel cysteine endopeptidase (RmLCE) involved in Vt proteolysis are described.

2. Materials and methods

2.1. Biological material

Porto Alegre strain of *R. microplus* (Acari: Ixodidae) ticks was maintained on Hereford calves confined in insulated stables. Fully engorged females were kept in Petri dishes at 28 °C and 80% relative humidity for oviposition. Eggs were collected and placed in glass tubes closed with cotton and maintained in the same conditions until hatching. Larvae were separated at 1, 3, 5, 7, 9, 11, 12 and 13 days after hatching and stored at −70 °C until processed.

2.2. Larval extracts

Larval extracts were prepared by homogenizing larvae in sodium phosphate (10 mM), pH 7.0, to a final concentration of 0.1 g of larva/mL, using pestle and mortar. Preparation was centrifuged (16,000 g for 5 min at 4 °C) and supernatant was filtered through a sequence of pre-filter AP20, 0.45 μm and 0.22 μm pore filters (Millipore, Bedford, MA, USA).

The amount of protein present in extracts and other samples was estimated by absorbance reading at 260 nm and 280 nm (Layne, 1957) in a spectrophotometer or by the bicinchonic acid (BCA) method reported by Smith et al. (1985), using bovine serum albumin (BSA; Pierce, Rockford, IL, USA) solution as standard. Protein content of column eluates was monitored by absorbance reading at 280 nm.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate extracts and active fractions protein content during purification steps. SDS-PAGE was also used to separate proteins for further analysis by Western blotting. Samples were applied in a gel containing 5% (w/v) polyacrylamide (stacking gel) and 12.5% (w/v) polyacrylamide (resolving gel), run at 100 V for approximately 3 h, and stained with Coomassie brilliant blue solution. For Western blotting analysis, samples were run in 4% (w/v) polyacrylamide stacking gel, followed by a run in polyacrylamide gradient ranging from 6% to 20% (w/v) polyacrylamide gel.

2.4. Western blotting

Proteins separated by SDS-PAGE were electrically transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany) using a semi-dry system (GE-Pharmacia, Uppsala, Sweden). Membrane was then blocked overnight with 5% (w/v) skim milk solution in sodium phosphate (10 mM) and NaCl (150 mM), pH 7.2 (phosphate buffered saline; PBS) at 4 °C. Afterwards, membrane was incubated with a specific anti-vitellin monoclonal mouse antibody for 2 h, followed by incubation with alkaline phosphatase-rabbit anti-mouse IgG conjugated (Sigma-Aldrich, St. Louis, MO, USA) for 1 h, and stained with chromogenic substrate for alkaline phosphatase NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt; USB Corporation, Cleveland, OH, USA). Between procedures each membrane was washed with PBS three times for 10 min.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

Microtitration plates (96 wells) were coated overnight at 4 °C with samples (diluted 1:3600 in carbonate buffer (50 mM), pH 9.6). Plates were then washed and blocked with skim milk solution (5%, w/v in PBS) for 1 h at 37 °C. After blocking, plates were washed three times with PBS before incubation with the anti-vitellin monoclonal antibody for 1 h at 37 °C, and subsequently washed three times with a 5% casein solution before incubation with secondary antibody goat anti-mouse IgG-peroxidase conjugated (Sigma-Aldrich) for 1 h at 37 °C. Enzymatic reaction was triggered following further three PBS washes by adding 100 μL/well of a solution containing peroxidase substrate (0.034% o-phenylenediamine; OPD;

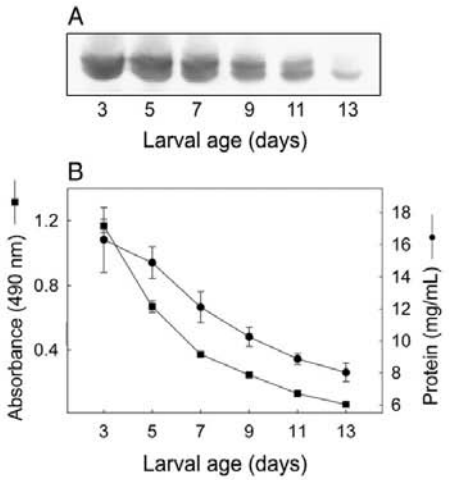

Fig. 1. Vitellin and total protein content in *R. microplus* larval extracts. (A) Larval protein extract (100 μg larvae/μL) analyzed by SDS-PAGE and transferred to a nitrocellulose membrane for analysis by western blot, hybridized with mouse anti-vitellin antibody (1:250), labeled with goat anti-mouse IgG-alkaline phosphatase-conjugated and stained for alkaline phosphatase activity. (B) Absorbance values (490 nm) in ELISA tests of larval extracts probed with anti-vitellin monoclonal antibody (1:1600) (squares), and total protein concentration of the same samples measured by BCA method (circles). Mean ± SE for three replicates. Data are representative of two independent experiments.

Sigma-Aldrich) and 0.015% (v/v) H_2O_2 in citrate-phosphate buffer (0.1 M, pH 5.0). Reaction was stopped with 50 µL/well of 12.5% (v/v) H_2SO_4 and absorbance was read at 490 nm in a Spectramax microplate spectrophotometer (Molecular Devices Corporation, CA, and USA).

2.6. Cysteine endopeptidase activity assay

Enzyme activity was tested with the fluorogenic substrate N–Cbz–Phe–Arg–MCA (Sigma-Aldrich) in sodium citrate/sodium phosphate (50 mM) buffer, pH 5.5, and DTT (Sigma-Aldrich) 10 mM solution, or under other experimental conditions, as specified in figure legends. A 10 µL aliquot of every sample was pre-incubated at 37 °C for 15 min with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.001 mM pepstatin A, and 0.5 mM ethylenediaminetetraacetic acid) prior to addition of DTT. Substrate (10 µL) was then added (final concentration of 1.4 µM) and activity was monitored by the fluorescence released at 370 $nm_{ex.}$–460 nm_{em}, measured in an fmax microplate fluorimeter (Molecular Devices Corporation, CA, USA) at 11 s intervals, during a 30 min period at 37 °C. All protease inhibitors and fluorogenic substrates used were purchased from Sigma-Aldrich and assayed under the same conditions.

2.7. Partial purification of cysteine endopeptidase activity

The RmLCE activity isolated from an extract of 1 to 12-day-old larva was partially purified using two chromatographic steps performed in a FPLC® system (GE-Pharmacia, Uppsala, Sweden): 1) ion-exchange in a HiTrap Q HP column (1.6×2.5 cm) equilibrated in sodium phosphate buffer (10 mM), pH 7.0, and eluted with a 0–0.8 M NaCl gradient in the same buffer; 2) gel filtration on Superose 12 column (10×300 mm), processed with the same buffer. After the first step, eluted fractions were tested upon a synthetic substrate (as described above) and those fractions showing high activity were pooled and concentrated in a Centricon-10 ultrafilter (Amicon Inc., Beverly, MA, USA) up to 200 µL in order to be processed in the gel-filtration step.

2.8. Inhibitory profile

Susceptibility to inhibitors was tested by incubating cysteine endopeptidase preparation for 15 min in citrate/sodium phosphate buffer (50 mM), pH of 5.5, in the presence or absence of the following classical peptidase inhibitors: chymostatin (0.1 mM); ethylenediaminetetraacetic acid (EDTA) (0.5 mM); leupeptin (0.01 mM); L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) (0.01 mM); Na-*p*-tosyl-lysine chloromethyl ketone (TLCK) (0.1 mM); pepstatin A (0.001 mM); or phenylmethylsulfonyl fluoride (PMSF) (0.1 mM). After pre-incubation with the inhibitors, the enzyme was activated with DTT (10 mM) solution for 10 min at 37 °C, and reaction started by adding the substrate N–Cbz–Phe–Arg–MCA to a final concentration of 1.4 µM. Activity was measured as a fluorescence increase at 370 $nm_{ex.}$–460 $nm_{em.}$ in an fmax microplate fluorimeter (Molecular Devices Corporation, CA, USA).

3. Results

3.1. Vitellin presence and digestion in R. microplus larva

Western blot analysis of crude extracts from larvae of different ages (days post hatching: 1, 3, 5, 7, 9, 11, 13) is shown in Fig. 1A. Vt subunits were recognized by anti-Vt antibody, demonstrating the presence of this reserve protein in unfed larva (Fig.1A). A decrease in Vt content with larval development was also observed (Fig. 1A). A quantitative analysis of larval extracts by ELISA with anti-Vt antibody confirmed a marked decrease in Vt content, suggesting Vt consumption by unfed larvae during development (Fig. 1B). Accordingly, total protein content had also decreased according larval aging, as verified by BCA quantification analysis (Fig. 1B). Indeed, acidic proteolytic activity rose during larval development, as observed by the hydrolysis of peptidase substrate (N–Cbz–Phe–Arg–MCA) (Fig. 2).

3.2. Purification of R. microplus larval cysteine endopeptidase (RmLCE)

A partially purified RmLCE preparation was obtained following two chromatographic steps, as shown in Fig. 3(A–B). Fractions containing high specific activity (eluted at 0.32 M NaCl) in the first ion-exchange resin (Fig. 3A) were pooled and subsequently fractioned by gel-filtration chromatography (Fig. 3B). The pooled active fractions from the gel-filtration step were, in effect, partially purified RmLCE. SDS-PAGE analysis of the protein content of endopeptidase-containing fractions at each purification step clearly showed removal of high-weight Vt polypeptides (Fig. 3C). The purification protocol is summarized in Table 1, from which it can be seen that RmLCE was purified 188 fold with a yield of 70%.

