UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

"Ação tóxica da urease de *Canavalia ensiformis* e do peptídeo recombinante Jaburetox-2Ec sobre *Oncopeltus fasciatus* (Hemiptera: Lygaidae)"

Marina Schumacher Defferrari

Dissertação submetida ao Programa de Pós-Graduação
em Biologia Celular e Molecular da UFRGS,
para a obtenção do título de Mestre em Ciências.

Orientação: Profª. Drª. Célia Regina Ribeiro da Silva Carlini
Co-orientação: Prof. Dr. Ian Orchard, University of Toronto at Mississauga

Porto Alegre
Março de 2010

BANCA EXAMINADORA

Dr. Carlos Peres Silva

Departamento de Bioquímica, Universidade Federal de Santa Catarina

---

Dr. Pedro Lagerblad de Oliveira

Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro

---

Dr. Carlos Termignoni

Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul

---

Dr. Diogo Ribeiro Demartini

Revisor da dissertação e membro suplente da banca examinadora

Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul

A primeira parte deste trabalho foi desenvolvida no Laboratório de Proteínas Tóxicas, Departamento de Biofísica e Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul, sob orientação da Profª. Drª. Célia Regina Ribeiro da Silva Carlini. A segunda parte foi desenvolvida no Laboratório de Neurofisiologia de Insetos, Departamento de Biologia da Universidade de Toronto em Mississauga (Canadá), sob orientação do Professor Ian Orchard, PhD, e no Centro de Investigação da Faculdade de Ciências Biomédicas, Universidade Austral (Argentina), sob orientação da Profª. Drª. Beatriz Patricia Settembrini.

As agências financiadoras do trabalho foram o Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), sendo este último o órgão responsável por conceder a bolsa de mestrado. No Canadá, o trabalho teve suporte do órgão de fomento NSERC (Natural Sciences and Engineering Research Council of Canada).

AGRADECIMENTOS

À minha orientadora Célia, agradeço por todas oportunidades, pelo aprendizado e pelo incentivo ao meu crescimento profissional. Muito obrigada por acreditar no meu trabalho e sempre estar pronta para discutir, acrescentar e melhorar minhas pesquisas.

Às agências brasileiras de financiamento, CAPES, CNPq, FAPERGS e FINEP, por fazerem possível o desenvolvimento da ciência em nosso país e em nosso estado; e em especial a CAPES, por me conceder a bolsa de mestrado.

Ao Ian e a Vicki, e a todo pessoal do laboratório de Neurofisiologia de Insetos, por me receberem em seu laboratório e em seu país de forma tão acolhedora. Aprendi e cresci muito como cientista e como ser humano ao longo dos seis meses que passei com vocês. E à agência canadense NSERC, por tornar toda essa experiência no Canadá possível.

Aos colegas e amigos do Laboratório de Proteínas Tóxicas, muito obrigada pela convivência diária, pelo apoio nos momentos complicados e pela parceria nos momentos de festa.

A todos meus amigos muito obrigada por existirem na minha vida e me fazerem uma pessoa mais feliz.

Aos meus irmãos muito obrigada por estarem sempre perto apesar das distâncias físicas. Vocês são, cada um com seu jeito especial de ser, as pessoas que eu quero ao meu lado para sempre.

Ao meu pai e à Helena obrigada por sempre terem me dado apoio, incentivo e serem um exemplo de amor, luta e perseverança.

À minha mãe obrigada por TUDO; pelas melhores oportunidades e pelo maior amor que alguém pode ter na vida. E ao Álvaro, muito obrigada por fazer ela feliz!

LISTA DE ABREVIATURAS

5HT: 5-hidroxitriptamina (serotonina)

A: absorbância

Abz: ácido orto-aminobenzóico

AMPc: adenosina monofosfato cíclico

BCIP: 5-bromo-4-cloro-3-indoil fosfato

EB: extrato bruto de intestinos

EDDnp: *N*-(2,4-dinitrofenil)-etilenodiamina

EDTA: ácido etilenodiamino tetracético

E-64: L-trans-epoxy succinil-L-leucilamido (4-guanidino)-butano

FITC: isotiocianato de fluoresceína

FPLC: fast protein liquid chromatography

IgG: imunoglobulina G

JBU: urease de *Canavalia ensiformis*

kDa: quilodaltons

MCA: 7-amido-4-metilcumarina

mU: miliunidade enzimática

NaAc: acetato de sódio

NBT: azul de nitrotetrazolina

PBS: tampão fosfato-salina

Pep-A: pepstatina A

PVDF: difluoreto de polivinilideno

URF: unidade relativa de fluorescência

SDS-PAGE: gel de poliacrilamida com dodecil-sulfato de sódio

TCA: ácido tricloracético

TTBS: tampão Tris-HCl salina contendo 0,05 % v/v Tween 20

RESUMO

A *Canavalia ensiformis* é uma leguminosa altamente resistente ao ataque de insetos, e contribuindo para essa resistência, a planta possui isoformas de urease, que são proteínas que apresentam atividade entomotóxica. Essa toxicidade é dependente de uma ativação proteolítica da molécula por enzimas digestivas do inseto, do tipo catepsinas, e subseqüente liberação de peptídeos internos, os quais possuem a ação inseticida. A partir dessa informação foi construído um peptídeo recombinante com base na seqüência da urease, chamado Jaburetox-2Ec, o qual é tóxico para insetos resistentes e suscetíveis a urease intacta. O hemíptero *Oncopeltus fasciatus* é um dos nossos modelos de estudo. O objetivo deste trabalho foi identificar as principais peptidases digestivas de ninfas de *O. fasciatus,* auxiliando na elucidação do mecanismo de ação inseticida dessas toxinas, além de estudar os efeitos da urease intacta e do peptídeo derivado sobre órgãos isolados. Quando alimentadas com diferentes concentrações da urease majoritária de *C. ensiformis* (JBU), ninfas de *O. fasciatus* apresentam uma taxa de mortalidade superior a 80% após duas semanas, assim como quando injetadas com Jaburetox-2Ec apresentam uma taxa de mortalidade de 100% após 48 horas. Ninfas de quarto instar foram dissecadas para retirada dos intestinos e obtenção de um extrato protéico bruto, o qual foi utilizado para a realização dos ensaios enzimáticos. O extrato bruto foi capaz de hidrolisar *in vitro* a JBU e liberar peptídeos com massa semelhante a peptídeos entomotóxicos já conhecidos, reconhecíveis pelo anticorpo anti-Jaburetox-2Ec. A atividade proteolítica majoritária sobre substratos protéicos, em pH 4.0, foi bloqueada por inibidores específicos de aspártico e cisteíno peptidases, não sendo afetada EDTA, inibidor de metalopeptidases. Ao utilizar-se um substrato específico para cisteíno peptidases, o pico majoritário observado foi em pH 5.0, sendo a atividade completamente inibida por E-64 (inibidor de cisteíno peptidases) em todos os valores de pH testados. Ao utilizar-se um substrato desenhado para aspártico peptidases, a atividade majoritária foi novamente em pH 5.0, sendo parcialmente inibida por Pepstatina A (inibidor de aspártico peptidases) é parcial em todos os valores de pH testados. Substratos sintéticos correspondentes às regiões N e C-terminal que flanqueiam o peptídeo inseticida na molécula de urease também foram testados. O primeiro não foi hidrolisado pelo extrato bruto, enquanto que o segundo apresentou um pico de atividade majoritário em pH 4.0-5.0, com inibição total por E-64. O extrato bruto foi submetido a um processo de purificação de duas etapas, uma troca iônica e uma cromatografia de gel filtração. A purificação foi monitorada utilizando-se um substrato específico para cisteíno peptidases, e a fração da gel filtração com maior atividade

apresentou massa molecular de 22 kDa. A fração ativa foi submetida a um gel de poliacrilamida, a banda foi excisada e digerida com tripsina, e os peptídeos resultantes foram então analisados por espectrometria de massas. Uma catepsina L foi identificada, com massa molecular semelhante ao previsto pela gel filtração. Os resultados sugerem que a susceptibilidade de ninfas de *O. fasciatus* a JBU é, como em outros modelos de insetos, relacionada à proteólise limitada da proteína ingerida e posterior liberação de peptídeos inseticidas por enzimas do tipo catepsinas. Diferentes tipos de catepsinas podem estar liberando os peptídeos inseticidas a partir da urease, sendo que um cisteíno peptidase, tipo Catepsina L, pode ter um papel importante na ativação da proteína em *O. fasciatus*.

Adicionalmente fizemos localização *in situ* de Jaburetox-2Ec após injeção na hemolinfa e observamos imunoreatividade no sistema nervoso central de ninfas de *O. fasciatus*, indicando que o peptídeo pode estar agindo neste local. Além dos efeitos tóxicos causados pelos peptídeos derivados da urease, já foram relatados em outros modelos, efeitos causados pela molécula intacta. Testamos, então, o efeito da JBU sobre contrações de intestinos *ex vivo* de *O. fasciatus*. Observamos que a urease tem a capacidade de inibir, em algumas concentrações, o efeito excitatório da serotonina sobre as contrações musculares, atividade que não é compartilhada com Jaburetox-2Ec. Concluímos com os dados gerados nesse estudo, e em estudos prévios, que a toxicidade das ureases não só é devida à ativação proteolítica e liberação de peptídeos, mas também à proteína intacta, que parece estar causando distúrbios em processos de sinalização celular.

ABSTRACT

Jackbean (*Canavalia ensiformis*) is a legume highly resistant to insects. Contributing to this resistance, the plant contains urease isoforms that are entomotoxic upon the release of internal peptides by insect's digestive cathepsin-like enzymes. A recombinant peptide, called Jaburetox-2Ec, was built based on urease's sequence and this peptide is poisonous to all insects tested. The hemipteran bug *Oncopeltus fasciatus* is one of our models of study. In this work we aimed to identify the main digestive peptidases of *O. fasciatus,* helping to elucidate the insecticidal mechanism of action of these toxins, and to test the effects of urease and derived peptides upon isolated organs. When fed with different Jackbean major urease (JBU) concentrations, *O. fasciatus* nymphs showed a mortality rate higher than 80% after two weeks. When injected with Jaburetox-2Ec the mortality rate was 100% after 48 hours. Homogenates of midguts dissected from fourth instars were used to perform proteolytic activity assays. The homogenates hydrolyzed JBU *in vitro,* yielding a fragment similar in size to known entomotoxic peptides. The major proteolytic activity at pH 4.0 upon protein substrates was blocked by specific aspartic and cysteine peptidases inhibitors, but was not affected by a metalopeptidase inhibitor (EDTA). The optimal activity upon a fluorogenic substrate specific for cysteine peptidases was at pH 5.0, being completely inhibited by E-64 at all pH values tested. The optimal activity upon a fluorogenic substrate designed for aspartic peptidases was again at pH 5.0, being partially blocked by Pepstatin A in the pH range 2 to 10. Fluorogenic substrates corresponding to the N- and C-terminal regions flanking the entomotoxic peptide within urease sequence were also tested. While the N-terminal peptide was not hydrolyzed by the midguts homogenate, the C-terminal peptide was cleaved maximally at pH 4.0-5.0, with complete inhibition by E-64. The midguts homogenate was submitted to ion exchange chromatography followed by gel filtration yielding a 22 kDa protein peak. After SDS-PAGE this band was excised from the gel, digested with trypsin and the peptides were analyzed by mass spectrometry. A Cathepsin L was identified. The results suggest that susceptibility of *O. fasciatus* nymphs to JBU is, like in other insect models, due to limited proteolysis of ingested protein and subsequent release of entomotoxic peptides by Cathepsin-like digestive enzymes.

Moreover, we performed *in situ* localization of Jaburetox-2Ec after injection in the hemolymph, and we observed immunoreactive processes in *O. fasciatus* nymphs' central nervous system, indicating that the peptide could be acting there. Besides the poisonous effects caused by urease derived peptides, effects caused by the intact molecule were already described. Hence we tested urease's effect upon *O. fasciatus*

isolated midguts contractions. We observed that urease is capable of inhibiting, at some concentrations, serotonin excitatory effect on muscular contractions, activity that was not shared with Jaburetox-2Ec. We concluded from the data of this study and previous studies, that urease's toxicity is not only due to proteolytic activation and release of toxic peptides, but also to the intact molecule, which seems to be causing disturbances in cell signalling.

ÍNDICE

I. Introdução 12

I.I. Mecanismos vegetais de defesa.. 12

I.II. Atividade entomotóxica das ureases 12

I.III. Digestão e excreção em insetos.. 15

I.IV. O sistema nervoso em insetos... 18

I.V. *Oncopeltus fasciatus*.. 21

II. Objetivos.. 23

II.I. Objetivos específicos.. 23

Primeira Parte

Manuscrito

"Insecticidal effect of *Canavalia ensiformis* major urease on nymphs of the milkweed bug *Oncopeltus fasciatus* and characterization of digestive peptidases"…………………………………………………………………… 24

Segunda Parte

"Interação da urease de *C. ensiformis* e de Jaburetox-2Ec com tecidos isolados de ninfas de *O. fasciatus"*.. 56

1. Materiais e Metodologia 57

1.1. Reagentes.. 57

1.2. Colônia de insetos.. 57

1.3. Salina para *O. fasciatus*... 57

1.4. Bioensaio.. 57

1.5. Imunohistoquímica.. 58

1.6. Quantificação e intensidade de contrações do intestino de *O. fasciatus*... 58

1.7. Análises estatísticas.. 58

2. Resultados 59

2.1. Atividade tóxica de Jaburetox-2Ec em ninfas de quinto instar de *O. fasciatus*... 59

2.2. Imunolocalização de Jaburetox-2Ec no sistema nervoso central de *O. fasciatus*... 60

2.3. Efeito da urease de *C. ensiformis* e do peptídeo recombinante Jaburetox-2Ec nas contrações de intestinos de *O. fasciatus*... 63

III. Discussão.. 69

IV. Conclusões.. 72
V. Referências Bibliográficas.. 73
ANEXO I
Curriculum Vitae.. 82
ANEXO II
Artigo publicado
"Stage-specific gut peptidases of the cotton stainer bug *Dysdercus peruvianus*: Role in the release of entomotoxic peptides from *Canavalia ensiformis* urease"……………………………………………………………... 88

I. Introdução

I.I. Mecanismos vegetais de defesa

As plantas produzem proteínas e peptídeos que participam dos mecanismos de defesa ao ataque de insetos, como por exemplo proteínas inibidoras de proteases e de glicohidrolases, lectinas, arcelinas e proteínas inativadoras de ribossomos. Outros componentes de natureza protéica envolvidos nesses mecanismos são as tiaminases, quitinases, vicilinas modificadas (Ryan, 1990; Felton, 1996; Bolter & Jongsma, 1997; Koiwa *et al.*, 1997; Stotz *et al.*, 1999) e as ureases (Polacco & Holland, 1993; Carlini & Grossi-de-Sá, 2002; Follmer 2008; Carlini & Polacco, 2008).