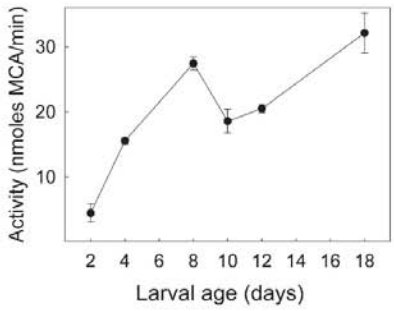

Fig. 2. Variation in cysteine endopeptidase activity during larval development. Activity assay was performed using a fluorogenic substrate (N–Cbz–Phe–Arg–MCA) at 37 °C in sodium citrate/sodium phosphate buffer (pH 5.5), 10 mM DTT solution, with protease inhibitors. Mean±SE for three replicates. Data are representative of two independent experiments.

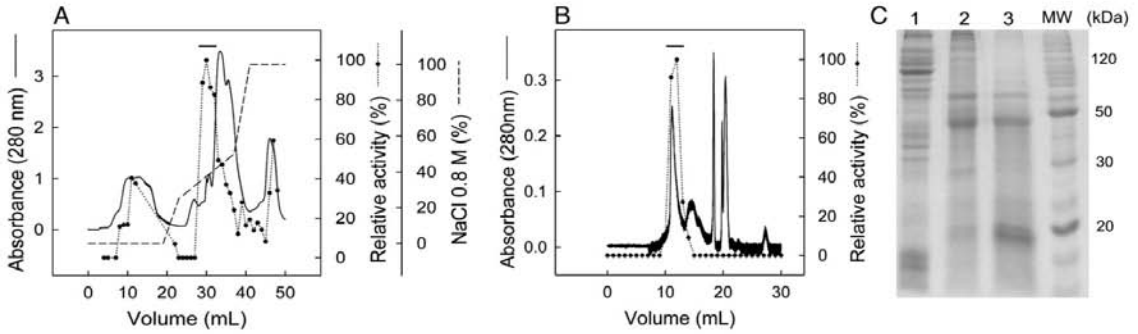

Fig. 3. Enzyme purification. (A) Larval extracts applied into an ion-exchange column (HiTrap, 1.6×2.5 cm) in sodium phosphate buffer (10 mM, pH 7.2); (B) selected fractions from the first step applied into a gel-filtration column (Superose 12, 10×300 mm) in the same buffer. Proteolytic activity of fractions was tested with N–Cbz–Phe–Arg–MCA at 37 °C in sodium citrate/sodium phosphate buffer (pH 5.5) and 10 mM DTT. Black bars indicate pooled fractions. (C) Analysis by SDS-PAGE of larval extracts (50 μg; lane1), pooled active fractions eluted from ion-exchange chromatography (25 μg; lane 2) and final enzyme preparation after gel-filtration chromatography (25 μg; lane 3). Molecular mass of standard proteins (kDa) is indicated.

3.3. Effect of protease inhibitors

Susceptibility to classical protease inhibitors is shown in Table 2, from which it can be seen that RmLCE was almost completely inhibited by the cysteine endopeptidase specific inhibitor E-64 and the serine/cysteine endopeptidase specific inhibitor TLCK, while the cysteine endopeptidase specific inhibitor leupeptin and the serine/cysteine endopeptidase inhibitor chymostatin also resulted in high inhibition of RmLCE. Inhibitors specific to aspartic and to metallo peptidases (pepstatin and EDTA, respectively) showed no inhibitory effect on enzyme activity. Additionally, peptidase activity increased 33% after 30 min incubation with 10 mM DTT solution (data not shown). The observed inhibitory profile as well as activation by DTT indicates that RmLCE is a cysteine endopeptidase.

3.4. Effect of temperature and pH

RmLCE showed a maximal activity at 37 °C (Fig. 4A) and an optimal pH in the range of 3.5–5.5 (Fig. 4B) when tested at different temperature and pH conditions. RmLCE was unstable and suffered a permanent loss of activity when incubated at pH ≥7.5 (Fig. 4C).

3.5. Activity upon synthetic substrates

Table 3 shows RmLCE activity upon different fluorogenic peptide substrates. Fluorescence occurs when the synthetic peptides are cleaved at the amide bond between the C-terminal amino acid and the methylcoumaryl-7-amide (MCA) fluorophore. High activities were observed upon N–Cbz–Phe–Arg–MCA and ε–NH_2–Cap–(SBzl)–Cys–MCA.

3.6. Vitellin-degrading activity

All Vt subunits were partially hydrolyzed and small hydrolysis products were observed after incubation with RmLCE, indicating the ability to digest Vt polypeptides. Furthermore, no degradation was seen in the presence of the cysteine endopeptidase specific inhibitor, E-64 (Fig. 5).

4. Discussion

Vitellin is the major yolk protein described in oviparous arthropods and can account for up to 95% of total egg protein (Maki and Yamashita, 1997). This maternally derived reserve protein has been reported in eggs of various arthropods, including *D. melanogaster*, *Locusta migratoria*, and *Rhodnius prolixus* (Kunkel and Nordin, 1985), and the ticks, *Dermacentor andersoni*, *D. variabilis*, *O. moubata*, *Argas hermanni*, *Hyalomma dromedarii*, *Rhipicephalus appendiculatus*, and *R. microplus* (James and Oliver, 1997). In fact, Vt has been found in all arthropod eggs where a search has been conducted. Vts are glycolipophosphoproteins with similar carbohydrate and lipid content in ticks and insects, the key difference in tick Vt being the heme component derived from digestion of host hemoglobin (James and Oliver, 1997). This feature of tick Vt seems to be of

Table 1
Partial purification process for a *R. microplus* larval cysteine endopeptidase (RmLCE)

Purification step	Volume (mL)	Protein (mg)	Activity		Yield (%)	Purification (fold)
			Total (nmol MCA min^{-1})	Specific (nmol MCA min^{-1} mg^{-1})		
Extract	8	136	2.92	0.02	100	1
HiTrap	1.9	5.3	2.02	0.38	69	18
Superose 12	1.9	0.51	2.07	4.06	70	188

Table 2
Enzyme sensitivity to protease inhibitors

Inhibitor	Specificity	Concentration (mM)	Residual Activity (pmol MCA min^{-1})	Inhibition (%)
None	–	–	95.4±10.4	–
E-64	Cysteine	0.01	0.4±0.4	100
Leupeptin	Cysteine	0.01	1.8±3.2	98
TLCK	Serine/ Cysteine	0.1	0.8±0.8	99
Chymostatin	Serine/ Cysteine	0.1	6.5± 1.6	93
PMSF	Serine/ Cysteine	0.1	112.0±19.7	0
Pepstatin	Aspartic	0.001	109.2±22.4	0
EDTA	Metallo	0.5	107.4±6.4	0

Activity on fluorogenic substrate (N–Cbz–Phe–Arg–MCA) measured at 37 °C in sodium citrate/sodium phosphate buffer (50 mM), pH 5.5 and 10 mM DTT after 15 min of pre-incubation in the presence of protease inhibitors. Mean±SE for three replicates.

great value to *R. microplus* due to its particular heme metabolic pathway (Braz et al., 1999).

Yolk proteolysis during egg development is a gradual and highly regulated process (Fagotto, 1995). Less than 50% of Vt in oocytes is used up during embryogenesis in *R. prolixus* (Oliveira et al., 1989) and *R. microplus* eggs (Logullo et al., 2002). Canal et al. (1995) verified the presence of Vt related peptides in *R. microplus* larvae. Immunological methods used in the present study confirmed the presence of Vt in *R. microplus* larval developmental stage. This reserve protein has also been found in the larvae of various species, such as the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* (Fagotto, 1995), the stick insect *Carausius morosus* (Masetti et al., 1998) and in the soft tick, *O. moubata* (Chinzei and Yano, 1985).

There is evidence that Vt is crucial in larval nutrition of many arthropods. Results reported by Fialho et al. (2005) showed that *R. prolixus* nymphs depend on Vt remaining in the midgut while seeking a host. This conclusion is supported by results of the present study that showed Vt consumption in unfed *R. microplus* larvae. A decrease in protein content along with a corresponding decrease in Vt content occurs soon after hatching and extends until day 13. This decrease in protein content suggests that amino acids resulting from Vt digestion are used in metabolic pathways other than protein synthesis. This data is consistent with results showing *R. microplus* eggs increase in guanine content after the 6th day of embryo development (Campos et al., 2006). Guanine is the product of nitrogen excretion in arachnids (Urich, 1990). Therefore, it is likely that at least part of the energy source for the embryo, and for the newly hatched larvae, comes from oxidation of vitellin-derived amino acids.

Vitellin degradation could be related to an increase in cysteine endopeptidase activity observed during *R. microplus* larval development. As described by Cho et al. (1999), cysteine endopeptidases are involved in yolk protein digestion in the yellow fever mosquito, *Aedes aegypti*, and in a number of other arthropods.

Maternally derived proteases have restricted specificity and are not able to fully degrade Vt; therefore, additional proteases are required to complete the hydrolysis of the remaining Vt peptides (Cecchettini et al., 2002). During *O. moubata* development, proteolytic activity of maternal origin is relatively constant during the embryonic stage, and decreases drastically after hatching. New synthesis of lysosomal enzymes in larval tissues is probable (Fagotto, 1990). Indeed, acid cysteine proteinase activity responsible for yolk degradation in brine shrimp increases 13-fold during larval development (Perona and Vallejo, 1985). Likewise, in the silkworm (*B. mori*) the yolk degrading protease becomes active only two days before

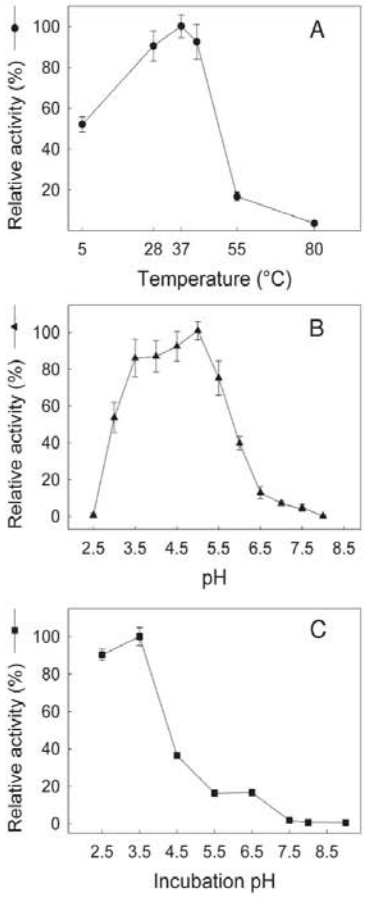

Fig. 4. Effect of temperature (A), and pH (B) on enzyme activity; effect of pH on enzyme stability (C). Activity tested using N–Cbz–Phe–Arg–MCA as substrate in sodium citrate/sodium phosphate buffer (pH 5.5) and 10 mM DTT. Experimental conditions: (A) pH 5.5, at different temperatures; (B) 37 °C, at different pH; (C) enzyme was incubated for 4 h at room temperature at pH 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.0 and 9.0 and then assayed in pH 5.5. Incubation buffers were: sodium citrate/sodium phosphate (50 mM) for pH 2.5 to 7.5 and sodium phosphate (10 mM) for pH 7.5 to 9.0. Mean±SE for three replicates.