A semente de *Canavalia ensiformis*, uma leguminosa altamente resistente ao ataque de insetos, popularmente conhecida como feijão-de-porco (Jackbean), é uma fonte rica em proteínas de interesse bioquímico e biotecnológico, como a urease (Sumner, 1926), a concanavalina A (Sumner & Howell, 1936), inibidores de tripsina (Ubatuba, 1955) e a canatoxina (Carlini & Guimarães, 1981). A urease de *C. ensiformis* (JBU) foi a primeira enzima a ser cristalizada e a primeira enzima níquel-dependente caracterizada (Sumner, 1926; Dixon *et al.*, 1975). A forma ativa mínima da JBU é um trímero de cadeias de 90 kDa, sendo encontrada na forma nativa como um hexâmero de 540 kDa (Zerner, 1991).

Carlini e Guimarães (1981) isolaram das sementes de *C. ensiformis* a canatoxina (CNTX), uma proteína indutora de convulsões, que causa morte de camundongos e ratos quando injetada intraperitonealmente (DL_{50}= 0,5 a 2,0 mg/ kg). A CNTX também é ativa por via intravenosa, intramuscular e subcutânea, porém não é capaz de induzir os efeitos letais se administrada por via oral, provavelmente devido à sua instabilidade em meio ácido (Carlini *et al.*, 1984; Carlini & Guimarães, 1991). Comparações de seqüências parciais indicaram alto grau de homologia (cerca de 85%) com a JBU, e estudos posteriores demonstraram que a CNTX é uma isoforma de urease, possuindo de 30 a 40% da atividade ureolítica da forma majoritária da enzima na semente (Follmer *et al.*, 2001). A forma ativa da CNTX apresenta uma massa molecular de 184 kDa quando analisada por gel-filtração. Em SDS-PAGE, em meio redutor ou não, sua massa molecular é cerca de 95 kDa, indicando que a forma nativa da proteína é um dímero ligado por forças não covalentes (Follmer *et al.*, 2001).

I.II. Atividade entomotóxica das ureases

Estudos sobre o mecanismo de ação da CNTX mostraram que esta apresenta potente efeito secretagogo em vários tipos de células de mamíferos, ativando a produção de eicosanóides e as vias de lipoxigenases, e induzindo alterações dos

níveis e fluxos intracelulares de Ca^{2+} (Carlini *et al.*, 1985; Barja-Fidalgo *et al.*, 1991a e 1991b; Ghazaleh *et al.*, 1992; Ghazaleh *et al.*, 1997). A CNTX induz em ratos bradicardia, hipertensão e hipotermia, fatos que precedem o fenômeno convulsivo tônico-clônico, característico da ação tóxica (Carlini *et al.*, 1984). Com exceção da toxicidade intraperitoneal em camundongos, a urease majoritária de *C. ensiformis* (JBU) parece compartilhar todas as outras propriedades farmacológicas descritas para a canatoxina (Follmer *et al.*, 2001). A exemplo de outras toxinas protéicas (Eidels *et al.*, 1983), JBU e CNTX se comportam como lectinas monovalentes ligando-se a glicoderivados complexos, como gangliosídeos e fetuína (Carlini & Guimarães, 1991; Follmer *et al.*, 2001; Real-Guerra, 2007).

O papel fisiológico das ureases na planta também foi investigado, buscando uma provável função protetora contra fitopatógenos e insetos fitófagos,. Ureases e proteínas canatoxina-like foram detectadas em diversas leguminosas através de ensaios de imunorreatividade com anticorpos policlonais anti-canatoxina, sugerindo um processo de conservação evolutiva dos determinantes antigênicos (Carlini *et al.*, 1988). Além disso, relatos de que o conteúdo da ureases aumenta progressivamente durante a maturação das sementes, reforça a idéia de que essas proteínas desempenham um importante papel de defesa na planta (Barcellos *et al.*, 1993). Estudos mais recentes demonstraram a atividade fungicida e fungistática, sobre fungos filamentosos, inclusive fitopatogênicos, tanto da CNTX (Oliveira *et al.*, 1999) quanto das ureases de *C. ensiformis*, de soja (*Glycine max*) e da bactéria *Helicobacter pylori* (Becker-Ritt *et al.*, 2007).

A CNTX foi administrada oralmente em insetos pertencentes a diferentes ordens, como por exemplo *Manduca sexta* (Lepidoptera: Sphingidae), *Schistocerca americana* (Orthoptera: Acrididae), *Drosophila melanogaster* (Diptera: Drosophilidae), *Callosobruchus maculatus* (Coleoptera: Bruchidae), *Aedes aegypti* (Diptera: Culicidae), *Rhodnius prolixus* e *Triatoma infestans* (Hemiptera: Reduviidae), *Nezara viridula* (Hemiptera: Pentatomidae) e *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) (Carlini *et al.*, 1997; Carlini & Grossi-de-Sá, 2002). Considerando-se que *C. maculatus*, *R. prolixus*, *T. infestans*, *N. viridula* e *D. peruvianus*, insetos suscetíveis à CNTX, possuem o trato digestório acídico e enzimas proteolíticas do tipo catepsinas, os resultados indicaram que a especificidade da ação tóxica está diretamente relacionada com o sistema digestório dos insetos (Carlini *et al.*, 1997; Ferreira-DaSilva *et al.*, 2000; Stanisçuaski *et al.*, 2005). Já os insetos resistentes à ingestão de ureases possuem o trato digestório alcalino e enzimas do tipo tripsinas, que digerem completamente a urease sem formar peptídeos tóxicos (Terra & Ferreira, 1994; Carlini *et al.*, 1997).

A CNTX não tem efeito inibitório sobre as principais proteases e α-amilases de *C. maculatus* e *R. prolixus* (Carlini *et al.*,1997). Descartada uma ação inibitória sobre as enzimas digestivas, a hipótese de ativação proteolítica da CNTX pelas peptidases do trato digestório dos insetos foi avaliada. Estudos com *R. prolixus* mostraram que a administração da canatoxina juntamente com inibidores clássicos de cisteíno e aspártico peptidases, como E-64 e Pepstatina-A respectivamente, aumentava significativamente a taxa de sobrevivência dos insetos (Carlini *et al.*, 1997; Ferreira-DaSilva *et al.*, 2000). Assim, a ativação da toxina envolveria enzimas com atividade em valores de pH acídicos (tipo aspártico e cisteíno-peptidases) e o efeito tóxico seria decorrente da liberação de peptídeos inseticidas. A hipótese foi comprovada a partir da hidrólise *in vitro* da CNTX por enzimas digestivas de *C. maculatus* – os peptídeos gerados mostraram efeito inseticida sobre *R. prolixus* e a administração conjunta dos inibidores não foi capaz de impedir a morte dos insetos (Gombarovits, 1999; Ferreira-DaSilva *et al.*, 2000).

Os fragmentos entomotóxicos gerados foram separados por gel-filtração e o mais ativo entre eles, chamado de pepcanatox, foi purificado e teve a seqüência N-terminal determinada (Gombarovitz, 1999; Ferreira-DaSilva *et al.*, 2000). Um peptídeo recombinante, equivalente ao pepcanatox, chamado Jaburetox 2-Ec, derivado de uma das isoformas de urease, foi obtido por expressão heteróloga em *Escherichia coli*, mostrando-se um bioinseticida em potencial (Pires-Alves, 2002; Mulinari, 2004). Jaburetox 2-Ec confirmou sua ação inseticida em ensaios biológicos com o percevejo *D. peruvianus* (Stanisçuaski *et al.*, 2005) e com a lagarta-do-cartucho do milho, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), mesmo sendo esta resistente às ureases – devido as suas enzimas digestivas tipo tripsinas (Mulinari *et al.*, 2007).

Follmer *et al.* (2004b) avaliaram as propriedades biológicas de ureases vegetais (*C. ensiformis* e *Glycine max*) e de uma microbiana (*Bacillus pasteurii*) em *D. peruvianus.* A JBU se mostrou tão tóxica quanto a CNTX, e ambas, na concentração de 0,052% (p/p), apresentaram uma potência inseticida três vezes maior que a urease de soja. Já urease de *B. pasteurii* não apresentou atividade inseticida, provavelmente por não possuir parte da seqüência amino acídica correspondente ao peptídeo entomotóxico.

Ferreira-DaSilva *et al.* (2000) e Stanisçuaski *et al.* (2005) relataram diferenças na susceptibilidade à CNTX entre formas adultas e jovens dos percevejos *R. prolixus* e *D. peruvianus.* Diferenças na resposta à toxinas protéicas durante o ciclo de vida de insetos já foram relatadas, como por exemplo, Keller *et al.* (1996) que observaram o aumento da resistência à toxina Cry1C, de *Bacillus thuringiensis* (*Bt*), nas últimas fases larvais de *Spodoptera littoralis* (Lepidoptera: Noctuidae) em relação às formas

mais jovens, fato atribuído a um processamento enzimático diferenciado da proteína ingerida. Piovesan *et al.* (2008), indicaram que em *D. peruvianus* a composição enzimática do trato digestório difere entre ninfas e adultos, sendo os picos de atividade majoritários deslocados para valores de pH mais básicos na forma adulta. Alterações na permeabilidade da membrana intestinal durante o ciclo de vida do inseto também poderiam ter um papel importante na toxicidade das ureases. Ferreira-DaSilva *et al.* (2000) demonstraram que, após um repasto de sangue de coelho, ninfas de *R. prolixus* apresentavam na hemolinfa imunoglobulinas de coelho intactas e fragmentadas, no entanto um padrão diferenciado de fragmentos e ausência da imunoglobulina não processada foi constatado na hemolinfa de adultos.

I.III. Digestão e excreção em insetos

A classe Insecta é composta por um grupo incrivelmente diverso de animais com mais de um milhão de espécies (Gallo *et al.*, 2002). Considerando-se todos os animais existentes hoje na Terra, cerca de 67% são insetos (Buzzi & Miyazaki, 1999). Estes se alimentam de substâncias orgânicas, sejam elas de origem animal ou vegetal - alguns consomem madeira, folhas, sementes, frutos ou fungos, enquanto outros se alimentam de tecidos animais, lã, sangue e até de outros membros do filo Arthropoda. Com relação aos hábitos alimentares praticados, os insetos podem ser classificados como fitófagos, zoófagos, incluindo predadores e parasitos, e saprófagos (Buzzi & Miyazaki, 1999).

A nutrição diz respeito aos componentes químicos necessários a um organismo para a manutenção dos tecidos, crescimento e reprodução, além da energia necessária para manter estas funções (Chapman, 1998). Os nutrientes necessários para o pleno desenvolvimento dos insetos podem vir do alimento ou de outras fontes, como tecidos de reserva (vitelo e corpo gorduroso), microrganismos simbiontes (bactérias, protozoários e fungos) ou da síntese do próprio organismo (vitaminas) (Buzzi & Miyazaki, 1999).

Grande parte do alimento ingerido pelos insetos é macromolecular, na forma de polissacarídeos e proteínas, enquanto lipídeos são ingeridos na forma de glicerídeos, fosfolipídios e glicolipídios. A digestão é o processo onde o alimento é degradado em componentes assimiláveis, ocorrendo no trato digestório através da ação hidrolítica de enzimas digestivas secretadas pelo intestino médio e glândulas salivares (Buzzi & Miyazaki, 1999). O sistema digestório de insetos é formado pelo tubo e pelas partes acessórias: glândulas salivares, cecos gástricos e túbulos de Malpighi. O tubo digestório é dividido em três regiões: intestino anterior, intestino médio e intestino posterior (Chapman, 1998). O intestino anterior serve como órgão de armazenagem, o

intestino médio é o principal órgão de digestão e absorção de nutrientes e o intestino posterior e os túbulos de Malpighi são órgãos de reabsorção e de excreção (Terra, 1988).

No intestino médio (Fig. 1) são encontradas as enzimas digestivas proteolíticas, que podem variar de acordo com a espécie e a fase do desenvolvimento do inseto (Terra & Ferreira, 1994). A partir da posição da ligação peptídica clivada as enzimas proteolíticas podem ser classificadas como endo ou exopeptidases. As primeiras clivam ligações peptídicas nas regiões internas da proteína, enquanto as exopeptidases agem nos extremos da cadeia protéica, que pode ser a porção amino (N) ou carboxi (C)-terminal (Barret *et al.*, 1998). Atualmente são reconhecidas 14 classes de enzimas capazes de clivar ligações peptídicas, e entre elas há pelo menos cinco classes de endopeptidases – aspárticas, cisteínicas, serínicas, treonínicas e metaloendopeptidases (www.chem.qmul.ac.uk/iubmb). Os mecanismos utilizados nos processos catalíticos das peptidases envolvem a ativação de uma molécula de água (como é o caso das endopeptidases aspárticas e das metaloendopeptidases), que adquire caráter nucleofílico e é capaz de atacar a carbonila da ligação peptídica, ou pela formação de um intermediário covalente em que as cadeias laterais dos aminoácidos (endopeptidases cisteínicas, serínicas e treonínicas) são os próprios nucleófilos (Barret *et al.,* 1998).

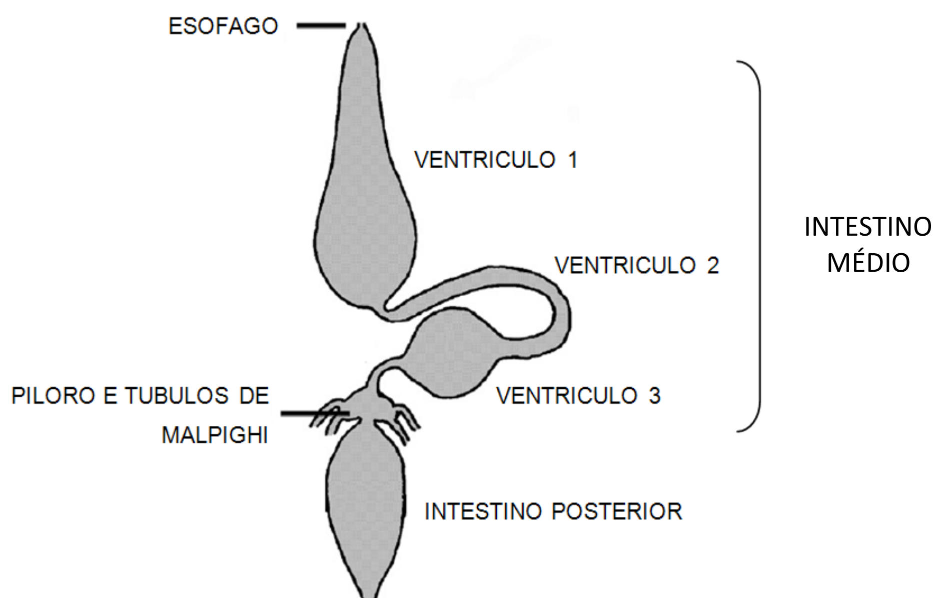


Figura 1. Esquema ilustrando o tubo digestório de *Oncopeltus fasciatus*. Adaptado de Te Brugge & Orchard, 2007.

Para seus processos de desenvolvimento e reprodução, os insetos necessitam dos mesmos aminoácidos essenciais que mamíferos: arginina, lisina, leucina, isoleucina, triptofano, fenilalanina, histidina, metionina, valina e treonina (Murdock & Shade, 2002). A ausência de certos nutrientes pode afetar o desenvolvimento dos insetos, comprometendo o crescimento, as ecdises e a reprodução. Além disso, também podem ocorrer alterações na coloração e na distribuição de castas nos insetos sociais (Chapman, 1998; Buzzi & Miyazaki, 1999).