Table 3
R. microplus larval cysteine endopeptidase (RmLCE) activity on fluorogenic substrates

Fluorogenic substrate	Activity (pmol MCA min^{-1})	Activity (%)
N–Cbz–Phe–Arg–MCA	102.2±12.2	100
ε–NH_2–Cap–(SBzl)–Cys–MCA	94.0±16.2	92
N–t–Boc–Glu–Ala–Arg–MCA	61.6±8.0	60
ε–NH_2–Cap–Leu–Phe–MCA	6.9±1.8	7
Nα–Benzoil–Lys–Arg–MCA	6.0±0.9	6
ε–NH_2–Cap–Leu–(OBzl)–Thr–MCA	5.4±1.1	5
N–t–Boc–Val–Pro–Arg–MCA	3.0±0.5	3
L–Phe–MCA	2.8±0.8	2.5
N–t–Boc–Gly–Arg–MCA	0.4±0.2	0
N–t–Boc–Ile–Glu–Gly–Arg–MCA	0±0.1	0
L–His–MCA	0±0	0

Proteolytic activity verified at 37 °C in sodium citrate/sodium phosphate buffer (50 mM), pH 5.5 and 10 mM DTT. After pre-incubation for 10 min different fluorogenic substrates were added at a final concentration of 100 μM. Mean±SE for three replicates.

hatching, when its activity increases dramatically (Maki and Yamashita, 1997). Increase in cysteine endopeptidase activity during *R. microplus* larval development strongly suggests RmLCE is synthesized at this stage of the life cycle, acting to complement Vt degradation initiated by maternally derived proteases in the embryo.

The *R. microplus* larval cysteine endopeptidase (RmLCE) activity found in *R. microplus* larval extracts was partially purified and characterized, the chromatographic protocol used resulting in a final preparation enriched in RmLCE. The inhibitory profile and activity upon synthetic substrates clearly demonstrated that the RmLCE is a cysteine endopeptidase but interestingly, this enzyme has different features from the vitellin-degrading cysteine endopeptidase (VTDCE) recovered from *R. microplus* eggs by Seixas et al. (2003). We found that while the optimal pH for RmLCE is higher than that for VTDCE the optimal temperature is lower. Moreover, RmLCE separated from vitellin in the first purification step and showed no strong association with vitellin, a particular characteristic of VTDCE (Seixas et al., 2003). A cysteine proteinase encoded by a gene cloned from a *R. microplus* larval cDNA library (Renard et al., 2000) and expressed in *Escherichia coli* presented some characteristics (pH range, synthetic substrate preference, and vitellin-degrading ability) observed in the native purified RmLCE, but no correlation between these two enzymes can be made before the RmLCE amino acid sequence is known.

Results clearly show that RmLCE is able to degrade Vt. As this storage protein is still present at larval stage, it is likely that Vt is the natural substrate for RmLCE. Other evidence supporting this hypothesis is the fact that cysteine endopeptidase activity was detected in gut dissected from *R. microplus* larvae (data not shown). Due to the tiny quantity of material available we made no attempt to purify the enzyme from larval gut. During arthropod embryological development, in yolk-rich eggs, an atypical gastrulation occurs in order to keep the yolk mass (previously located in the blastocoele) enclosed in the endoderm which forms the archenteron, the structure that will form the digestive tract of the developing embryo (Snodgrass, 1935; Gillott, 1980). It is reasonable to suppose that a protease with the primary function of digesting yolk-derived reserve proteins would be co-localized in the larval gut. In fact, in *B. mori* eggs, the protease responsible for Vt degradation is located in the embryo midgut (Furusawa et al., 1993).

Considering results of this study it can be concluded that RmLCE takes part actively in Vt digestion, providing amino acids and energy for larval survival until a host is encountered and feeding process starts. RmLCE is also able to degrade hemoglobin (data not shown) suggesting that RmLCE activity may be important not only during unfed larval stage but also at the beginning of blood feeding, degrading the most abundant protein present in the new food source. This hypothesis requires further investigation.

A better understanding of tick larval proteins could result in the identification of molecules that can be targets for host antibodies which can disturb the parasitic life cycle at this stage (Untalan et al., 2005). If RmLCE remains in the tick gut, acting as a digestive protease at different stages of this tick's life cycle, this protein could be an important target for antibodies ingested by the larvae when they start to feed on the host. Moreover, the selective inhibition of digestive enzymes is currently considered an important aspect for the control of many insect pests, including the yellow mealworm *Tenebrio molitor* and the fruit fly *Ceratitis capitata* (Silva et al., 2006; Vinokurov et al., 2006).

In summary, the data presented in this work adds to the present knowledge on yolk degrading processes and describes a novel *R. microplus* larval cysteine endopeptidase (RmLCE) likely to be involved in vitellin digestion in *R. microplus* larvae, widening the range of possible targets to a vaccine against ticks.

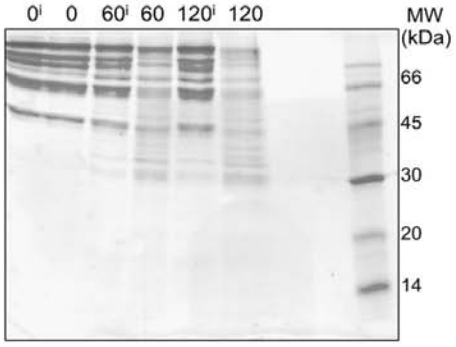

Fig. 5. *R. microplus* larval cysteine endopeptidase (RmLCE) vitellin-degrading activity. Partially purified enzyme was incubated with vitellin (21 μg) in sodium citrate/sodium phosphate buffer (50 mM, pH 4.0) at 37 °C in the absence or presence of inhibitor (E-64 (10 μM), indicated by "i"). Reactions stopped by boiling, at 0, 60 or 120 min and proteolysis products analyzed by SDS-PAGE. Molecular mass standards (kDa) are indicated. Data are representative of two independent experiments.

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ANEXO 2

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Localization and function of *Rhipicephalus (Boophilus) microplus* Vitellin-Degrading Cysteine Endopeptidase

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Abstract

The tick *Rhipicephalus (Boophilus) microplus* is the major bovine parasite in the southern hemisphere. Characterization of molecules involved in mechanisms, such as vitellogenesis and embryo development, can contribute to a better understanding of this parasite physiology. The Vitellin-Degrading Cysteine Endopeptidase (VTDCE) is the most active enzyme involved in vitellin hydrolysis in *R. microplus* eggs. Here we show an association between VTDCE and vitellin by an additional site on top of the active site. This enzyme is present in different tissues as ovary, midgut, fat body, salivary gland and female hemolymph, where it is controlled by a physiological inhibitor. In *R. microplus* female midgut, VTDCE is located in areas of protein synthesis and trafficking with the underlying hemolymph. VTDCE it is also located in ovary basal region, in vesicles membranes of ovary pedicel cells and in oocyte citosol. With these data, we suggest that VTDCE is produced by tick midgut and transported through hemolymph, associated with a physiological inhibitor, before being taking up by oocytes.

Keywords: tick; VTDCE; cysteine endopeptidase; vitellin; hemolymph; cysteine endopeptidase inhibitor.

1. Introduction

The tick ***Rhipicephalus (Boophilus) microplus*** is a one host parasite that affects bovines, in tropical and subtropical regions, causing huge economical losses (Willadsen, 2006). Tick control methods are based on application of chemical acaricides, which induce selection of drug-resistant populations and present a potential risk of food and environmental contamination (Willadsen, 2004). Vaccines have been shown to be a feasible tick control method, offering a cost-effective, environmentally friendly alternative to chemical control. However, identifying tick-protective antigens remains a limiting step in the development of a vaccine able to replace chemical acaricides (de la Fuente et al., 2007). In this context, a better knowledge of tick physiological important mechanisms may be helpful in finding new vaccine targets.

Cysteine endopeptidases is a widely distributed group of enzymes, with a broad range of putative functions (McGrath, 1999, Sajid and McKerrow, 2002; Carnevali et al., 2006). The cathepsin-L like protease from the tick ***Ornithodoros moubata*** has a role in the digestion of vitellin (Vt), the major reserve protein in arthropod eggs, providing nutrients during embryogenesis (Fagotto, 1990). In ***R.(B.) microplus***, two cysteine endopeptidases involved in Vt hydrolysis have been studied: Vitellin-Degrading Cysteine Endopeptidase (VTDCE; Seixas ***et al.***, 2003) and ***Riphicephalus microplus*** Larval Cysteine Endopeptidase (RmLCE; Estrela ***et al.***, 2007). VTDCE was previously purified from ***R. microplus*** eggs by a purification protocol in wich an autolysis step to hydrolyze Vt is crucial (Seixas et al., 2003). Contrarily to VTDCE, the larval cysteine endopeptidase RmLCE elutes from an anionic resin free of Vt.

Vitellin, a polydisperse protein, is derived from a maternal protein, vitellogenin (Vg), which is synthesized by the tick fat body and midgut after adult females obtain their blood meal. In general, the carbohydrate, lipid and amino acid composition of tick Vg is similar to that of insect Vg, excepting that tick Vg contains heme from digestion of host hemoglobin (Logullo et al., 2002). After synthesis, Vg is released into the hemolymph and uptake, via receptor-mediated endocytosis, by the growing oocytes. Vg is partially processed in the endosomal compartment and then stored as Vt in specialized organelles, yolk granules (Raikhel and Dhadialla, 1992; Fagotto, 1995; Mitchell et al., 2007).

The *R. microplus* ovary is classified as the panoistic type (Saito *et al.*, 2005). In this type of ovary, nurse cells are absent and oocytes are attached to the ovarian wall through a cellular pedicel. Balashov (1983) suggests that pedicel cells play the role of nurse cells, normally present in meroistic type ovaries, incorparating the material that subsequently will be uptake by the oocytes. Pedicel cells present a fine layer of cytoplasm in which the presence of vacuoles is observed. The basal lamina that supports the more external cells of the ovary wall is absent at the point of contact between oocyte and pedicel cells, and this contact membrane presents interdigitations that increase the contact surface between these two cells types. The vitellogenesis process in *R. microplus* occurs through endogenous production of lipids and proteins until the oocytes reach developmental stage III, beyond which there is also incorporation of material from the hemolymph (Saito *et al.*, 2005).

In the present work we sought to investigate the presence of cysteine endopeptidase activity in *R. microplus* eggs, larvae and adult female hemolymph and tissues . As well as to investigate the association between the vitellin-degrading cysteine endopeptidase (VTDCE) with its natural substrate Vt.

2. Material and Methods

2.1. Ticks

Ticks from the Porto Alegre strain were reared in bovines, which were brought from a tick-free area and maintained in insulated individual boxes protected from any contact with other ticks and insects. Bovines were infested with 15 day-old (from hatching) *R. microplus* larvae. Partially engorged tick females (20 days of life on the host) were forcibly removed from the host and fully engorged females (spontaneously detached from the host at the 22th day) were collected. These ticks were used for experiments. Also, fully engorged females were incubated at 28° C and 85% relative humidity for laying eggs. Eggs from different days after oviposition were collected and stored at -70° C until to be used or maintained in glass tubes closed with cotton plugs at the same conditions for larvae hatching. Larvae were separated, 5 or 20 days after hatching, and stored at -70°C until use.

2.2. Preparation of tissues, eggs, and larvae extracts

Fully and partially engorged females were washed with 70% ethanol, immobilized with glue on Petri dishes and flooded in cold phosphate-buffered-saline [PBS; sodium phosphate (10 mM), NaCl (150 mM), pH 7.2]. The dorsal cuticle was removed using a microscalpel and midmidgut, salivary glands, ovary, synganglio, and fat body were dissected with forceps. Tissues were homogenized in a tube with a disposable grinder (GE Helthsciences, Uppsala, Sweden) in 300 µl of PBS. Homogenates were centrifuged at 16000 x g / 10 min to pellet insoluble material. After removing the soluble fraction, 300 µl of PBS, containing 2.5 % deoxicolate were added and the insoluble material was again homogenized and centrifuged at 16000 x g / 10 min. This supernatant constitutes the insoluble proteins extract. Tissue extracts were stored at −20 °C until use.

2.3. Collecting hemolymph and saliva

Hemolymph was collected from immobilized ticks, (chilled for 15 min at 4 °C) to avoid midgut contractions and hemolymph contamination with blood. A small incision was made in the cuticle, and hemolymph was collected using a micropipette. Tick saliva was collected as described previously (Horn *et al.*, 2000). Briefly, adult engorged females recently detached from host were rinsed, fixed to glass plates with adhesive tape and induced to salivate by injecting 5 µl pilocarpine (2% w/v in PBS). Ticks were maintained in a humid chamber and saliva was collected for a period of 2 h with a small vacuum apparatus. Hemolymph and saliva were kept at −20 °C until use.

2.4. Size exclusion chromatography

Samples (200 µl) were applied onto a size exclusion column Superose 12 (GE Healthcare, Uppsala, Sweden), that was previously equilibrated in 10 mM sodium phosphate buffer pH 7.0 in FPLC System (GE Healthcare, Uppsala, Sweden). Fractions of 1 ml were collected and protein concentration of column eluate was monitored by absorbance reading at 280 nm. Column was previously calibrated with same buffer using aprotinin (6.5 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa), alcohol dehydrogenase (150 kDa), catalase (232 kDa), and ferritin (440 kDa) as standards.

2.5. Cysteine endopeptidase activity assay

Cysteine endopeptidase activity was tested as previously described (Seixas et al., 2003). Briefly, 10 l-aliquots of column fractions, tissues extracts, hemolymph and saliva, or other samples, were incubated with 50 mM sodium citrate/sodium phosphate buffer pH 3.5 and 10 mM DTT at 37^{o} C in the presence or absence of cysteine endopeptidase inhibitor E-64 (10 M; L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane). After 10 min, a fluorogenic substrate, N-Cbz-Phe-Arg-MCA (Cbz, carboxibenzoyl, MCA amido-4-methyl coumarin) was added to a final concentration of 1.4 M. Hydrolysis was monitored at 11-seconds intervals by fluorimetry in a M2e Microplate Reader (Molecular Devices Corporation, Sunnyvale, U.S.A.). The wavelength pair for excitation and emission was 370 nm / 460 nm (Oliveira ***et al.***, 1992). Enzyme activity is given by the enzyme initial rate from kinetic measurements, where one enzyme unity (U) corresponds to an increase by one relative fluorescence unit (RFU) per second.

2.6. Protein determination

Protein concentration was determined by the bicinchoninic acid (BCA) method, according to Smith ***et al.*** (1985), using bovine serum albumin (BSA) as standard.

2.7. Polyclonal antibodies against VTDCE

Anti-VTDCE antiserum was obtained as described by Seixas ***et al***. (2008). Briefly, rabbit was inoculated subcutaneously with 100 g of purified VTDCE emulsified in Freund's complete adjuvant followed by three boosters of VTDCE (100 g) in Freund's incomplete adjuvant with an interval of 15 days between each dose.

2.8. Immunobloting

Extracts were separated by SDS-PAGE using a Bio-Rad Mini-Protean Cell II unit. Resolving and stacking gels were 12.5 and 5% polyacrylamide, respectively. Proteins were transferred to a nitrocellulose membrane (0.45 m, Schleicher & Schuell, Germany) in a semi-dry system (GE-Healthcare, Uppsala, Sweden) using 25 mM Tris, 192 mM aminoacetic acid, 30% methanol, pH 8.4. Nitrocellulose membranes were blocked with

blotto [5% cow non-fat dry milk in sodium phosphate (10 mM), NaCl (150 mM), pH 7.2] and probed with rabbit polyclonal antibodies against VTDCE (1:100). After 3 washes with blotto, anti-rabbit IgG alkaline phosphatase conjugate was used as a secondary antibody. Development was performed with NBT/BCIP (nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt; USB Corporation, Cleveland, USA). Dot blot was performed in the same conditions. Monoclonal antibodies anti-VT were kindly supplied by Sandra E. Farias, Centro de Biotecnologia do Estado do Rio Grande do Sul, Brazil.

2.9. Transmission Electron Microscopy immunohistochemistry

Fully engorged tick ovaries were dissected as described in section 2.2. and fixed at 4° C for 12 h in 0.25% glutaraldehyde, 4% paraformaldehyde, 10 mM calcium chloride, 0.1 M cacodylate buffer, pH 7.3. Following that, the material was dehydrated in an ascending series of ethanol concentration, and embedded in LR-White resin **(**Electron Microscopy Sciences, Hatfield, PA**)** at 4° C. Ultrathin sections (70 nm) were caught in formvar cover cooper grids, blocked by incubation with blotto for 15 min, and then incubated with antiserum (anti-VTDCE) in the same buffer for 1 h. Anti-rabbit IgG conjugated to 10 nm colloidal gold was used as secondary antibody. Finally, grids were stained with saturated uranyl acetate and lead citrate (Glauert, 1974), and observed through a transmission electron microscope 900 Zeiss (Zeiss, Oberkochen, Germany) at 80 kV.

2.10. Immunofluorescence

Fully engorged females were dissected at third day after blood meal. Tissues were fixed as described above, embedded with PBS-sucrose 20% during 12hs / 4^0C, O.C.T. polymer (TissueTek, Minnesota, USA) during 2hs and then frozen in liquid nitrogen. Thin sections of 5 m were cut, exposed to rabbit polyclonal antibody anti-VTDCE (1:100) or pre-immune serum and then to a goat anti-rabbit antibody conjugated with fluorescein (1:500) (DAKO, Glostrup, Denmark). Samples were visualized under an Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany)**.**

2.11. Binding assay

Purified VTDCE (260 g) was iodinated with ^{125}I-sodium iodide (17.4 Ci/mg, GE Healthcare, UK) using Iodo-gen (Pierce, Rockford, USA) as described elsewhere (Gondim and Wells, 2000). To remove free iodide, reaction mixture was extensively dialyzed against PBS, pH 7.4. An ^{125}I-VTDCE specific activity around 150,000 cpm/ g of protein was obtained. For binding assay, 20 µg of sample were fixed in pieces of nitrocellulose membrane that were placed into wells of a 96-well plate. Membranes were blocked with BSA (25 mg/ml), washed twice with PBS, incubated with 1 g of ^{125}I-VTDCE in 100 l of BSA 2.5 mg/ml in PBS for 2 h in the presence or absence of leupeptin, and then washed througly with the same buffer. Assays were performed in triplicates. Radioactivity associated with the filters was determined in a γ-counter. Radioactivity in control membranes was subtracted from values of experimental membranes, containing vitellin. Results were expressed as mean ± S.D. Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Significance level was 0.05, and *p*-values are indicated in legend of Figure 4.