A diversidade de fontes de alimento dos insetos resultou em modificações profundas do tubo digestório e dos processos enzimáticos em certas espécies. As enzimas que realizam a digestão dos alimentos exibem diversidade correspondente e variam com a natureza dos mesmos (Terra & Ferreira, 1994; Murdock & Shade, 2002). Devido ao reduzido conteúdo protéico dos tecidos vegetais, a obtenção de nitrogênio é fator limitante na nutrição de insetos fitófagos, e por isso a eficiência na hidrólise das proteínas ingeridas na dieta é fundamental para que sobrevivam.

A ampla distribuição de proteínas de defesa nas plantas reflete a importância do processo de proteólise para os insetos predadores, pois a ingestão de inibidores de proteases interfere diretamente na biodisponibilidade de aminoácidos necessários para o crescimento, desenvolvimento e reprodução dos insetos (Broadway & Duffey, 1986; Broadway, 1995; Koiwa *et al.*, 1998; Zhu-Salzman *et al.*, 2003). Possivelmente, a diversidade das enzimas proteolíticas descritas em insetos fitófagos seja uma resposta para a presença abundante de inibidores de protease nos tecidos vegetais (Broadway, 1996).

I.IV. O sistema nervoso em insetos

O sistema nervoso dos insetos é formado por um cérebro e um cordão nervoso dividido em gânglios. O cérebro é composto de um protocérebro com olhos, um deutocérebro com antenas e um tritocérebro, posicionado dorsalmente na cabeça, acima do intestino anterior. Os gânglios nervosos segmentares, divididos em torácicos e abdominais, freqüentemente encontram-se fundidos, e são ventralmente posicionados. O primeiro gânglio da cadeia ventral é o subesofágico, formado pela fusão dos gânglios dos segmentos mandibular, maxilar e labial. Os corpos cardíacos e alados são corpos glandulares associados ao gânglio hipocerebral, encontram-se sobre o intestino anterior logo abaixo do cérebro e, juntamente com as glândulas protorácicas, são os principais centros endócrinos nos insetos. Controlam crescimento e metamorfose, regulação da reabsorção hídrica, ritmo cardíaco e outros processos metabólicos. Tipicamente existem três gânglios torácicos, mas em alguns insetos estão fusionados e formam um único gânglio. O gânglio metatorácico é comumente fusionado com um ou mais gânglios abdominais anteriores. Cada gânglio torácico tem cinco ou seis nervos em cada lado, que enervam músculos e órgãos sensoriais do tórax e dos apêndices. Algumas vezes, a maioria dos gânglios, ou todos eles, são fusionados e formam um único gânglio composto, como nos hemípteros *Rhodnius prolixus* e *Oncopeltus fasciatus* (Fig. 2). (Ruppert & Barnes, 1996; Chapman, 1998)

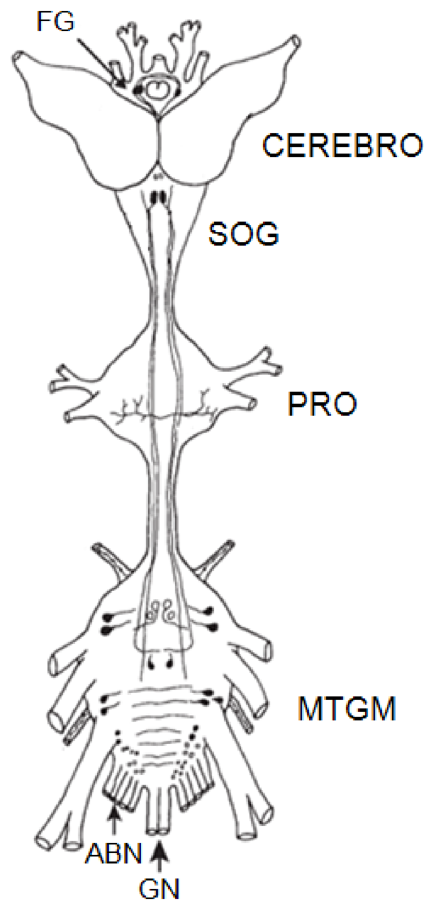


Figura 2. Esquema ilustrando a anatomia do sistema nervoso central de *Oncopeltus fasciatus*. MTGM – massa gangliônica mesotorácica; ABN – nervos abdominais; GN – nervos genitais; SOG – gânglio subesofágico; PRO – gânglio protorácico; FG – gânglio frontal. Adaptado de Miggiani *et al*., 1999.

Diversas substâncias atuam como transmissores no sistema nervoso central (SNC) de insetos, sendo uma das mais estudadas a serotonina (5HT) (Miggiani *et al.*, 1999), que age como neurotransmissor convencional ou como modulador de funções integradas. Atua também em órgãos periféricos como o coração, as glândulas salivares, os músculos bucais, os túbulos de Malpighi e a cutícula (Settembrini & Villar, 2004). Insetos tratados com urease apresentam vários processos com controle neurohormonal afetados (Stanisçuaski *et al.*, 2009), o que pode significar que ureases e peptídeos derivados estejam competindo com neurotransmissores, ou alterando a transdução de sinal envolvida, causando um prejuízo em processos mediados pelos mesmos.

Estudos foram realizados com órgãos isolados de *Rhodnius prolixus* para avaliar o mecanismo de ação das ureases e peptídeos derivados sobre sistemas específicos. Stanisçuaski *et al.* (2009) investigaram a ação antidiurética dessas moléculas em Túbulos de Malpighi isolados, e observaram que tanto JBU quanto Jaburetox-2Ec afetaram a secreção induzida por serotonina, em concentrações de 10^{-9} a 10^{-12} M. Stanisçuaski *et al.* (submetido) obervaram uma potenciação causada pela JBU no efeito excitatório da serotonina em contrações de intestinos. No mesmo estudo, foi observada após administração oral de JBU, a presença de moléculas com atividade ureolítica preservada na hemolinfa de *R. prolixus*. Também no lepidóptero *Bombyx mori* (Bicho-da-seda) havia sido demonstrado que moléculas de urease enzimaticamente ativas atravessam o epitélio do intestino médio e podem ser detectadas na hemolinfa (Hirayama *et al.,* 2000; Kurahashi *et al.,* 2005). A presença de urease intacta na hemolinfa sugere que o efeito inseticida possa não ser exclusivamente dependente da liberação de peptídeos entomotóxicos, e que os efeitos da JBU observados *in vitro* também possam contribuir para a toxicidade.

I.V. *Oncopeltus fasciatus*

Insetos da espécie *Oncopeltus fasciatus* (Hemiptera: Lygaeidae) possuem uma coloração de alerta vermelha e se alimentam, na natureza, preferencialmente de plantas da família Asclepiadaceae, que possuem, no látex e na seiva, altos teores de glicosídios cardioativos, compostos tóxicos para muitos vertebrados e invertebrados (Meredith *et al* 1984). Por ser uma das poucas espécies de insetos que se alimentam dessas plantas, o controle das populações de *Asclepias* spp. é feito em grande parte por *O. fasciatus*. Este inseto possui poucos predadores naturais, pois acumula em seu corpo substâncias impalatáveis que retira das plantas (www.life.uiuc.edu/ib/109/Insect %20rearing/milkweedbug). Exemplares da espécie podem ser encontrados em diversas regiões da América do Norte, principalmente nas proximidades das Montanhas Rochosas, e também no Brasil (Feir, 1974).

Seu ciclo biológico é incompleto e curto, durando cerca de 50 dias. Dos ovos eclodem ninfas, que passam por 5 estágios larvais até tornarem-se adultos. Colônias desse inseto podem ser facilmente estabelecidas em laboratório, sendo mantidas com dietas compostas basicamente de sementes de girassol (*Helianthus annuus*) (Feir, 1974), em temperaturas entre 25 e 27 ^{o}C, como descrito por Romeiro *et al.* (2000). Apesar de não ser economicamente importante como uma praga, a espécie tem sido largamente utilizada como modelo para estudos em pesquisas entomológicas e como conseqüência disto, muitos aspectos da sua fisiologia são conhecidos em detalhes (Feir, 1974). No presente estudo, demonstramos que ninfas de *O. fasciatus* (Fig. 3) são suscetíveis à ação tóxica da JBU, após administração oral, e à ação tóxica do peptídeo Jaburetox-2Ec após injeção na hemolinfa. *In vitro* as enzimas do trato digestório são capazes de clivar a urease e liberar peptídeos reconhecíveis pelo anticorpo anti-Jaburetox-2Ec. Por essas razões, o modelo em questão se torna útil para o estudo dos efeitos entomotóxicos dessas proteínas, possibilitando a comparação com outros modelos já estudados.

Figura 3. Ninfas de quinto instar de *Oncopeltus fasciatus*. Foto por Mike Quinn, TexasEnto.Net.

II. Objetivos

II.I. Objetivo geral

O objetivo deste trabalho foi estudar a atividade entomotóxica e o mecanismo de processamento da urease majoritária de *Canavalia ensiformis* (JBU) e seus efeitos sobre sistemas isolados do modelo hemíptero *Oncopeltus fasciatus.* Os dados obtidos foram analisados em paralelo com dados prévios de outros modelos, auxiliando na compreensão e elucidação do modo de ação das ureases em insetos.

II.II. Objetivos específicos

- Testar a ação inseticida da urease de *C. ensiformis* e do peptídeo recombinante Jaburetox-2Ec em ninfas, por via oral ou por injeção na hemolinfa.
- Isolar e identificar enzimas envolvidas na hidrólise da urease de *C. ensiformis* no trato digestório de ninfas de *O. fasciatus.*
- Estudar os efeitos da urease de *C. ensiformis* e do peptídeo recombinante Jaburetox-2Ec em tecidos musculares por monitoramento de freqüência de contrações em intestinos isolados.
- Imunolocalizar após injeção o peptídeo recombinante Jaburetox-2Ec em diferentes tecidos no organismo do inseto.

Primeira Parte

“Insecticidal effect of *Canavalia ensiformis* urease on nymphs of the milkweed bug *Oncopeltus fasciatus* and characterization of digestive peptidases”*

**Manuscrito submetido ao Journal of Insect Biochemistry and Molecular Biology*

Elsevier Editorial System(tm) for Insect Biochemistry and Molecular Biology
Manuscript Draft

Manuscript Number: IB-D-10-00107

Title: Insecticidal effect of *Canavalia ensiformis* urease on nymphs of the milkweed bug *Oncopeltus fasciatus* and characterization of digestive peptidases

Article Type: Full Length Article

Keywords: Cysteine endopeptidase; cathepsin-L; proteinase; purification; insecticide; insect digestion; class specific proteinase inhibitor

Corresponding Author: Professor Celia R Carlini, Ph.D.

Corresponding Author's Institution: Universidade Federal do Rio Grande do Sul

First Author: Marina S Defferrari, M.Sc.

Order of Authors: Marina S Defferrari, M.Sc.; Thiago B Marcelin; Paulo M Pinto, Ph.D.; Celia R Carlini, Ph.D.

Abstract: Jackbean (*Canavalia ensiformis*) ureases are entomotoxic upon the release of internal peptides by insect's digestive enzymes. Here we studied the digestive peptidases of *Oncopeltus fasciatus* (milkweed bug) and its susceptibility to jackbean urease (JBU). JBU-fed O. fasciatus nymphs showed 90% mortality after two weeks. The major proteolytic activity of MH upon protein substrates at pH 4.0 was blocked by E-64 or pepstatin-A, but it resisted EDTA, ortho-phenantroline or PMSF. Hydrolysis by MH of fluorogenic substrates designed for cysteine or aspartic peptidases was optimal at pH 5.0, with inhibition by E-64 or by pepstatin A, respectively. Nymph's midgut homogenates (MH) hydrolyzed JBU in vitro. Fluorogenic substrates corresponding to the N- and C-terminal regions flanking the entomotoxic peptide within JBU were tested. While the N-terminal peptide was not hydrolyzed, MH cleaved the C-terminal peptide (optimal pH 4.0-5.0), with complete inhibition by E-64. MH submitted to ion exchange chromatography and gel filtration yielded a major activity peak (22 kDa). In gel trypsin digestion of this protein band followed by mass spectrometry analysis identified a cathepsin-L. Our data suggest that after ingestion by O.fasciatus, JBU is subjected to limited proteolysis by cathepsin-like digestive enzymes yielding entomotoxic peptide(s) that ultimately kill the insect.

Insecticidal effect of *Canavalia ensiformis* urease on nymphs of the milkweed bug *Oncopeltus fasciatus* and characterization of digestive peptidases

Marina S. Defferrari[a], Thiago B. Marcelin[c], Paulo M. Pintoa[b], and Celia R. Carlini [a, c, *]

[a]Graduate Program of Cellular and Molecular Biology, Center of Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Building 43421, CEP 91501-970 Porto Alegre, Rio Grande do Sul, Brasil

[b]Institute of Biotechnology, Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas 1130, Bloco 57, Caixa Postal 1352, Caxias do Sul, Rio Grande do Sul, Brasil

[c]Department of Biophysics and Center of Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Building 43422, CEP 91501-970 Porto Alegre, Rio Grande do Sul, Brasil

*Corresponding author: Prof. Célia R. Carlini
Department of Biophysics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil, CEP 91501-970, tel: +55-51-3308-7606, fax: +55-51-3308-7003.
E-mail: ccarlini@ufrgs.br or celia.carlini@pq.cnpq.br

Abstract

Jackbean (*Canavalia ensiformis*) ureases are entomotoxic upon the release of internal peptides by insect's digestive enzymes. Here we studied the digestive peptidases of *Oncopeltus fasciatus* (milkweed bug) and its susceptibility to jackbean urease (JBU). JBU-fed *O. fasciatus* nymphs showed 90% mortality after two weeks. The major proteolytic activity of MH upon protein substrates at pH 4.0 was blocked by E-64 or pepstatin-A, but it resisted EDTA, ortho-phenantroline or PMSF. Hydrolysis by MH of fluorogenic substrates designed for cysteine or aspartic peptidases was optimal at pH 5.0, with inhibition by E-64 or by pepstatin A, respectively. Nymph's midgut homogenates (MH) hydrolyzed JBU *in vitro*. Fluorogenic substrates corresponding to the N- and C-terminal regions flanking the entomotoxic peptide within JBU were tested. While the N-terminal peptide was not hydrolyzed, MH cleaved the C-terminal peptide (optimal pH 4.0-5.0), with complete inhibition by E-64. MH submitted to ion exchange chromatography and gel filtration yielded a major activity peak (22 kDa). *In gel* trypsin digestion of this protein band followed by mass spectrometry analysis identified a cathepsin-L. Our data suggest that after ingestion by *O.fasciatus*, JBU is subjected to limited proteolysis by cathepsin-like digestive enzymes yielding entomotoxic peptide(s) that ultimately kill the insect.

Keywords: Cysteine endopeptidase, cathepsin-L, proteinase, purification, insecticide, insect digestion, class specific proteinase inhibitor

Introduction

Plants have diverse physicochemical defense mechanisms to resist insect attack, among which are insecticidal proteins such as lectins, arcelins, ribosome inactivating proteins, peptidase- and amylase inhibitors, thiaminases, chitinases, modified storage proteins (Ryan *et al.*, 1990; Felton *et al.*, 1996; Bolter & Jongsma, 1997; Koiwa *et al.*, 1997; Stotz *et al.*, 1999). More recently ureases were characterized as part of the arsenal of plant entomotoxic proteins (Polacco & Holland, 1993; Carlini & Grossi-de-Sá, 2002; Follmer *et al.*, 2004a; Carlini & Polacco, 2008). Ureases (urea amidohydrolase; EC 3.5.1.5) are nickel-dependent enzymes that catalyze urea hydrolysis into ammonia and carbon dioxide, and are synthesized by plants, fungi and bacteria (Mobley *et al.*, 1995; Follmer, 2008). In plants, ureases are homotrimers or hexamers of 90 kDa subunits and contribute to the use of urea as nitrogen source (Polacco & Holland, 1993; Sirko & Brodzik, 2000; Follmer, 2008). Canatoxin is a less abundant urease isoform isolated from *Canavalia ensiformis* seeds (Carlini & Guimarães, 1981; Follmer *et al.*, 2001) and, similar to the seed's major urease, both proteins display insecticidal properties (Carlini *et al.*, 1997) independent of ureolytic activity (Follmer *et al.*, 2004a; Follmer *et al.*, 2004b; Stanisçuaski *et al.*, 2005). This toxicity is partly due to the release of urease's internal peptides by the insect's digestive enzymes; insects with cathepsin-like peptidases are susceptible to this effect, while insects with trypsin-like enzymes are not (Carlini *et al.*, 1997; Ferreira-DaSilva *et al.*, 2000; Piovesan *et al.*, 2008). Nymphs of the hematophagous kissing bug, *Rhodnius prolixus* (Hemiptera:Reduviidae), larvae of the cowpea weevil, *Callosobruchus maculatus* (Coleoptera:Bruchidae), and nymphs of the cotton stainer bug, *Dysdercus peruvianus* (Hemiptera: Pyrrochoridae) are examples of susceptible insects, all relying on cathepsin-like digestive enzymes (Ferreira-DaSilva *et al.*, 2000; Stanisçuaski *et al*, 2005). Acidic metallopeptidases were also shown to be involved in urease breakdown in *D. peruvianus* nymphs (Piovesan *et al.*, 2008). A 10 kDa entomotoxic peptide named pepcanatox was obtained from in vitro hydrolysis of canatoxin by *C. maculatus* digestive enzymes (Ferreira-DaSilva *et al.*, 2000). Jaburetox-2Ec, a recombinant peptide equivalent to pepcanatox (Mulinari *et al.*, 2004, 2007), is a potent insecticide but does not affect mice or neonate rats when administered by oral or intraperitoneal routes (Mulinari *et al.*, 2007). This recombinant peptide also kills insects that are resistant to intact ureases, such as *Spodoptera frugiperda* (Mulinari *et al.*, 2007) or *Triatoma infestans* adults (Tomazzeto *et al.*, 2007).

Digestive peptidases play critical roles in insect physiology: breaking down proteins into essential amino acids for growth and development, and inactivating

ingested protein toxins (Terra *et al.*, 1996). There are six groups of endopeptidases according to the amino acid residue, or metal ion, present in the active site, involved in peptide bond hydrolysis (Barret *et al.*, 1998): serine, cysteine, aspartic, glutamic, threoninic and metallo peptidases. Insects that have neutral to alkaline digestive tracts usually use serine peptidases for digestion, while insects with acidic midguts generally have cysteine and aspartic peptidases (Terra & Ferreira, 2005; Howe & Jander, 2008). The large milkweed bug, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae), is found east of the Rocky Mountains in Canada and the United States, feeding on milkweed seeds, *Asclepias syriaca*, but occasionally they suck juices from other plants. *O. fasciatus* has been used in physiological studies, and recently has proven to be a useful insect model system for developmental studies, since they are easy to rear and have a short life cycle (egg to adult in approximately 50 days) (Feir, 1974; Te Brugge & Orchard, 2007). Acidic pH values for the digestive tract of *O. fasciatus* were previously reported (Bongers, 1970), as well as the presence of cysteine peptidases and the absence of serine peptidases trypsin and chymotrypsin (Woodring *et al.*, 2007). In this study, we aimed to investigate urease toxicity and processing by nymphs of *O. fasciatu*s and to characterize the profile of their digestive enzymes. Because the release of entomotoxic peptides from urease could be due to a concerted or sequential action of more than one enzyme, as is the case for *D. peruvianus* (Piovesan et al.,2008), we decided to study the enzymatic activity profile of *O. fasciatus* whole midguts' homogenates.

Material and Methods

Reagents

Azocasein, bovine hemoglobin, trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64), Pepstatin-A, ethylenediamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), methylcoumarin amide (MCA)-coupled peptide substrates, anti-rabbit IgG conjugated with alkaline phosphatase, nitro-blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Jackbean urease were from Sigma Chemicals Company (Saint Louis, USA). *o*-phenantrolin was from Pro Analysis, Merck (Darmstadt, Germany). Hybond-P membrane (Polyvinylidene Fluoride – PVDF, 0.45 μm) was from Amersham Biosciences (Little Chalfont, England). CM-Sepharose Fast Flow and Sephadex G-50 were from GE Healthcare, Amersham Biosciences (Little Chalfont, England). Centriprep devices with YM-3 membranes were from Amicon Inc. (Beverly, MA, USA). Dialysis membranes were from SpectraPor (California, USA). Fluorogenic peptide substrates coupled to aminobenzidine and ethylenediamine 2,4-dinitrophenyl (Abz-EDDnp) were supplied by Dr. Luis Juliano Neto and Dr. Maria

Aparecida Juliano (Dept. Biophysics, Universidade Federal de São Paulo, São Paulo, Brazil). The recombinant peptide Jaburetox-2Ec and anti-Jaburetox-2Ec polyclonal antibodies raised in rabbits were prepared as described previously (Mulinari *et al.*, 2007; Tomazzeto *et al.*, 2007).

Insects

A colony of *O. fasciatus* was established in our laboratory from individuals initially supplied by Dr. Patrícia Azambuja Penna (Fundação Oswaldo Cruz, Rio de Janeiro) and by Dr. Denise Feder (Universidade Federal Fluminense, Rio de Janeiro). The colony is kept under controlled temperature (22 ^{o}C ± 2 ^{o}C) and humidity (70% +/- 5%) and a 14/10 hours (light/dark) photoperiod, according to Milano *et al.* (1999). The insects feed on sunflower seeds (*Helianthus annuus*) and cotton seeds (*Gossipium hirsutum*) and have free access to water, developing from eggs to adults in about 30-35 days.

Bioassay of urease insecticidal activity

The bioassay was carried out essentially as described by Stanisçuaski *et al.* (2005). Groups of 14 nymphs (third instars) of *O. fasciatus* were fed on artificial cotton seeds, consisting of gelatin capsules (size 2, Elli Lilly Co.) containing cotton seed flour mixed to 0.01 or 0.2% (w/w) freeze-dried urease. Survival rates and molting were recorded daily until day 15.

Midgut homogenates

Homogenates of *O. fasciatus* whole midguts were used for determination of proteolytic activities. Non-starved fourth instars were anaesthetized in ice, whole midguts were removed and stored at -20 ^{o}C in 0.02 M sodium phosphate buffer NaPB) pH 7.0 with 0.02% sodium azide, in a proportion of 50 intestines per mL. To prepare the midgut homogenates (MH), the material was thawed, homogenized manually with an ice-cold Potter pestle, centrifuged at 4 ^{o}C at 8,000 g for 5 minutes and then at 8,000 g for 30 minutes. The final supernatants were filtered through a 0.22 μm membrane, dialyzed against 2 liters of NaPB and kept frozen at -20 ^{o}C until use.

Protein determination

Protein concentration was determined by the method of Layne (1957), using a spectrophotometer (UV/Vis Spectrophotometer Hitachi, California, USA) at 280/260 nm absorbances according to Layne, 1957.

Enzyme assays

For all enzyme assays the buffers used were sodium phosphate for pH 2.0, 6.0 and 7.0, sodium acetate for pH 4.0 and 5.0, ammonium formiate for pH 3.0, TRIS base for pH 8.0 and 9.0, and sodium carbonate for pH 10.0. For determination of proteolytic activity upon protein substrates, MH (2 mg protein/mL) aliquots were incubated with 0.2 % azocasein or 0.2 % hemoglobin in 300 μL reaction volume in buffers (0.1 M) with pH varying from 2.0-10.0, at 37°C. When present, inhibitors were preincubated for 30 min with MH before adding the substrate. After 1 h at 37 °C, 250 μL of 10% trichloroacetic acid (TCA) was added, followed by centrifugation (20 min, 8000 g). To 450 μL supernatant 250 μL NaOH (2 M) was added, mixed and then absorbance was read at 420 nm (azocasein) or 280 nm (hemoglobin). One activity unit was defined as the amount of enzyme releasing 1 A420 or 1 A280 of trichloroacetic acid-soluble peptides, per hour at 37 °C. Different fluorogenic substrates were employed to characterize the enzymatic activities in the midgut extracts. Aliquots of MH were incubated in microplates, at 37 °C, with 20 μM Abz-AIAFFSRQ-EDDnp or 2 μM N-Cbz-Phe-Arg-MCA, in 100 μL final volume. Peptide substrates corresponding to the N and C-terminal regions flanking the entomotoxic peptide within urease sequence (Mulinari *et al.*, 2007) were also tested, at 20 μM final concentration. The substrates were Abz-NAIADGPVQ-EDDnp (equivalent to the N-terminal region) and Abz-KVIRDGMGQ-EDDnp (equivalent to the C-terminal region). The pH was varied between 2.0 and 8.0 using different buffers (0.05 M final concentration). Reactions were monitored for 30 minutes with a Spectra-Max M2*e* (Molecular Devices Inc.), using 320 nm excitation – 420 nm emission filters for EDDnp substrates and 370 nm excitation – 460 nm emission filters for the MCA substrate. Results are shown as relative fluorescence units (RFU) per hour per mg of MH, taken the highest activity peak as 100%.

Urease *in vitro* hydrolysis

Urease digestion by *O. fasciatus* peptidases was performed as described in Ferreira-DaSilva *et al.* (2000) with minor modifications, using 1.5 mU of MH azocaseinolytic activity per microgram of urease, in 0.05 M ammonium formate, pH 5.6, at 37 °C, under continuous stirring for 24 hours. The reaction was stopped by freezedrying the samples.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Samples were mixed 4:1 (v/v) with the SDS-PAGE sample buffer and loaded on 5%-stacking/12%-separating gels. After the run, gels were stained with Coomassie brilliant blue R-250 and destained with 10% acetic acid. Molecular mass markers used were Bench Mark Protein Ladder (Invitrogen Life Technologies, California, USA).

Western blot

Urease hydrolysis was followed by Western blot (Towbin *et al.,* 1979) after SDS-PAGE in 12% polyacrylamide gels. After being transferred to 0.22 μm PVDF membranes, they were incubated overnight with anti-Jaburetox-2Ec antibodies (1:25,000) and then exposed for 2 hours to anti-rabbit IgG coupled to alkaline phosphatase (1:50,000). The color reaction was developed with NBT and BCIP in buffer containing MgCl2, pH 9.6.

Purification of a cysteine peptidase from midguts homogenate

All purification steps were analyzed using the fluorogenic substrate 2 μM NCbz-Phe-Arg-MCA, in 100 μL final volume, pH 5.0, at 37 °C, as described above.

Cation exchange chromatography

For cation exchange chromatography, a column packed with 10 mL of CM Sepharose Fast-Flow resin was equilibrated in 20 mM sodium acetate buffer pH 5.0 (buffer A). After sample application (91.8 A280), the column was washed with buffer A o remove non-retained proteins and then step-wise eluted with buffer A containing 0.1, 0.3, 0.5 and 1 M NaCl. Non-retained and eluted materials were collected in 10 ml fractions. Enzymatically active fractions were pooled, dialyzed against buffer A in a 3.5 kDa cut-off membrane, and concentrated using a Centriprep ultrafiltration device (3 kDa cut-off).

Size exclusion chromatography

Gel filtration was performed using a Sephadex G-50 column (1.2 x 30 cm) equilibrated in buffer A. Active pools (7.84 A280) from cation exchange chromatography were applied to the column. Protein peaks, monitored at 280 nm, were individually collected in 0.5 mL fractions and assayed for enzymatic activity. For molecular mass determination, the column was previously calibrated with protein markers from a gel filtration LMW Calibration Kit (GE Healthcare, Amersham Biosciences, Little Chalfont, England).

In-gel trypsin digestion and mass spectrometry analysis

Protein bands were manually excised from Coomassie stained gels and *in-gel* digested with trypsin. Gel slices were reduced (10 mM DTT) and alkylated (50 mM iodoacetamide) at room temperature and washed in three steps with 180 µL of 50% acetonitrile and 50 mM ammonium bicarbonate for 15 min, followed by one washing step with 180 µL of acetonitrile. After the washing procedures, gel plugs were dried by vacuum centrifugation and digested for 18–24 h at 37 ºC using 12 µL of 10 µg per mL modified porcine trypsin (sequencing grade modified trypsin, Promega Corporation, Wisconsin, USA) diluted in 25 mM NH4HCO3. After tryptic digestion, peptides were extracted in two washing steps with 50 µL of 50% acetonitrile and 5% trifluoroacetic acid (TFA) for 1 h. Extracted peptides were dried and resuspended in 10 µL of 0.1% formic acid. To identify proteins, a liquid chromatography (LC) separation (reversed-phase HPLC) coupled with tandem mass spectrometry (MS/MS) strategy was used. MS/MS analysis were performed in an electrospray ionisation (ESI) quadrupole time-of-flight (Q-TOF) Q-TOF Micro™ mass spectrometer (Micromass, Waters, Milford, USA) coupled to a nanoAcquity UltraPerformance LC® (UPLC®, Waters, Milford, USA). A nanoflow ESI source was used with a lockspray source for lockmass measurement during all the chromatographic runs. The samples were separated in a Nanoease C18 (75 µm ID) capillary column eluted with a water/acetonitrile 0.1% formic acid gradient. Data were acquired in data-dependent mode (DDA), and multiple charged peptide ions (+2 and +3) were automatically mass selected and dissociated in MS/MS experiments. Typical LC and ESI conditions were flow of 600 nL/min, nanoflow capillary voltage of 3.5 kV, block temperature of 100°C, and cone voltage of 100 V. The MS/MS spectra were processed using Proteinlynx v.2.0 software (Waters,Milford, US) and the generated PKL files were used to perform database searches using the MASCOT software v. 2.2 (Matrix Science, London, UK) against the nonredundant NCBI database (9,868,855 sequences and 3,366,351,629 residues, as per Oct. 15, 2009). Search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 0.2 Da, and MS/MS tolerance of 0.2 Da. The significance threshold was set at $p < 0.05$, and identification required that each protein contained at least one peptide with an expected value <0.05.

Statistical analysis

Results are expressed as means + standard deviation or standard error of means. Significance of differences between means was determined using one-way ANOVA. Data were considered statistically different when $p < 0.05$.