3. Results

3.1. Cysteine endopeptidase activity in R. microplusfemale tissues, saliva and hemolymph

Cysteine endopeptidase activity was present in fat body, midmidgut, salivary glands, and ovary extracts from partially and fully engorged females (Table 1). In partially engorged females, midgut was the richest source of enzyme comparing to other tissues (904 ± 100 U / mg tissue). In contrast, after female full engorgement, midgut cysteine endopeptidase specific activity reduces almost 500 times (1.9 ± 0.1U / mg tissue). Other female tissues extracts like ovary (1063 ± 69 U / mg tissue), fatty body (27 ± 0.8 U / mg tissue), and salivary gland (21 ± 0.2 U / mg tissue), showed an increase in the cysteine endopeptidase specific activity after engorgement. At this life stage, ovary was the richest source of this enzyme. Significantly, all activity was inhibited by E-64, confirming that all endopeptidase activity detected is due to cysteine endopeptidases.

Cysteine endopeptidase activity was also detected in hemolymph of partially and fully engorged ***R. (B) microplus*** females (Table 1). In partially engorged females this activity was 278 ± 12 U / ml, increasing to 589 ± 73 U / ml after full engorgement (host

detachment day; Table 1). During the days preceding oviposition, the activity in hemolymph gradually decrease (Table 1). Variation in total hemolymph protein content was also observed. Protein content in partially engorged female hemolymph was 7.97 mg / ml, increasing to 10.93 mg / ml in fully engorged females (first day post detachment). At the second day post detachment, the highest protein level was achieved (31.03 mg / ml), remaining almost constant until the third day (29.76 mg / ml). In contrast to all other samples tested, no activity was detected in saliva (Table 1).

3.2. Cysteine endopeptidase activity of partially and fully engorged female tissues

Figure 1 shows the filtration profile of soluble proteins extracted from partially and fully engorged female fat body (A, B), midmidgut (C, D), salivary gland (E, F) and ovary (G, H). Protein patterns differ according to the tissue studied, althought having a major common peak (at 9 ml) in all tissues. Cysteine endopeptidase patterns are more consistent. In fat body, salivary gland, and ovary, cysteine endopeptidase activity eluted at ca. 11 ml, and in midmidgut at 13.5 ml.

Major differences between protein profiles from fully and partially engorged female tissues were observed in midmidgut (Figure 1 C and D) and ovary (Figure 1 G and H). In fully engorged midmidgut, three major groups of proteins elute at 9, 11 and 16 ml and only the first peak co-elutes with one from the earlier lifestage (partially engorged, Figs. 1 C and D). Partially engorged female ovary proteins that eluted between 14 and 18 ml (Figure 1 G) almost disappear in the fully engorged female (Figure 1H). The fully engorged midmidgut cysteine endopeptidase activity elutes in two distinct peaks, one at the same volume observed in partially engorged female (13.5 ml) and a new one eluting at 11.5 ml, suggesting stage specific changes (Figure 1D). In fully engorged female ovaries, in addition to the same peak observed in partially engorged females (eluting at 10.5 ml), two new small activity peaks appear, eluting at 18.5 and 24.5 ml (Figure 1 H).

3.3. Enzyme activity profile in egg and larva

Egg cysteine endopeptidase elutes from gel filtration chromatography as a single major peak (at 9.5 ml) and a minor peak detectable in a single fraction at 12.5 ml (Figure 2 A). Larval cysteine endopeptidase activity also elutes at one major and one minor peak but

the major peak elutes at 12.5 ml and the minor at 9.5 ml (Figure 2 B). Therefore, changes in enzymatic profile according to life stage are observed again.

Gel filtration analysis of egg and larval extracts showed Vt eluting at the major peak of protein (9.5 ml; Figure 2). Although, Vt in larva seems to be degraded and proteins of lower molecular weight, or Vt fragments, are detected. Here, Vt identification was done based on (i) tick Vt property of containing haem (Sonenshine, 1991) which confers a brownish color to the sample; (ii) similarity to the standard of purified Vt (Figure 2 C) and (iii) qualitative dot-blot using anti-Vt antibodies.

3.4. VTDCE and Vt distribution over *tick development*

VTDCE and Vt distribution over tick development were investigated by Western blot (Figure 3). Analysis with anti-Vt showed Vt different polypeptides present in all samples tested: ovaries, eggs from different days, young and old larvae (Figure 3 B). The presence of VTDCE was verified (i) in ovaries of females recently detached from the host (Figure 3 B, ovary 1); (ii) in ovaries of 3 days-post-detachment females (Figure 3 B, ovary 3), (iii) during the embryonic development (1 day-egg , 3 days-egg, 7 days-egg, 12 days-egg, 20 days-egg); (iv) in young larvae (5 days-larvae), and (v) in old unfed larvae (20 days-larvae). In addition to the enzyme, proteins of high molecular weight, similar to that recognized by anti-Vt, were also recognized by antibodies anti-VTDCE prepared from a checked pure VTDCE preparation (Figure 6B). This suggests that VTDCE binds to different Vt polypeptides. Therefore, this protein interaction was further investigated.

3.5. VTDCE-Vt association

The VTDCE capacity to bind Vt was verified using ^{125}I-VTDCE and membrane-fixed Vt in a radio-binding assay. Figure 4 shows that VTDCE binds to Vt in a dose-dependent manner (Figure 4 A). The enzyme was able to bind Vt also in the presence of leupeptin (a cysteine endopeptidase inhibitor) (Figure 4 B). This association was shown to be specific since the binding to immobilized Vt was reduced by adding an excess of soluble Vt (in the presence or absence of leupeptin; Figure 4 B).

3.6. VTDCE localization in fully engorged female midgut and ovary

Immunofluorescence analysis of ***R. microplus*** female midgut sections showed VTDCE presence in basophilic cells. Additioning, an intense signal was observed in basal lamina (Figure 5, panel I). In the ovary, labeling was observed in the pedicel cells , oocyte cytosol, chorium, and basal lamina (Figure 5 panel II). The region close to germinal vesicle was also labeled (Figure 5, panel II A, B). No signal was observed in yolk granules and in controls with non-immune serum (Figure 5 panel I – D, panel II - D).

Using gold-immunohistochemistry in electron microscopy, we have observed that VTDCE is clearly located in typical material-exchanging areas in ovary (Figure 5, panel III). The enzyme was immunolocalized at the membrane of vesicles present in the pedicel cells, which appear highly decorated with gold particles (Figure 5, panel III - A), and in the ovary basal region in close contact with haemocoel (Figure 5, panel III - B).

3.7. A possible endogenous VTDCE inhibitor

Surprisingly, a 90-fold increase in hemolymph cysteine endopeptidase activity was observed after gel filtration fractionation (Table 2 and Figure 10 A). This finding suggests that tick hemolymph contains a cysteine endopeptidase inhibitor. Presence of an inhibitor was investigated by incubating 10 µl of each gel filtration fraction with purified egg VTDCE, before substrate addition. A cysteine endopeptidase inhibitory activity eluted from gel filtration column between 13 and 15 ml (Figure 6 A). These pooled fractions (Mw between 34 and 11 kDa) inhibited VTDCE in a dose dependent manner (Figure 6 B).

4. Discussion

Previous studies demonstrated the presence of acidic peptidase activity in ***R. microplus*** ovaries, eggs and larva (Seixas ***et al.***, 2003; Estrela ***et al.***, 2007). The egg peptidase (vitellin degrading cysteine-endopeptidase; VTDCE) was purified and characterized as a cathepsin-L-like enzyme, active at acidic pH and totally inhibited by E-64 (Seixas ***et al.***, 2003). Here, we investigated the distribution of VTDCE in tissues and hemolymph of the cattle tick ***R. microplus*** female and VTDCE association with its natural substrate vitellin.

Data presented here show that cysteine endopeptidase activity is widely distributed in tick, being found in engorged female fat body, ovary, midmidgut, salivary glands and hemolymph. Tissues differ in stage-specific protein patterns. Although, enzyme profile has a constant pattern in all tissues, a cysteine endopeptidase activity peak eluting at 11.5 ml (Figure 1). Peculiar profiles were observed in egg and larva. Most egg peptidase activity elutes at 9.5 ml (≅244 kDa) followed by a small activity peak eluting at 12.5 ml (≅ 45 kDa; Figure 2A). In larva, on the other hand, this 9.5 ml activity peak is reduced and most cysteine endopeptidase activity elutes at 12.5 ml (Figure 2B), that indicates stage specific changes in the enzyme profile. Active egg extract fractions reacted positively to anti-VTDCE antibodies and co-eluted with Vt. So, variations in VTDCE gel filtration elution profile can be correlated with its association with multiple Vt subunits. A Vt-VTDCE association have been suggested previously (Seixas *et al.*, 2003), and is well observed in western blot analysis localizing VTDCE in ovary, eggs and larvae (Figure 3). During embryogenesis, VTDCE is observed in a low molecular weight form corresponding to a free enzyme, and several high molecular weight forms, that are also recognized by anti-Vt antibodies and correspond to VTDCE associated with different Vt subunits and/or Vt partial digestion products. This kind of association that alters the apparent Mw was previously described for other arthropod enzymes and could be related to Vt-degradation control (Giorgi *et al.*, 1999).

The VTDCE purification protocol includes an autolysis step in acidic pH in which Vt is hydrolyzed by VTDCE and products of degradation precipitates while the enzyme stays soluble (Seixas *et al.*, 2003). Actually, it is hard to establish unambiguously the Mw of pure VTDCE. Earlier studies described the presence of a 22 and a 17 kDa protein (Seixas *et al.*, 2003). However, these characteristics may change as result of further protein processing during autolysis, and only one subunit of low molecular weight remains, suggesting that the 22 kDa protein corresponded to a distinct processing stage. Indeed, difficulties in isolation, purification and characterization of cathepsin-L, due to autolysis, are frequently observed in final purifications steps (Cristofoletti *et al.*, 2005). In the case of VTDCE this problem is even more crucial, since autolysis is an essential step to digest Vt, and enzyme must be subsequently maintained in acid conditions to avoid eventual inactivation.