Results

Urease insecticidal effect

In order to evaluate the insecticidal effect of *C. ensiformis* urease on third instar *O. fasciatus,* nymphs were fed with the freeze-dried protein mixed to cotton flower in artificial seeds. Figure 1 shows that a 90% mortality rate is reached after 14 days for the tested two concentrations (0.01 and 0.2% w/w). These results are similar to previous observations for both isoforms of urease isolated from *C. ensiformis* seeds, canatoxin and JBU, in *Dysdercus peruvianus* nymphs (Follmer *et al.*, 2004b; Stanisçuaski *et al.*, 2005).

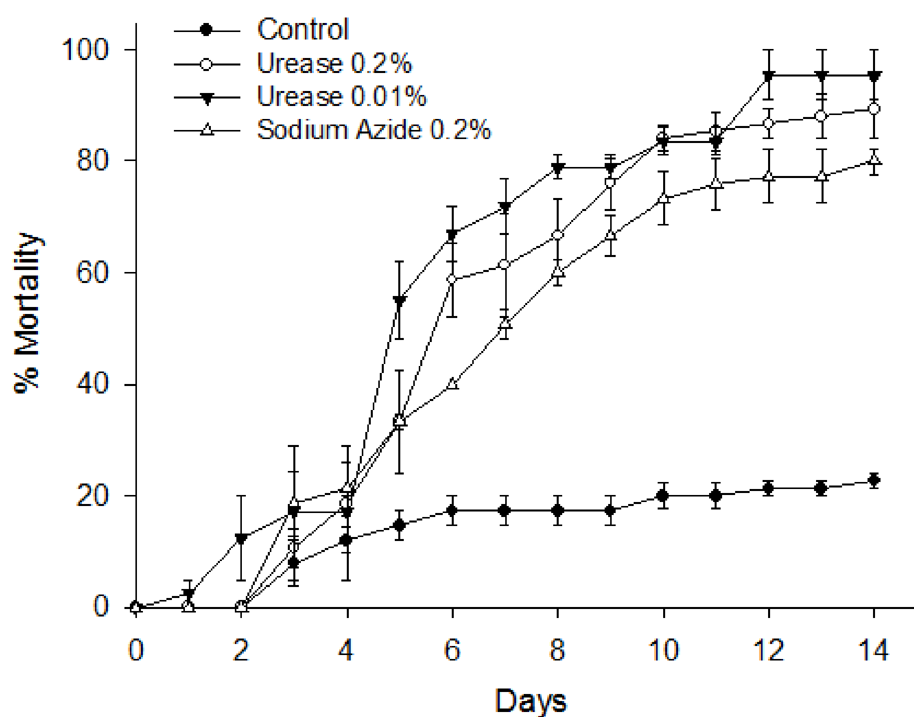


Figure 1. Insecticidal effect of urease on third instar *Oncopeltus fasciatus* nymphs. Insects (N=14) were fed on artificial seeds containing 0.01 and 0.2% (w/w) freeze-dried jackbean urease. Artificial seeds containing 0.2% (w/w) sodium azide was used as a positive control. Mortality rate was observed daily. Results are expressed as means ± s.e.m. of triplicates.

Urease *in vitro* hydrolysis

As we previously showed for *D. peruvianus* (Piovesan *et al*., 2008), the release of the entomotoxic peptide(s) from urease could be due to the concerted or sequential action of more than one enzyme. Because of that, here we decided to analyze homogenates of whole midguts. Urease was hydrolyzed by MH after a 24 hours incubation at 37 °C, pH 5.6, forming a fragment between 10 and 15 KDa, recognized by anti-Jaburetox-2Ec antibodies (Fig. 2). By size (Mr) and cross-immunological reactivity criteria this peptide is similar to the entomotoxic peptide characterized by Ferreira-DaSilva *et al*. (2000).

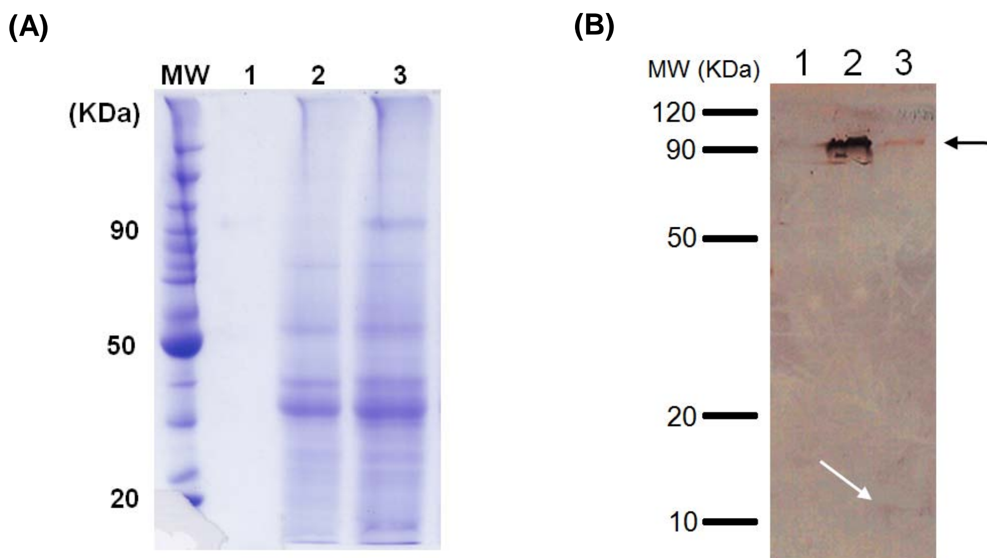


Figure 2. Urease *in vitro* hydrolysis by MH. (A) Analysis by SDS-PAGE. Lane 1, urease (1 μg) alone; lane 2, MH (50 μg) alone; lane 3, urease reaction with MH (1.5 azocaseinolytic units of MH per μg urease) after 24 hours at pH 5.6, 37 °C. Molecular masses of standard proteins (kDa) are indicated. (B) Western blot analysis developed with anti-Jaburetox antibodies. Lane 1, MH (50 μg) alone; lane 2, urease (1 μg) alone; lane 3, urease reaction with MH (same as above). The black arrow indicates intact urease and the white arrow indicates the fragment recognized by anti-Jaburetox antibodies.

Enzymatic activity upon protein substrates

Hemoglobin is protein substrate preferentially cleaved by aspartic proteinases (Barret, 1998). The activity of MH upon this substrate (Fig. 3) was seen in pH ranging from pH 3.0 to 6.0, peaking at pH 4.0, being strongly inhibited by E-64 (a cysteine peptidase inhibitor) (Fig. 3A) and by Pepstatin A (an aspartic peptidase inhibitor) (Fig. 3B), below pH 6.0. Azocasein is protein substrate preferentially cleaved by cysteine peptidases (Barret, 1998). Fig. 4 shows that MH cleaved this substrate at pH ranging from pH 3.0 to 8.0, peaking at pH 4.0 and again at pH 6.0, being strongly inhibited by E-64 (Fig. 4A) and poorly inhibited by Pepstatin A (Fig. 4E) in all pH values tested. No significant inhibition by the metallopeptidases inhibitors EDTA (Fig. 3C and 4C) or *o*-phenantrolin (Fig. 4D), was detected for the MH activity upon hemoglobin or azocasein at pH 5.0 or lower, although some inhibition was detected at neutral to alkaline pHs. PMSF, a serine peptidase inhibitor, also did not significantly inhibited MH activity at pH 5.0 or lower, producing 40-540% inhibition of MH azocaseinolic activity in pH 6.0 and 7.0 (Fig. 4B).

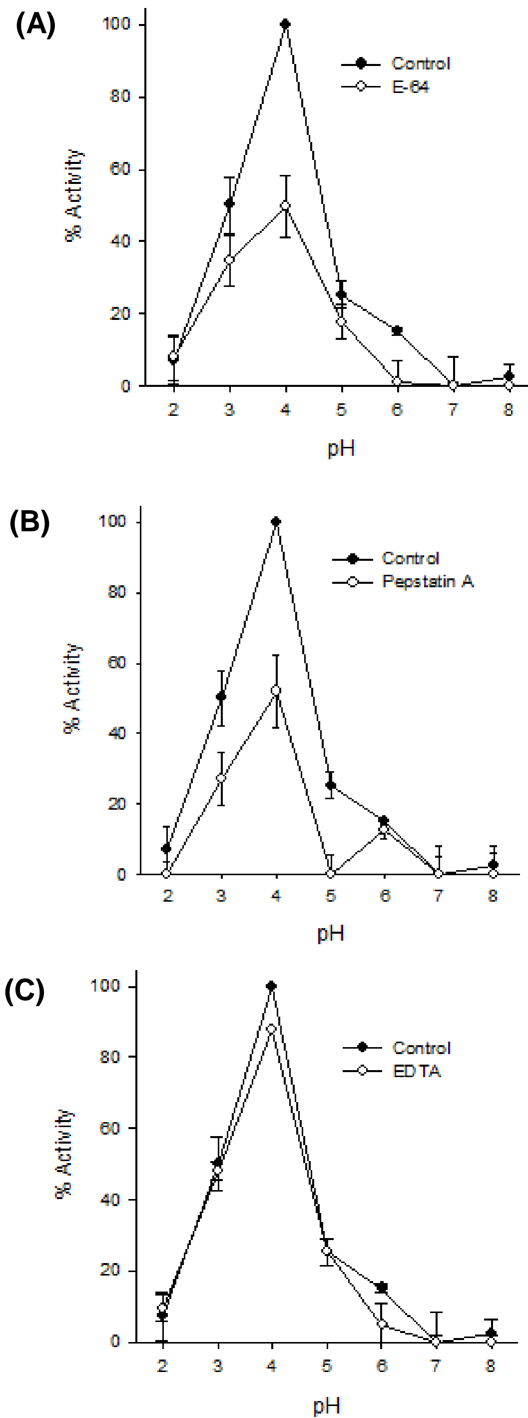


Figure 3. Effect of proteinases inhibitors on MH hydrolysis upon hemoglobin as substrates. Activities were assayed in buffers with different pH at 37 °C for 1 h, in presence of 10 μM E-64 (A), 10 μM Pepstatin A (B) or 1 mM EDTA, with a 0.2% final substrate concentration. Results are expressed as means ± s.d. of triplicates.

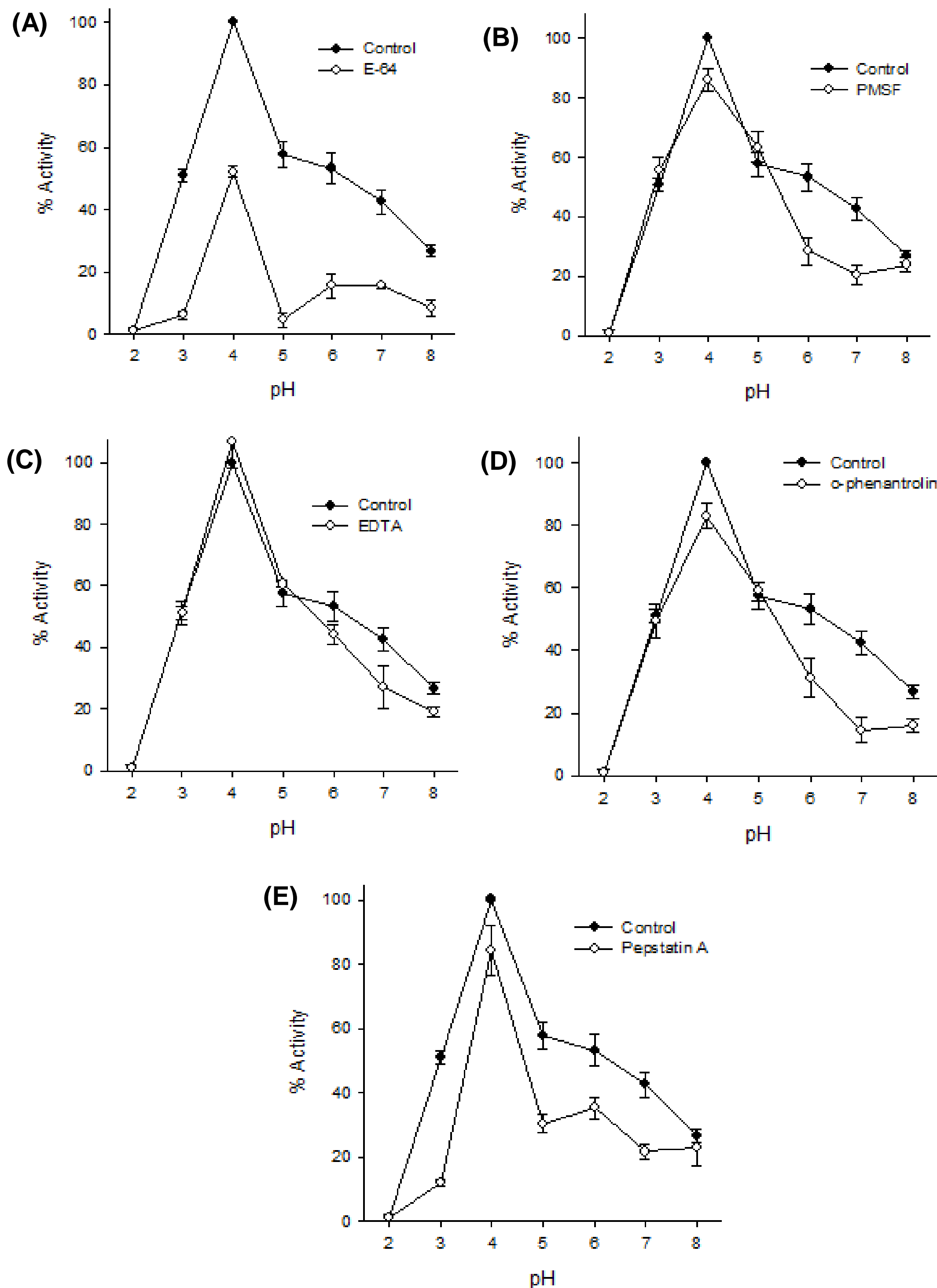


Figure 4. Effect of proteinase inhibitors on MH hydrolysis upon azocasein as substrates. Activities were assayed in buffers with different pH at 37 °C for 1 h, in presence of 10 μM E-64 (A), 1 mM PMSF (B), 1 mM EDTA (C), 1mM *o*-phenantrolin (D) or 10 μM Pepstatin A (E), with a 0.2% final substrate concentration. Results are expressed as means ± s.d. of triplicates.