Experiments using ^{125}I-radiolabeled VTDCE confirm VTDCE associates with Vt, its natural substrate. In fact, results show that purified VTDCE is able to associate with Vt in a dose-dependent manner (Figure 4 A).It is important to stress that association VTDCE-Vt can occur regardless the presence of a cystein endopeptidase inhibitor (leupeptin). This finding arises a query of a possible mechanism that allow the coexistence of VTDCE and Vg/Vt with no premature Vg/Vt polypeptide clivage during vitellogenesis. Association between Vg and a cysteine endopeptidase has been previously described in ***Blatella germanica*** (Yin et al, 2001). In this cockroach, the enzyme colocalizes with Vg in fat body granules, suggesting that the enzyme and Vt are secreted by this tissue together in order to be transported through the hemolymph and incorporated into the oocytes.

Some considerations helps to understand the significance of Vg-VTDCE interaction. At first, Vg may act as a potential inhibitor, maintaining the enzyme as a zymogen throughout vitellogenesis (Kucera and Turner 1981, Yin ***et al.***, 2001). Secondly, enzyme activation may be delayed until pro-protease and Vg are fully dissociated from each other due to acidification of the yolk granules (Nordin ***et al.***, 1990). Finaly, a physiological inhibitor may be part of this complex acting to control enzymate activity (Kucera and Turner 1981). It is also important to consider that copackaging of ennnzyme to Vg/Vt responds to the requirements of a general reproductive strategy, whereby the developing embryo is provided with a reserve protein and an associated enzyme able to mobilise this protein (Fagotto, 1990; Giorgi ***et al.***, 1999).

The VTDCE biological function can be clarified by the elucidation of its distribution in tick tissues. In midgut, the enzyme is present in basophilic cells and at high levels in the basal lamina. Midgut basophilic cells in female ticks are derived from basal remnants of type 2 secretory cells (Agbede & Kemp, 1987). Cytoplasm of these cells is filled with well organized rough endoplasmic reticula, Golgi complexes and secretory granules evidencing their synthetic capacity. Moreover, the contact of this cell with the underlying hemolymph is extended by basal labyrinth infoldings, that facilitates material exchange. During the final rapid phase of engorgement, basophilic cells seem to assume an active role in water transport across the midgut wall, but subsequent to this phase, its rough endoplasmic reticulum cisternae are reorganized and resume a secretory role. After the fat body, the midgut epithelium is the second source of Vt synthesis in ticks (Coons ***et al.***,

1982). Another enzyme involved in Vt digestion in eggs, the *Boophilus* aspartic pro-cathepsin (BYC), is also synthesized in midgut and fat body cells (Logullo *et al.*, 1998, Nascimento-Silva et al, 2008). Thus, the distribution profile of VTDCE in midgut suggests it can have the same synthesis site as Vt and BYC, and is also secreted into hemolymph to be transported to ovary. The stronger signal in basal lamina, compared to basophilic cells, makes sense considering that analysis was done at the third day post-detachment, the stage of protein export.

In the ovary, VTDCE is located in the basal region and in membranes of vesicles present in ovarian pedicel cells. Pedicel is a structure resulting from proliferation of ovary wall epithelial cells, which attach oocytes to the ovary (Till, 1961; Ricardo *et al.*, 2007). *R. microplus* ovary is devoid of nurse cells, a characteristic of panoistic-type ovaries (Saito *et al.*, 2005). Thus, pedicel cells act producing and/or incorporating proteins from the hemolymph, which will then be transported to the oocytes. In *Amblyomma triste*, proteins are accumulated at the regions of contact among pedicel cells and at the pedicel cells/oocytes interface, showing that protein exchange among these cells in fact occurs (Ricardo *et al.*, 2007). Therefore, the pedicel participates as an active structure that supplies yolk components for oocytes. In *R. microplus* oocytes, VTDCE is located at the basal lamina/chorium, in the cytoplasm and close to the germinal vesicle. No labeling was observed in the yolk granule similarly to *Aedes aegypti* yolk-degrading cysteine endopeptidase (Vitellin cathepsin B; VCB), which is present in a narrow layer between yolk and yolk body membrane in developing oocytes (Cho, et al. 1999). Therefore, immunolocalization results together with the presence of VTDCE in the hemolymph and other tissues, such as midgut and fat body, indicate VTDCE has an extra-ovarian origin, and it is internalized into the oocytes through the pedicel cells. However, a concomitant ovarian synthesis can not be discarded.

As mentioned above, cysteine endopeptidase activity is present in female hemolymph. This activity was found in partially and in fully engorged females, at the first day post-detachment up to the third day, when oviposition starts. The highest activity occurs in recently detached fully engorged female hemolymph. Interestingly, cysteine endopeptidase activity decreases gradually in the two-day period before oviposition onset. The high activity in fully engorged female hemolymph at the first day after detachment can

result from active synthesis by tissues such the midgut, which secretes the enzyme into hemolymph. In addition, the activity decrease in the following days can result from enzyme uptake by the ovary.

The hemolymph cysteine endopeptidase activity increases almost one hundred times after gel filtration chromatography, indicating the presence of a cysteine endopeptidase inhibitor. The existence of such inhibitor was confirmed by inhibition of cysteine endopeptidase activity by whole hemolymph, as well as some gel filtration fractions. Indeed, a partially purified inhibitor preparation (pooled gel-filtration fractions) inhibits egg-VTDCE in a dose-dependent manner. This inhibitor would prevent premature degradation of Vt during transport through hemolymph, as well as in oocytes. It was suggested that the latency of yolk granules is reinforced by other regulatory mechanisms (Fagotto, 1995). Actually, VTDCE and most lysosomal enzymes are active under mild acidic conditions and display residual activity even at pH close to the neutrality, while digestion of Vt polypeptides only occurs at quite low pH. Thus, controlling the luminal pH alone is probably not enough to prevent all premature yolk granule proteolysis during the long period of oogenesis (Fagotto, 1995). Indeed, a cystatin, Bmcystatin, was cloned from a *R. microplus* fat body cDNA library and the recombinant protein inhibits VTDCE (Lima et al., 2006). VTDCE inhibitory activity showed here eluted from gel filtration with a molecular mass between 34 kDa and 11 kDa (Figure 6), and the recombinant Bmcystatin has a molecular mass of 11 kDa (Lima et al., 2006). It is possible that the cysteine endopeptidase inhibitory activity found in *R. microplus* hemolymph is the same Bmcystatin. Besides the well described role of cystatins in haematophagy, this new data showing the presence of an active cysteine endopetidase inhibitor in *R.(B.) microplus* hemolymph is the first demosntration relating a cysteine endopeptidase inhibitor to vitellogenesis control.

In conclusion, VTDCE is a vitellin-associated enzyme, present in *R. microplus* eggs, larvae, partially and fully engorged females with a role in embryogenesis. Data also suggested that VTDCE is a maternally derived endopeptidase involved in a route including extra-ovarian synthesis, transport, and ovary uptake, before internalization by tick oocytes. Moreover, as VTDCE is the most active of all vitellin-degrading enzymes so far described in *R. microplus*eggs (Seixas *et al*., 2008) the strict regulation of its activity seems to have a

key role in the control of yolk mobilization, ensuring nutrient providing at the right time to the developing embryo.

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Figures and Tables

Table 1 - Cysteine endopeptidase activity in partially and fully engorged female tissues, saliva and hemolymph of *R. (B.) microplus.*

Tissue	Partially Engorged Female Activity (U)			Fully Engorged Female Activity (U)		
	Soluble	Insoluble	Total	Soluble	Insoluble	Total
Fatty body[a]	5.8 ± 0.16	2.2 ± 0.30	8	27 ± 0.8	2.5 ± 0.06	29.5
Gut[a]	904 ± 100	17 ± 0.47	921	1.9 ± 0.1	0.8 ± 0.02	2.7
Ovary[a]	18 ± 1.38	0.6 ± 0.04	18.60	1063 ± 69	117 ± 3.6	1180
Salivary glands[a]	5.3 ± 0.11	0.7 ± 0.07	6.00	21 ± 0.2	1.6 ± 0.17	22.6
Saliva[b]	–	–	0	–	–	0
Hemolymphb	–	–	278	–	–	–
Hemolymph 1[b]	–	–	–	–	–	589
Hemolymph 2[b]	–	–	–	–	–	310
Hemolymph 3[b]	–	–	–	–	–	250

[*] U = Relative fluorescence Units / sec.
[a] = U / mg of tissue.
[b] = U / ml.
Numbers 1, 2 and 3 in hemolymph indicate days after female engorgement.

Table 2 - Fractioning of *R. (B.) microplus* engorged female hemolymph by gel filtration chromatography and analysis of cysteine endopeptidase activity profile.

Sample	Volume (ml)	Protein (mg/ml)	Activity		Yield (%)	Purification Fold
			Total (U)	Specific (U/mg)		
Hemolymph	0.20	12.36	0.82	0.14	100	1
Superose 12	1.00	0.40	2.01	12.58	245	90

U = Relative Fluorescence Units / sec.

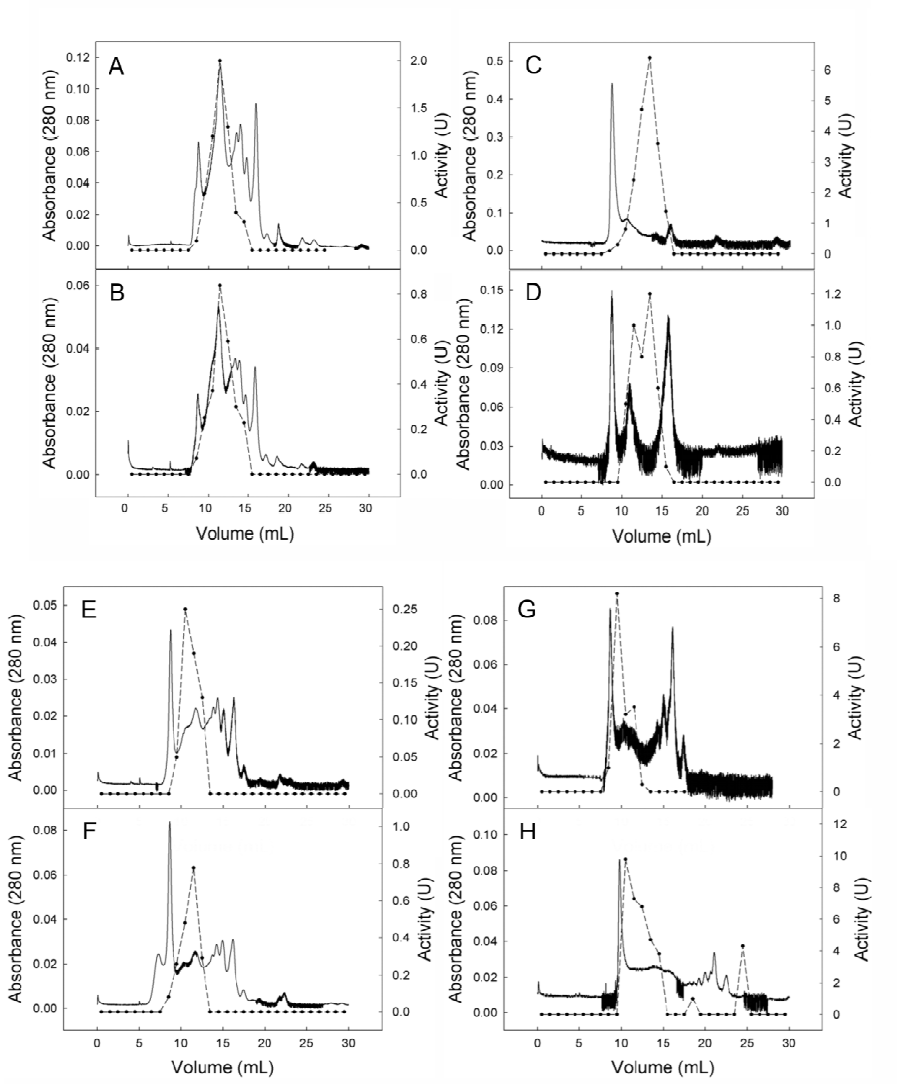

Figure 1 – Gel filtration fractioning profile of soluble proteins from partially and fully engorged female tissues. Protein loaded from each tissue was: A- partially engorged female fat body, 800 g; B- fully engorged female fat body 390 g, C- partially engorged female midmidgut, 800 g; D- fully engorged female midmidgut, 800 g; E- partially engorged female salivary gland, 460 g; F- fully engorged female salivary gland 526 g; G- partially engorged female ovary, 800 g; H- fully engorged female ovary, 800 g. Protein elution (A 280 nm ——); proteolytic activity (U ---●---).

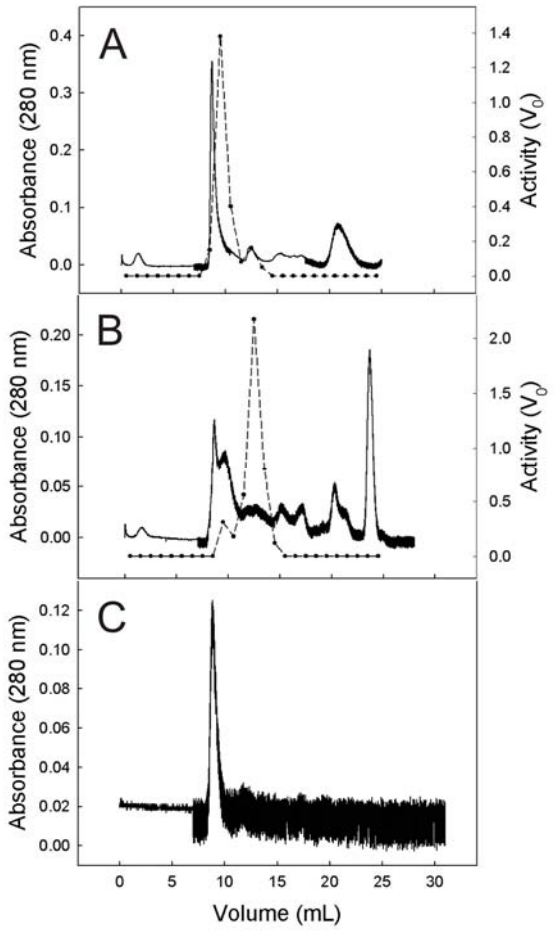

Figure 2 – Gel-filtration analysis of egg and larva protein extracts and purified vitellin. Samples were applied onto a Superose 12 column in sodium phosphate buffer (10 mM, pH 7.0) at 0.5 ml/min in FPLC system. Protein loaded from each sample was: A- egg extract, 3.6 mg; B- larva extract, 4.1 mg; C- purified Vt, 750 g. For A and B, proteolytic activity of fractions was tested on a fluorogenic substrate (N-CBz-Phe-Arg-MCA) at 37 °C in sodium citrate/sodium phosphate buffer (pH 3.5) and 10 mM DTT. Protein elution (A 280 nm ——); proteolytic activity (U ---●---).

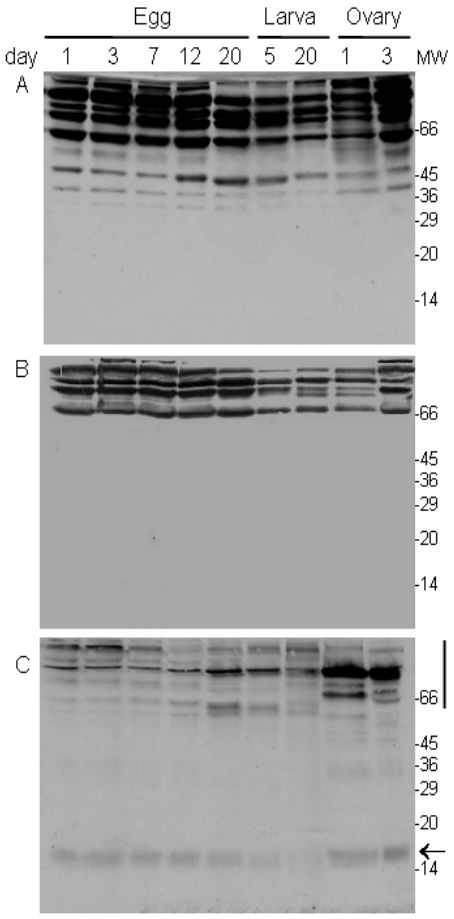

Figure 3 – VTDCE and VT immunolocalization in different developmental stages of ***R. microplus***. Extracts of 1 day-egg (1), 3 days-egg (3), 7 days-egg (7), 12 days-egg (12), 20 days-egg (20), 5 days-larva (5), 20- days larva (20), ovary of fully engorged female 1 day post-detachment (1) and 3 days post-detachment (3) were separated by (A) SDS-PAGE, (B) transferred to nitrocellulose and probed with anti-VT and (C) anti-VTDCE. Molecular weights in kDa are shown (MW). Replicate membranes probed with pre-immune serum showed no reactivity. VTDCE was recognized by anti-VTDCE antibodies in two forms: with high molecular weight (vertical bar) and with low molecular weight (arrow).

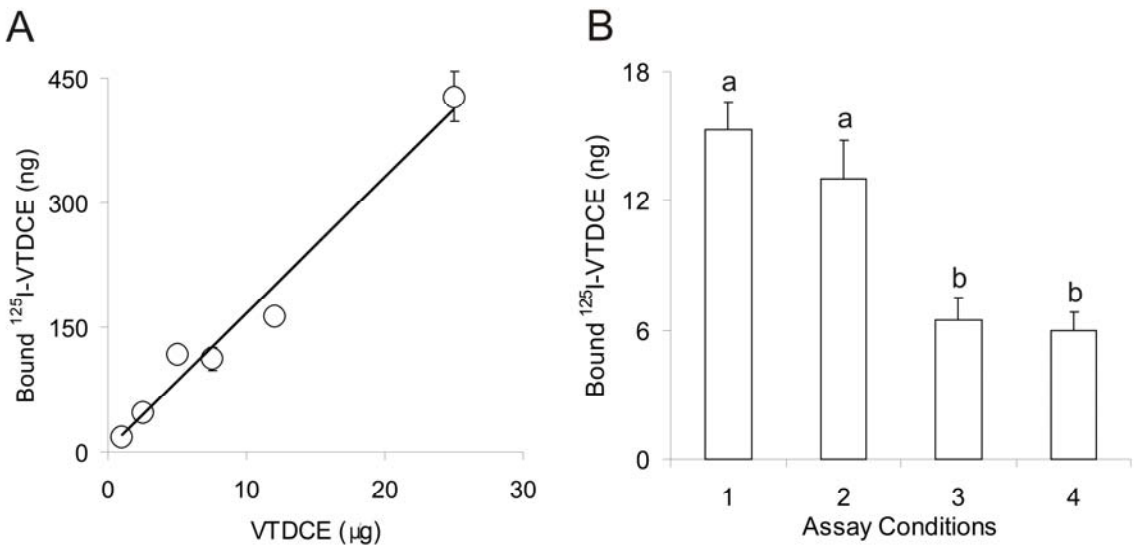

Figure 4 – VTDCE-vitellin binding. The association of soluble ^{125}I-VTDCE with VT (20 µg), fixed on nitrocellulose membrane, was tested (see Materials and Methods). In (A), binding assay performed in the presence of different amounts of ^{125}I-VTDCE. In (B), VT (20 µg, fixed on membrane) was incubated with: (1) 1 g ^{125}I-VTDCE; (2) 1 g ^{125}I-VTDCE + 100 M leupeptin; (3) 1 g ^{125}I-VTDCE + 1 mg VT (in solution); (4) 1 g ^{125}I-VTDCE + 1 mg VT (in solution) + 100 µM leupeptin. Results are expressed as total bound ^{125}I-VTDCE, and are means ± S.D. Different letters above bars denote statistically significant differences for $p<0.05$.