Enzymatic activity upon synthetic substrates

Four synthetic substrates were used to characterize the peptidases present in the midgut homogenates. N-Cbz-Phe-Arg-MCA is a substrate suitable for the cysteine peptidases cathepsins B and L (Renard *et al.*, 2000; Estrela *et al.*, 2000). Figure 5 illustrates a major activity peak of MH at pH 5.0 upon this substrate, which is abolished by E-64 in all pH tested (Fig. 5A) while Pepstatin A inhibited only 20% of the activity detected at pH 5.0 (Fig. 5B). Abz-AIAFFSRQ-EDDnp was developed for cathepsin-D like aspartic peptidases (Sorgine *et al.*, 2000). Upon this substrate MH showed an optimal activity at pH 4.0-5.0, strongly inhibited by Pepstatin-A between pH 3.0 and 6.0 (Fig. 6B). On the other hand, inhibition by E-64 was less significant, in all pH values tested (Fig. 6A). MH peptidase activity was assayed upon synthetic substrates corresponding to N- and C-terminal regions flanking the entomotoxic peptide within urease to gain further insights into the processing of urease within the gut. MH efficiently cleaved with optimal pH at 4.0-5.0 the C-terminal substrate, Abz-KVIRDGMGQ-EDDnp. The activity was blocked by E-64 (Fig. 7A) while Pepstatin A did not affect it at all (Fig. 7B). On the other hand, the substrate Abz-NAIADGPVQ-EDDnp (equivalent to the N-terminal flanking region) was not significantly hydrolyzed by the homogenate (Fig. 8).

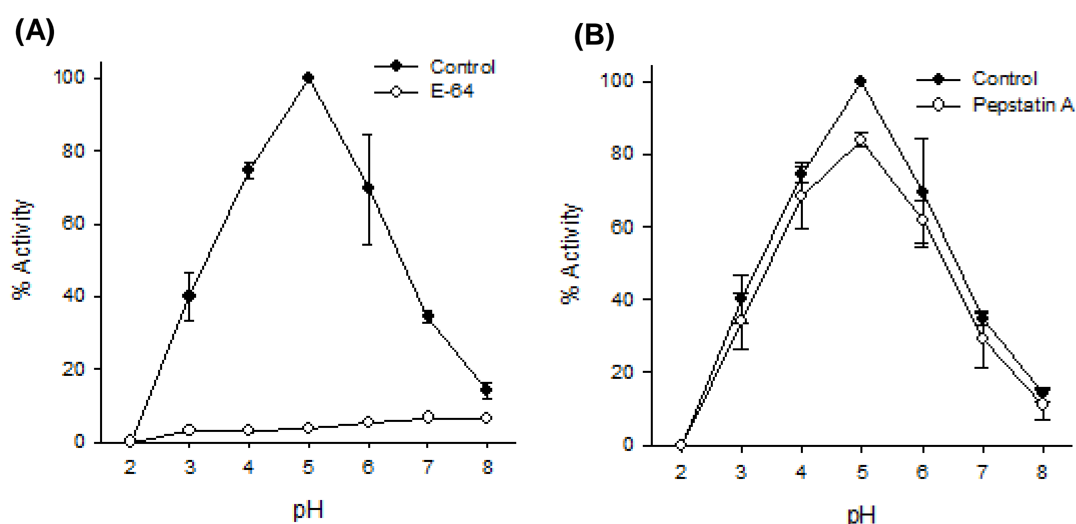


Figure 5. Effect of inhibitors on Mh hydrolysis upon the synthetic fluorogenic substrate N-Cbz-Phe-Arg-MCA hydrolysis by MH. Activities were assayed in buffers with different pH at 37 °C for 30 minutes, at 2 μM final substrate concentration, in presence of 10 μM E-64 (A) or 10 μM Pepstatin-A (B). Results are expressed as means ± s.d. of triplicates.

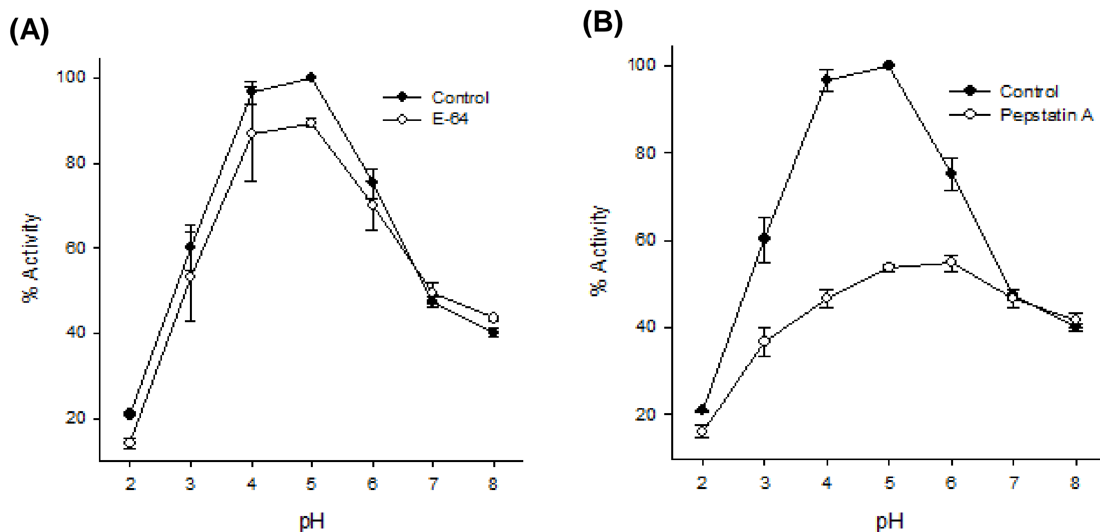


Figure 6. Effect of inhibitors on MH hydrolysis upon the synthetic fluorogenic substrate Abz-AIAFFSRQ-EDDnp hydrolysis by MH. Activities were assayed in buffers with different pH at 37 °C for 30 minutes, at 20 μM final substrate concentration, in presence of 10 μM E-64 (A) or 10 μM Pepstatin-A (B). Results are expressed as means ± s.d. of triplicates.

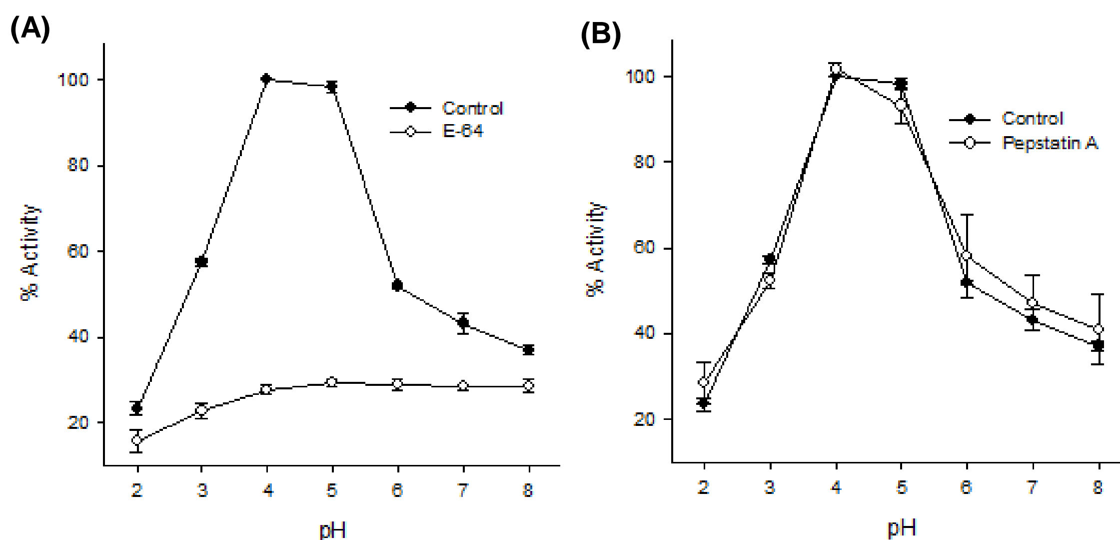


Figure 7. Effect of inhibitors on MH hydrolysis upon the synthetic fluorogenic substrate Abz-KVIRDGMGQ-EDDnp. Activities were assayed in buffers with different pH at 37 °C for 30 minutes, at 20 μM final substrate concentration, in presence of 10 μM E-64 (A) or 10 μM Pepstatin-A (B). Results are expressed as means ± s.d. of triplicates.

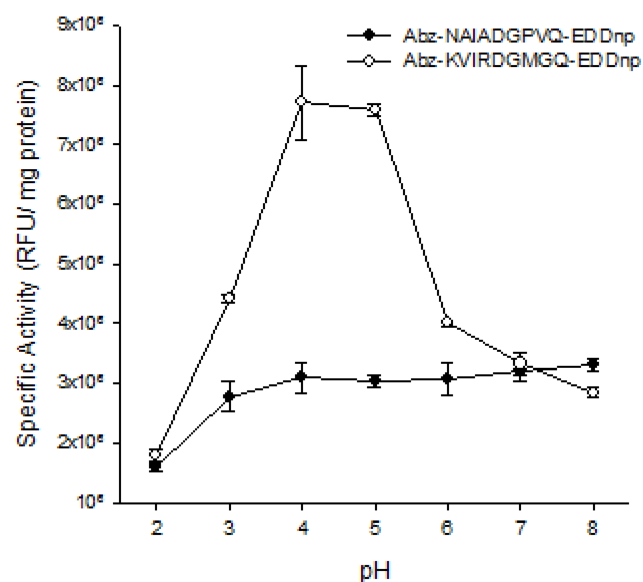


Figure 8. Hydrolysis by MH of the synthetic fluorogenic substrates Abz-NAIADGPVQEDDnp and Abz-KVIRDGMGQ-EDDnp. Activities were assayed in buffers with different pH at 37 °C for 30 minutes, at 20 μM final substrate concentration. Results are expressed as means ± s.d. of triplicates.

Purification and identification of a cathepsin L from midguts of *O. fasciatus* nymphs

Purification of a major peptidase active upon N-Cbz-Phe-Arg-MCA was accomplished by a combination of ion-exchange chromatography and gel filtration. As shown in Figure 9, activity concentrated in the fraction of the first elution (0.1 M NaCl) of the ion-exchange chromatography. This material was concentrated and then applied into a pre-calibrated Sephadex G-50 column. The major cysteine peptidase activity eluted between 13 and 15 mL, peaking at 14 mL (Fig. 10). A molecular mass of 22 kDa was calculated from the gel-filtration, and later confirmed by SDS-PAGE (Fig. 10 inset). The band close to 40 KDa seen in the same gel-filtration fraction, probably represents a dimer of the 22 kDa protein. Table 1 summarizes the purification data for this peptidase. After a SDS-PAGE, the corresponding band was excised from the gel, digested with trypsin and the peptides were analyzed by an electrospray ionisation (ESI) quadrupole time-of-flight (Q-TOF) Q-TOF Micro™ mass spectrometer (Micromass, Waters, Milford, US) coupled to a nanoACQUITY UltraPerformance LC® (UPLC®, Waters, Milford, US) (Fig. 11). A cathepsin-like enzyme was identified, matching with a cathepsin L zymogen from the nematode *Brugia malayi* (Table 2).

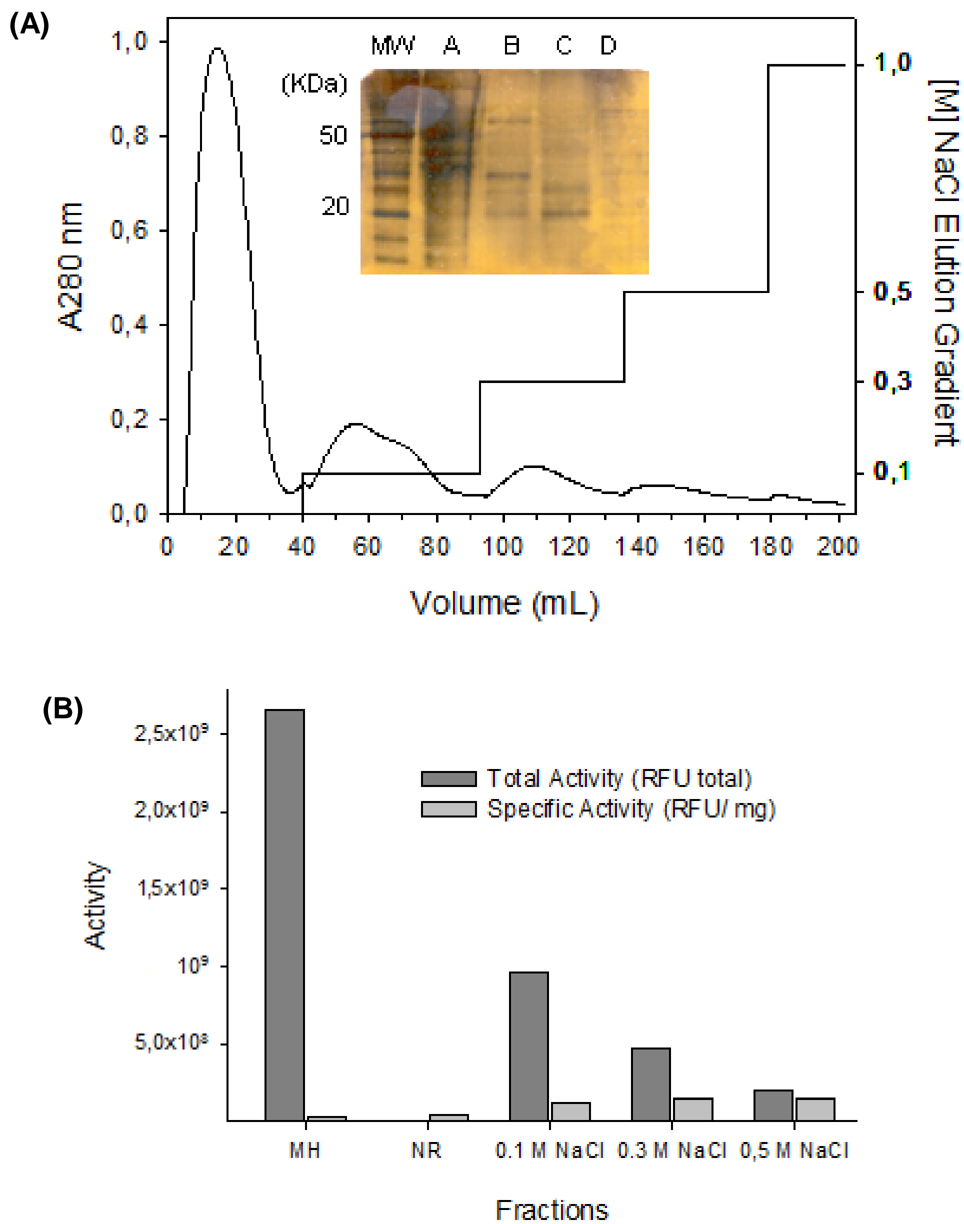


Figure 9. Cation-exchange chromatography of MH on CM-Sepharose. The resin was equilibrated with 20 mM sodium acetate, pH 5.0. (A) Chromatogram showing absorbance at 280 nm as a continuous line, and the stepwise NaCl gradient. Inset: silver-stained SDS-PAGE of eluted fractions (2 µg protein each); lane A, non-retained fraction; lane B, (100 mM NaCl)-eluted fraction; lane C, (300 mM NaCl)-eluted fraction; lane D, (500 mM NaCl)-eluted fraction. Molecular masses of standard proteins (kDa) are indicated. (B) Total and specific activity of fractions upon N-Cbz-Phe-Arg-MCA assayed at 37°C for 30 minutes in 20 mM sodium acetate, pH 5.0.