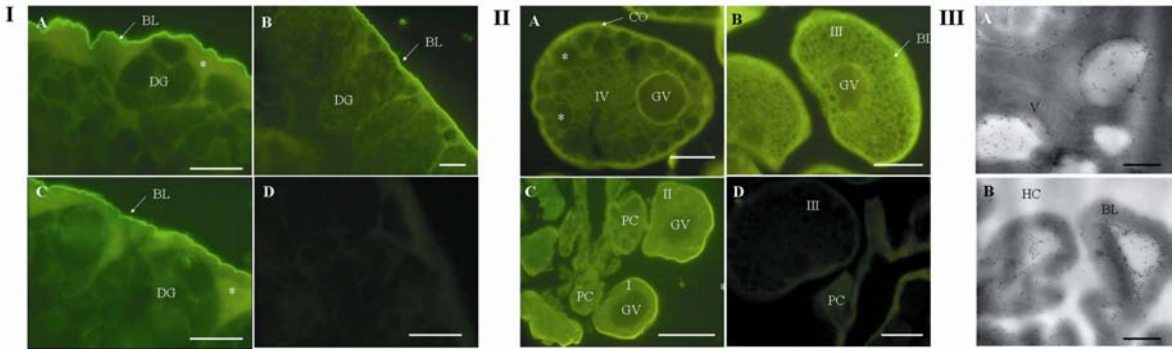

Figure 5 – VTDCE localization by immunofluorescence in ***R.*** (B.) ***microplus*** midgut and ovary. Fully engorged females were dissected at third day after blood meal and organs reacted with anti-VTDCE as described in material and methods. Midgut tissue showed a stronger signal in the basal lamina (BL; Panel I, A and B) contrasting with the signal in the basophilic cells (asterisk; Panel I, C). Scale bars represent 10 µm. Panel II, ovary thin sections presenting a strong labeling at the oocyte cytosol and basal lamina/chorium (CO) (Panel II, A and B), and also at the laminal region of the pedicel cells (PC) (Panel II, C); yolk granules (asterisk) do not present a significant VTDCE signal. Scale bars represent 20 µm. No labeling was observed in controls with non-immune serum (Panel I and II letter D). I, II, III, IV – oocyte developmental stage; gv – germinal vesicle. Panel III, detailed view of the VTDCE distribution in ***R. microplus***ovary by immuno-electron micrograph. The enzyme was immunolocalized in the pedicel cells vesicles membrane (A) and in ovary basal region (B). Scale bars represent 1 µm in A and 0.8 µm in B. V – pedicel cell vesicle; Hc – haemocoel, BL – basal lamina.

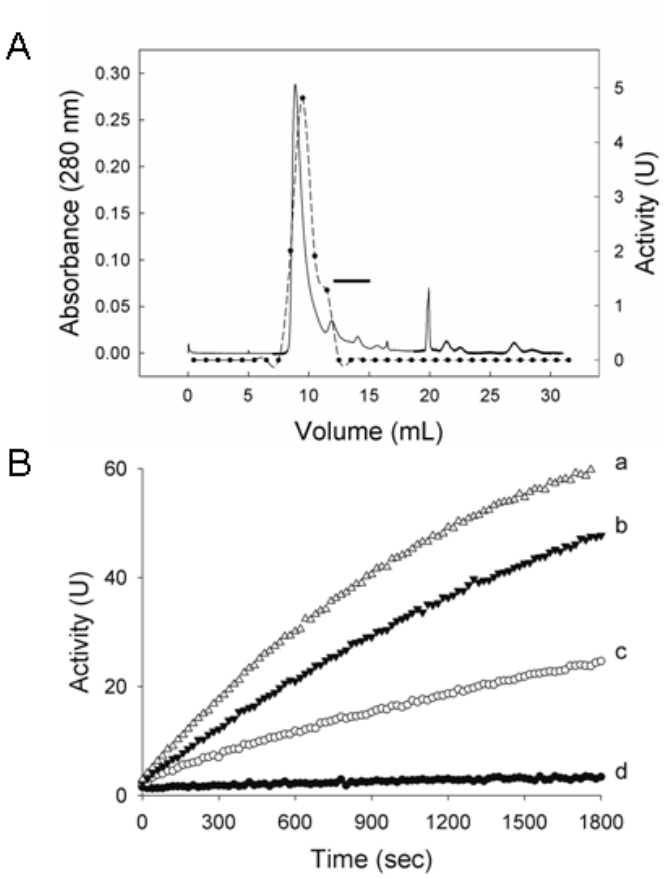

Figure 6 – A) Gel-filtration fractioning profile of engorged female hemolymph. Hemolymph (1:5 in PBS; 200 µl) was applied onto a Superose 12 column in sodium phosphate buffer (10 mM, pH 7.0) at 0.5 ml/min in FPLC system. Proteolytic activity of fractions tested upon the fluorogenic substrate N-CBz-Phe-Arg-MCA is shown as U ---●---; the black bar shows the cysteine endopeptidase inhibitor activity. Protein elution (A 280 nm ——). B) Dose dependent inhibition of VTDCE (1 µg) by the partially purified hemolymph cysteine endopeptidase inhibitor (gel filtration pool). Small letters indicate volumes of inhibitor fraction used: a- control, b – 2 µl, c – 5 µl, d - 8µl.

Andréia Bergamo Estrela
Curriculum Vitae

Junho/2008

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Estudos Comportamentais Associados a Semioquímicos Utilizando Instrumentação Avançada para o Desenvolvimento de Metodologias de Manejo de Insetos-Praga

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Situação: Concluído Natureza: Pesquisa
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2002 - Atual Cisteíno endopeptidase de larva do carrapato bovino Boophilus microplus

Descrição: O carrapato bovino Boophilus microplus é de grande importância econômica na pecuária, setor onde causa grandes prejuízos. Na tentativa de identificar peptídeos com potencial antigênico, estamos estudando a fisiologia deste parasita. O projeto visa a purificação e caracterização de uma cisteíno endopeptidase de larvas do carrapato bovino Boophilus microplus com atividade sobre vitelina, a principal proteína de reserva de embrões de arthropoda.
Situação: Em Andamento Natureza: Pesquisa
Alunos envolvidos: Graduação (1); Especialização (0); Mestrado acadêmico (1); Mestrado profissionalizante (0); Doutorado (1);
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3. Imunologia Aplicada

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Espanhol Compreende Razoavelmente , Fala Razoavelmente, Escreve Razoavelmente, Lê Razoavelmente

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2005 Destaque do XVII Salão de Iniciação Científica, PROPESQ- UFRGS

Produção em C, T & A

Produção bibliográfica

Artigos completos publicados em periódicos

1. ESTRELA, Andréia., SEIXAS, Adriana, TERMIGNONI, Carlos
A cysteine endopeptidase from tick (Rhipicephalus (Boophilus) microplus) larvae with vitellin digestion activity.. Comparative Biochemistry and Physiology. B, Biochemistry & Molecular Biology. , v.148, p.410 - 416, 2007.

Comunicações e Resumos Publicados em Anais de Congressos ou Periódicos (resumo)

1. ESTRELA, Andréia., SEIXAS, Adriana, CEOLATO, Juliana, TEIXEIRA, V. O., TERMIGNONI, Carlos
Digestive enzyme association in the cattle tick Rhipicephalus (Boophilus) microplus midgut In: XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology and 10th International Union of Biochemistry and Molecular Biology (IUBMB) Conference , 2007, Salvador.
XXXVI Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology. , 2007.
Referências adicionais : Brasil/Inglês. Meio de divulgação: Meio digital

2. ESTRELA, Andréia., SEIXAS, Adriana, TERMIGNONI, Carlos
Degradação de vitelina e hemoglobina por cisteíno endopeptidase no carrapato bovino Rhipicephalus (Boophilus) microplus. In: 14º Congresso Brasileiro de Parasitologia Veterinária, 2006, Ribeirão Preto.
14º Congresso Brasileiro de Parasitologia Veterinária - Programa -Resumos. , 2006.
Áreas do conhecimento : Bioquímica,Enzimologia,Parasitologia
Referências adicionais : Brasil/Português. Meio de divulgação: Impresso

3. ESTRELA, Andréia., SEIXAS, Adriana, TERMIGNONI, Carlos
Participação de cisteíno endopeptidase na digestão de proteínas no carrapato Rhipicephalus (Boophilus) microplus In: XVIII Salão de Iniciação Científica, 2006, Porto Alegre.
XVIII Salão de Iniciação Científica. , 2006.
Referências adicionais : Brasil/Português. Meio de divulgação: Vários

4. ESTRELA, Andréia., SEIXAS, Adriana, CEOLATO, Juliana, TERMIGNONI, Carlos
Boophilus microplus larvae cysteine endopeptidase: an improved purification protocol. In: XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005, Águas de Lindóia, SP.
XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. , 2005.
Palavras-chave: Boophilus microplus, tick larvae, vitellin, cysteine-endopeptidase
Áreas do conhecimento : Bioquímica,Enzimologia,Parasitologia
Referências adicionais : Brasil/Inglês. Meio de divulgação: Vários

5. ESTRELA, Andréia., SEIXAS, Adriana, TERMIGNONI, Carlos
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Eventos

Participação em eventos

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Totais de produção

Produção bibliográfica

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Participações em eventos (encontro)	3
Participações em eventos (outra)	6