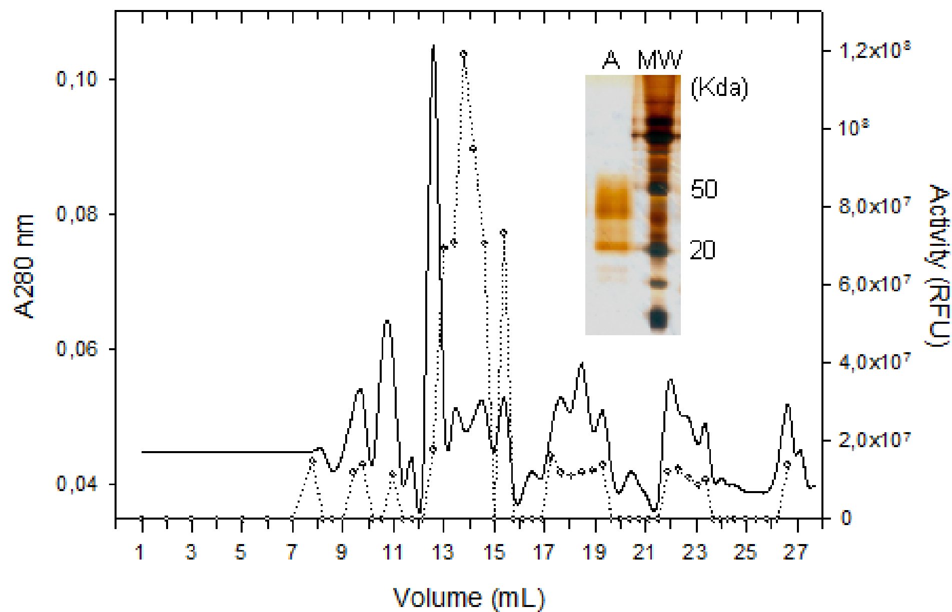


Figure 10. Gel filtration chromatography on Sephadex G-50. The column was equilibrated with 20 mM sodium acetate pH 5.0. Absorbance at 280 nm showed as a continuous line and activity of fractions upon N-Cbz-Phe-Arg-MCA as a dotted line. Protein peaks were assayed at 37°C for 30 minutes in 20 mM sodium acetate pH 5.0. The major active peak corresponds to 22 kDa. Inset: silver-stained SDS-PAGE; lane A, 14 mL (1 μg).

Table 1. Purification of a cathepsin L-like digestive enzyme from *O. fasciatus* nymphs.

Sample	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Activity (RFU/mL)	Specific Activity (RFU/mg)	Total Activity (RFU)	Yield (%)	Fold Purified
Midgut Homogenate	9	10.2	91.8	295 $x10^6$	28 $x10^4$	2651 $x10^6$	100	1
CM Sepharose	17.5	0.448	7.84	55 $x10^6$	122 $x10^6$	9625$x10^5$	36.3	424.97
Sephadex G50	3	0.053	0.159	11 $x10^6$	207 $x10^6$	3300 $x10^4$	1.24	718.44

*Enzymatic activity of samples was assayed upon 2 μM N-Cbz-Phe-Arg-MCA at 37 °C for 30 min, in 20 mM Sodium Acetate pH 5.0 and shown as Relative Fluorescence Units. Midgut homogenate of *O. fasciatus*; CM Sepharose (fraction eluted at 100mM NaCl); Sephadex G-50 (fraction eluted at 14 mL).

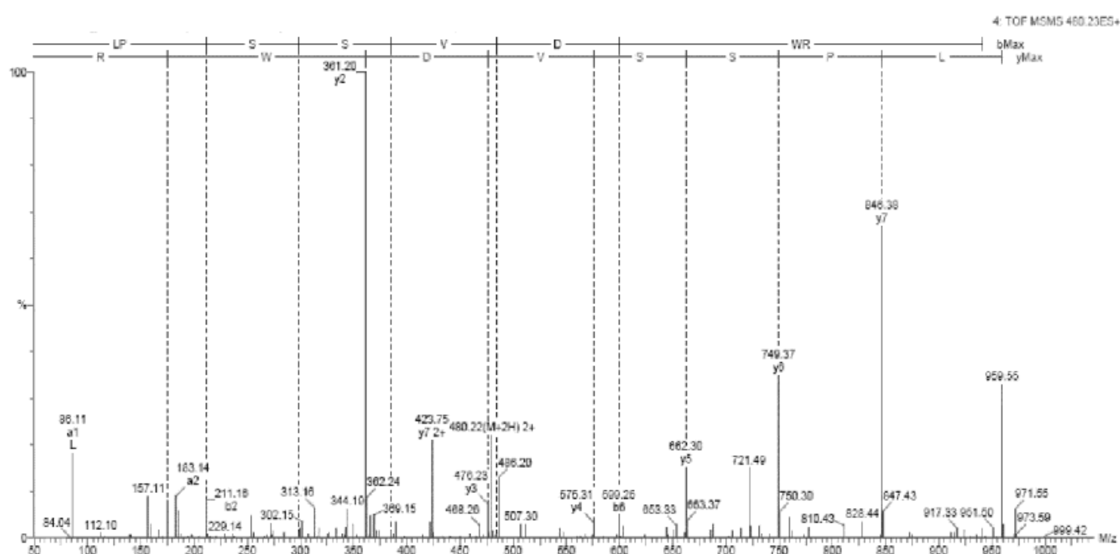


Figure 11. Peptide analysis by LC-MS/MS of the 22 kDa protein band. The protein band (lane A, fig. 10 inset) was excised from the gel, trypsin-digested and the peptides were analyzed by an electrospray ionisation (ESI) quadrupole time-of-flight (Q-TOF) TOF Micro™ mass spectrometer (Micromass, Waters, Milford, US) coupled to a nanoACQUITY UltraPerformance LC® (UPLC®, Waters, Milford, US).

Table 2. Protein identification by LC-MS/MS.

Acession Number[1]	Protein Description[2]	Organism	Mascot Score[3]	Peptides	Theoretical pI/MW (kDa)
gi|13242025	cathepsin L-like cysteine peptidase (precursor)	***Brugia malayi***	76	L.PSSVDWR.K R.LPSSVDWR.K	9.03/45.0

[1]Protein identification according to NCBI databank.

[2]CDS access number in the NCBI databank.

[3]MASCOT score is −10 × log(P), where P is the probability that the observed match is a random event.

Discussion

Proteolytic enzymes present in the digestive systems of insects include aminopeptidases, carboxypeptidases and proteinases of the families of the cysteine-, aspartic-, serine- and metalloendopeptidases (Terra & Ferreira, 1994; Bown et al., 1997; Girard & Jouanin, 1999; Patankar et al., 2001; Murdock & Shade, 2002; Ferry et al., 2004). This wide diversity implies multiple functions of insect enzymes, including involvement in developmental processes besides interactions with food components. Hemipteran proteolytic enzymes are predominantly acidic, belonging to aspartic and cysteine peptidases classes (Houseman, 1978; Houseman & Downe, 1981; Houseman & Downe, 1982a e 1982b; Houseman & Downe, 1983). Extracellular protein digestion by cathepsin-like enzymes, usually found intracellularly, in insects (Houseman et al., 1984; Terra & Ferreira, 1994; Cristofoletti et al., 2003) is probably consequent to evolution and adaptation (Houseman et al., 1985; Cristofoletti et al., 2003). Terra *et al.* (1996) suggested that ancestral floem-feeding bugs modified lysosomal cathepsins to digest dietary proteins instead of using the serine peptidases trypsin and chymotrypsin because seeds are, in general, rich sources of trypsin inhibitors. In this work we investigated the digestive peptidase profile of the phytophagous hemipteran *Oncopeltus fasciatus*, and tested the susceptibility of young forms of this insect to Jackbean (*C. ensiformis*) major urease. Third instars *O. fasciatus* were highly susceptible to low doses of urease (0.01% w/w) given orally - deaths began 72 hours after feeding and reached almost 100% mortality two weeks later. We also showed that *C. ensiformis* major urease is hydrolyzed *in vitro* by midgut homogenates of *O. fasciatus* releasing peptide(s) similar in size and also recognized by anti-Jaburetox-2Ec antibodies. Although intact urease may also contribute to entomotoxic effect (Stanisçuaski *et al.*, 2009; 2010), the release of peptides equivalent to jaburetox-2Ec from urease is enough to kill the insect (Mulinari *et al.*, 2007). Because the release of entomotoxic peptides from urease could be due to a concerted or sequential action of more than one enzyme (Piovesan *et al.*, 2008), studies of urease in vitro hydrolysis and enzymatic assays to characterize the presence of proteinases were done using whole midgut homogenates. Intestinal homogenates of *O. fasciatus* nymphs contain a major predominance of cysteine and aspartic cathepsin-like enzymes, demonstrated by using different substrates and specific inhibitors. The maximal activities in acidic pH values found in the present work confirmed previous studies with this species (Bongers, 1970; Woodring *et al.*, 2007), and many other heteropterans, including the cotton stainer bug *D. peruvianus* (Silva & Terra, 1994; Piovesan *et al.*, 2008). As reported by Woodring *et al.* (2007), we also did not find proeminent serine or metallo peptidases, with only low

proteolytic activity being detected at neutral to alkaline pH for some of the tested substrates. While no inhibition of MH activities at pH 5.0 or lower was seen for EDTA, *o*-phenantrolin, or PMSF, these compounds blocked partially the low residual activity detected in neutral to alkaline media, suggesting metallo- and serino-peptidase(s) as minor components of *O. fasciatus* digestive proteinase profile. In previous study we demonstrated the presence of acidic metallopeptidase(s) in nymphs and serine peptidase(s) in adult *D. peruvianus* (Piovesan *et al*., 2008). We have previously shown that *in vitro* hydrolysis of canatoxin by digestive enzymes from *C. maculatus* larvae produced a family of entomotoxic peptides, being pepcanatox the most toxic one (Ferreira-DaSilva *et al*., 2000). The N-terminal sequence GPVQ- of pepcanatox was determined by Edman degradation and based on the molecular mass, its C-terminal was deduced to be –KVIRD (Mulinari *et al*., 2007). *O. fasciatus* midgut homogenates did not cleave the substrate containing the Nterminal flanking sequence. In contrast, midgut homogenates of *D. peruvianus* nymphs hydrolyzed this substrate at pH 4.0, being strongly inhibited by EDTA, indicating that a metallopeptidase activity could be responsible for cleavage of this specific sequence (Piovesan *et al*., 2008). As no acidic metallopeptidase activity was detected in *O. fasciatus* homogenates, we can infer that the N-terminal cleavage site for releasing urease-derived entomotoxic peptides by *O. fasciatus* nymphs is probably different from that reported for *D. peruvianus*. The cleavage of the C-terminal substrate Abz-KVIRDGMGQ-EDDnp was optimal at pH 5.0, with complete inhibition by E-64 at all pH values tested. Previously we saw that the cleavage of the same substrate by *D. peruvianus* digestive enzymes occurred between the arginine and the aspartic acid residues (RD), with a significant inhibition only by E-64 (Piovesan *et al.,* 2008). Cathepsin L-like cysteine peptidases act as endopeptidases and the cleavage of RD bonds by human cathepsin L was previously described (Dahl *et al*., 2001). It is interesting to note that in *D. peruvianus*, metallopeptidase(s) apparently has(ve) a key role in releasing the toxic peptide (Piovesan et al., 2008), while in *O. fasciatus* this class of enzymes appears not to be relevant for urease toxicity. On the other hand, previous studies showed that simultaneous administration of canatoxin with either E-64 or Pepstatin A to *Rhodnius prolixus* led to significant reduction in the mortality rate, suggesting that cysteine and aspartic peptidases are hydrolysing the protein in this insect (Carlini *et al*., 1997). These data suggest that the cleavage sites of urease releasing toxic fragments in different insects may vary, thus generating slightly different peptide sequences, without interfering in the entomotoxicity. Recently, Barros *et al.* (2009) showed that the toxic peptide Jaburetox-2Ec displays membrane-disruptive properties and might adopt a well-defined β-hairpin conformation, similar to those found in some neurotoxic and antimicrobial pore-forming

peptides. This information is consistent with the idea that the N-terminal portion of the insecticidal peptide(s) is less important for toxicity than the amino acids forming the β-hairpin domain, which is located within the C-terminal half of the peptide. Previous studies showed that acidic digestive enzymes are present in the midgut of *O. fasciatus* (Bongers, 1970; Woodring *et al.*, 2007), more specifically cysteine peptidases (Woodring *et al.*, 2007). Here we demonstrate that besides cysteine peptidases, aspartic peptidases are also present. Furthermore, a cathepsin L from *O. fasciatus* midguts was isolated by a two step purification protocol. The purified enzyme was identified by mass spectrometry, matching a 45 KDa cathepsin L-like cysteine peptidase precursor from the nematode *Brugia malayi*. This zymogen is converted into a 23 KDa active enzyme (Guiliano *et al.*, 2004), which is very similar in size to the 22 kDa proteinase isolated from *O. fasciatus* midgut. In summary, the data presented here aggregate valuable information to our present knowledge on the mechanism of urease processing by insects digestive enzymes, and emphasize the need of a better comprehension of insect digestive physiology and how insecticidal proteins affect them, looking forward to develop biotechnological strategies to protect crops against pests.

Acknowledgments

This project was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil), by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and by Fapergs (Fundação de Amparo à Pesquisa do Rio Grande do Sul). M.S.D is a recipient of a CAPES fellowship; P.M.P. and T.B.M. are recipient of CNPq fellowships.

References

Barret, A.J., Rawlings, N.D., Woessner, J.F. 1998. Handbook of Proteolytic Enzymes. Academic Press, San Diego, 1666 pp.

Barros, P. R., Stassen, H., Freitas, M.S., Carlini, C. R., Nascimento, M. A. C., Follmer, C. 2009. Membrane-disruptive properties of the bioinsecticide Jaburetox-2Ec: Implications to the mechanism of the action of insecticidal peptides derived from ureases. Biochim. Biophys. Acta. 1794, 1848–1854.

Billingsley, P.F., Downe, A.E.R. 1985. Cellular-localization of aminopeptidases in the midgut of *Rhodnius prolixus* Stal (Hemiptera:Reduviidae) during blood digestion. Cell Tissue Res. 241(2), 421–428.

Billingsley, P.F., Downe, A.E.R. 1988. Ultrastructural-localization of cathepsin B in the midgut of *Rhodnius prolixus* Stal (Hemiptera:Reduviidae) during blood digestion. Int. J. Insect Morph. Emb. 17(4-5), 295-302.

Bolter, C., Jongsma, M.A. 1997. The adaptation of insects to plant peptidase inhibitors. J. Insect Physiol. 43(10), 885-895.

Bongers, J. 1970. Die Carbohydrasen und Esterasen in Speicheldrüsen und Mitteldarm von *Oncopeltus fasciatus* (Lygaeidae). Zeitschrift für Vergleichende Physiologie. 70, 382–400.

Bown, D.P., Wikinson, H.S., Gatehouse, J.A. 1997. Differentially regulated inhibitor sensitive and insensitive peptidases genes from the phytophagous insect pest, *Helicoverpa armigera*, are member of complex multigene families. Insect Biochem. Mol. Biol. 27(7), 625-638.

Carlini, C.R., Guimarães, J.A. 1981. Isolation and characterization of a toxic protein from *Canavalia ensiformis* (Jackbean) seeds, distinct from concanavalin A. Toxicon 19, 667–676.

Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J., Wells, M.A. 1997. Biological effects of canatoxin in different insect models. Evidence for a proteolytic activation of the toxin by insect cathepsin-like enzymes. J. Econ. Entomol. 90, 340–348.

Carlini, C.R., Grossi-de-Sá, M. F. 2002. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon. 40(11), 1515-1539.

Carlini, C.R. & Polacco, J.C. 2008. Toxic properties of urease. Crop Science. 48, 1665-1672

Cristofoletti, P.T., Ribeiro, A.F., Deraison, C., Rahbe, Y., Terra, W.R. 2003. Midgut adaptation and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrthosiphon pisum*. J. Insect Physiol. 49, 11–24.

Dahl, S.W., Halkier, T., Lauritzen, C., Dolenc, I., Pedersen, J., Turk, V., Turk, B. 2001. Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. Biochemistry 40, 1671–1678.

Estrela, A., Seixas, A., Termignoni, C. 2007. A cysteine endopeptidase from tick (*Rhipicephalus (Boophilus) microplus*) larvae with vitellin digestion activity. Comp. Biochem. Physiol, 148B, 410–416.

Feir, D. 1974. *Oncopeltus fasciatus*: a research animal. Annual Review of Entomology 19, 81–96.

Felton, G.W. 1996. Nutritive quality of plant protein: Sources of variation and insect herbivore responses. Arch. Insect Biochem. Physiol. 32(1), 107-130.

Ferreira-DaSilva, C.T., Gombarovits, M.E., Masuda, H., Oliveira, C.M., Carlini, C.R. 2000. Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. Arch. Insect Biochem. Physiol. 44, 162–171.

Ferry, N., Edwards, M.G., Gatehouse, J.A. 2004. Plant-insect interactions: molecular approaches to insect resistance. Current Opinion Biotech. 15(2), 155-161.

Follmer, C., Barcellos, G.B.S., Zingali, R.B., Machado, O.L.T., Alves, E.W., Barja-Fidalgo, C., Guimarães, J.A., Carlini, C.R. 2001. Canatoxin, a toxic protein from Jackbean (*Canavalia ensiformis*), is a variant from of urease (EC. 3.5.1.5): biological effects of urease independent of its ureolytic activity. Biochem. J. 360, 217–224.

Follmer, C., Real-Guerra, R., Wassermann, G.E., Olivera-Severo, D., Carlini, C.R. 2004a. Jackbean, soybean and *Bacillus pasteurii* ureases: biological effects unrelated to ureolytic activity. Eur. J. Biochem. 271, 1357–1363.

Follmer, C., Wassermann, G., Carlini, C.R. 2004b. Separation of Jackbean (*Canavalia ensiformis*) urease isoforms by immobilized metal affinity chromatography and characterization of insecticidal properties unrelated to ureolytic activity. Plant Sci. 167, 241–246.

Follmer, C. 2008. Insights into the role and structure of plant ureases. Phytochemistry 69, 18–28.

Girard, C., Jouanin, L. 1999. Molecular cloning of cDNAs enconding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae*. Insect Biochem. Mol. Biol. 29(12), 1129-1142.

Guiliano, D.B., Hong, X., McKerrow, J.H., Mark.L. Blaxter, M.L., Yelena Oksov, Jing Liu, Elodie Ghedin and Sara Lustigman. 2004. A gene family of cathepsin L-like peptidases of filarial nematodes is associated with larval molting and cuticle and eggshell remodeling. Mol. Biochem. Parasitology. 136 (2): 227-242.

Houseman, J. 1978. A thiol-activated digestive peptidase from adults of *Rhodinus prolixus* Stal (Hemiptera:Reduviidae). Can. J. Zool. 56(5), 1140–1143.

Houseman, J.G., Downe, A.E.R. 1981. Identification and partial characterization of digestive peptidases from *Triatoma phyllosoma pallidipennis* (Hemiptera:Reduviidae). Comp. Biochem. Physiol. 70B (4), 713-717.

Houseman, J.G., Downe, A.E.R. 1982a. Identification and partial characterization of digestive peptidases from two species of bedbug (Hemiptera:Cimicidae). Can. J. Zool. 60(8), 1837-1840.

Houseman, J.G., Downe, A.E.R. 1982b. Characterization of acidic peptidases from the posterior midgut of *Rhodnius prolixus* Stal (Hemiptera:Reduviidae). Insect Biochem. 12(6), 651-655.

Houseman, J.G., Downe, A.E.R. 1983. Cathespin D-like activity in the posterior midgut

of hemipteran insects. Comp. Biochem. Physiol. 75B (3), 509-512.

Houseman, J.G., Macnaughton, W.K., Downe, A.E.R. 1984. Cathepsin B and aminopeptidase in the posterior midgut of *Euschistis euschistoides* (Hemiptera: Pentatomidae). Can. J. Entomol. 116(10), 1393-1396.

Houseman, J.G., Morrison, P.E., Downe, A.E.R. 1985. Cathepsin B and aminopeptidase in the posterior midgut of *Phymata wolffii* Stal (Hemiptera: Phymatidae). Can. J. Zool. 63(6), 1288-1291.

Howe, G.A. & Jander, G. (2008). Plant Immunity to Insect Herbivores. Annual Rev. Plant Biol. 59: 41-66

Koiwa, H., Kato, H., Nakatsu, T., Oda, J., Yamada, Y., Sato, F. 1997. Purification and characterization of tobacco pathogenesis-related protein PR-5d, an antifungal thaumatin-like protein. Plant Cell Physiol. 38(7), 783-791.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the bacteriophage T4. Nature. 227, 680-685.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzimol. 3, 447-454.

Milano, P., Cônsoli, F.L., Zério, N.G., Parra, J.R.P. 1999. Exigências térmicas de *Dysdercus peruvianus* Guérin-Méneville (Heteroptera: Pyrrhocoridae), o percevejo manchador do algodoeiro. Anais da Sociedade Entomológica do Brasil. 28(2), 233-238.

Mobley, H.L., Island, M.D., Hausinger, R.P. 1995. Molecular biology of microbial ureases. Microbiol. Rev. 59, 451–480.

Mulinari, F., Freitas-Silva, M.A., Grossi-De-Sa, M.F., Moraes, M.G., Kurtenbach, E., Carlini, C.R. 2004. Toxina Praguicida, Construção Gênica e Método de Controle de Pragas. Patent registered at National Institute for Intellectual Property (INPI). Brazil; 08/04/2004 [protocol BRPI0403435A].

Mulinari, F., Stanisçuaski, F., Bertholdo-Vargas, L.R., Postal, M., Oliveira-Neto, O.B., Ridgen, D.J., Grossi-de-Sa , M.F., Carlini, C.R. 2007. Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the plant *Canavalia ensiformis*. Peptides 28, 2042–2050.

Murdock, L.L., Shade, R.E. 2002. Lectins and peptidase inhibitors as plant defenses against insects. J. Agric. Food Chem. 50(22), 6605-6611.

Patankar, A.G., Giri, A.P., Harsulkar, A.M., Sainani, M.N., Deshpande, V.V., Ranjekar, P.K., Gupta, V.S. 2001. Complexity in specificities and expression of *Helicoverpa armigera* gut peptidases explains polyphagous nature of the insect pest. Insect Biochem. Mol. Biol. 31(4-5), 453-464.

Piovesan, A.R, Stanisçuaski, F., Marco-Salvadori, J., Real-Guerra, R., Defferrari, M.S., Carlini, C.R. 2008. Stage-specific gut peptidases of the cotton stainer bug *Dysdercus peruvianus*: Role in the release of entomotoxic peptides from *Canavalia ensiformis* urease. Insect Biochem. Mol.Biol. 38, 1023–1032.

Polacco, J.C., Holland, M.A. 1993. Roles of urease in plant cells. Int. Rev. Cytol. 145, 65–103.

Renard, G., Garcia, J.F., Cardoso, F.C., Richter, M.F., Sakanari, J.A., Ozaki, L.S., Termignoni, C., Masuda, A. 2000. Cloning and functional expression of a *Boophilus microplus* cathepsin L-like enzyme. Insect Biochem. Mol. Biol. 30, 1017–1026.

Ryan, C. A. 1990. Peptidase inhibitors in plants: genes for improving defenses against insects and pathogens. Ann. Rev. Phytopathol. 28, 425-449.

Silva, C.P., Terra, W.R. 1994. Digestive and absorptive sites along the midgut of the cotton seed sucker bug *Dysdercus peruvianus* (Hemiptera:Pyrrhocoridae). Insect Biochem. Mol. Biol. 24, 493–505.

Sirko, A., Brodzik, R. 2000. Plant ureases: role and regulation. Acta Biochim. Pol. 47, 1189–1195.

Sorgine, M.H., Logullo, C., Zingali, R.B., Paiva-Silva, G.O., Juliano, L., Oliveira, P.L.

2000. A heme-binding aspartic peptidase from the eggs of the hard tick *Boophilus microplus*. J. Biol. Chem. 275, 28659–28665.

Stanisçuaski, F., Ferreira-Dasilva, C.T., Mulinari, F., Pires-Alves, M., Carlini, C.R. 2005. Insecticidal effects of canatoxin on the cotton stainer bug *Dysdercus peruvianus* (Hemiptera:Pyrrhocoridae). Toxicon 45, 753–760.

Stanisçuaski, F., Te Brugge, V., Carlini, C. R., Orchard, I. 2009. In vitro effect of Canavalia ensiformis urease and the derived peptide Jaburetox-2Ec in *Rhodnius prolixus* Malpighian tubules. J. Insect Physiol. 55, 255-263.

Stanisçuaski, F., Te Brugge, V., Carlini, C. R., Orchard, I. 2010. Jack bean urease alters serotonin-induced effects on *Rhodnius prolixus* anterior midgut. J. Insect Physiol., E-pub Mar16, 2010 (*http://dx.doi.org/10.1016/j.jinsphys.2010.03.002)*.

Stotz, H.U., Kroymann, J., Mitchell-Olds, T. 1999. Plant-insect interactions. Cur. Opinion Plant Biol. 2(4), 268-272.

Te Brugge, V.A., Orchard, I. 2007. Distribution and activity of a Dippu DH31-like peptidein the large milkweed bug *Oncopeltus fasciatus*. Peptides. 29, 206–213.

Terra, W.R., Ferreira, C. 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109, 1–62.

Terra, W.R., Ferreira, C., Jordão, B.P., Dillon, R.J. 1996. Digestive Enzymes. In: Biology of the Insect Midgut. Chapman & Hall, London, pp. 153–194.

Terra, W. R., Ferreira, C., 2005 . Biochemistry of Digestion. In: Gilbert, L. I.; Iatrov, K.; Gill, S.. (Org.). Comprehensive Molecular Insect Science. Oxford: Elsevier, 2005, v. 4, pp. 171-224.

Tomazzeto, G., Mulinari, F., Staniscuaski, F., Settembrini, B.P., Carlini, C.R., Ayub, M.A.Z. 2007. Expression kinetics and plasmid stability of recombinant *E. coli* encoding urease-derived peptide with bioinsecticide activity. Enzyme Microb. Technol. 41, 821–827.

Towbin, H., Staehelin, T., Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.

Woodring, J., Hoffman, K. H., Lorenz, M.W. 2007. Feeding, nutrient flow, and digestive enzyme release in the giant milkweed bug, *Oncopeltus fasciatus.* Physiol. Entomol., 32, 328–335.

Segunda Parte

Interação da urease de *C. ensiformis* e de Jaburetox-2Ec com tecidos isolados de ninfas de *O. fasciatus*

1. Materiais e Metodologia

1.1. Reagentes

Os anticorpos anti-IgG de coelho produzidos em 'asno' (*Equus asinus*), conjugados com a sonda fluorescente FITC foram adquiridos da Jackson Immuno Research (Suffolk, UK). Serotonina (5HT), 8-bromo-AMPc e JBU tipo III foram adquiridos da Sigma Chemicals Company (Saint Louis, USA). O peptídeo Jaburetox-2Ec foi obtido através da expressão heteróloga em *E. coli* seguido de purificação em resina com níquel imobilizado, segundo Mulinari *et al.*, 2007. Os anticorpos policlonais anti-Jaburetox-2Ec foram obtidos mediante imunização de coelhos com o peptídeo recombinante Jaburetox-2Ec (Mulinari et al., 2007).

1.2. Colônia de insetos

Ninfas de quinto instar de *O. fasciatus* foram retiradas de uma colônia estabelecida na Universidade de Toronto em Mississauga, para os ensaios de contração de intestinos e de toxicidade do Jaburetox-2Ec. Para os ensaios de imunohistoquímica, ninfas de quinto instar de *O. fasciatus* foram retiradas de uma colônia estabelecida no Laboratório de Proteínas Tóxicas da Universidade Federal do Rio Grande do Sul. Em ambas as colônias, os insetos são mantidos a uma temperatura de 22 ^{o}C +/- 2 ^{o}C, fotoperíodo de 14/10 horas (claro/escuro), com água e sementes de girassol à vontade.

1.3. Salina para *O. fasciatus*

A salina para *O. fasciatus* foi preparada segundo Meredith *et al.*, 1984: NaCl 20 mM, KCl 24 mM, $CaCl_2$ 2 mM, $MgCl_2$ 2 mM, $NaHCO_3$ 4 mM, glicose 6.7 mM e HEPES 10%, e o pH ajustado para 6,9.

1.4. Bioensaio

Ninfas de quinto instar foram injetadas na região ventral do abdômen (seringa Hamilton, agulha calibre 20), com solução salina contendo Jaburetox-2Ec ou somente com solução salina, para controle negativo. Os insetos foram injetados em uma proporção de 0,02 μg/ mg peso, em um volume máximo de 2,5 μL. Controles foram injetados com solução salina, em volume máximo de 2,5 μL. Após as injeções os insetos foram mantidos nas mesmas condições normais de manutenção (temperatura, fotoperíodo, umidade e água e alimento à vontade).

1.5. Imunohistoquímica

Ninfas de quinto instar foram injetadas com solução salina contendo o peptídeo recombinante Jaburetox-2Ec (0,04 μg/ mg peso) e os controles receberam injeções apenas de solução salina. Após 12 horas, os insetos foram imobilizados com fita adesiva, pelas patas, abertos pela cutícula dorsal e fixados (paraformaldeído 4%) por 24 h. Após a fixação, os insetos foram dissecados para o isolamento do sistema nervoso central, compreendendo o Protocérebro, o Gânglio Protorácico, a Massa Gangliônica Mesotorárica e os nervos adjacentes. Após a dissecção, os tecidos foram incubados com o anticorpo anti-Jaburetox-2Ec (1:2500) por 48 h, lavados com PBS diversas vezes, incubados com o anticorpo secundário conjugado com a sonda fluorescente FITC (1:250) por 24h e lavados com PBS diversas vezes novamente. Após a última lavagem, os tecidos foram montados em lâminas com glicerol e analisados em microscópio de epifluorescência ou por microscopia confocal (Centro de Investigação da Faculdade de Ciências Biomédicas, Universidade Austral, Argentina).

1.6. Quantificação e intensidade de contrações do intestino de *O. fasciatus*

Para avaliar o efeito da urease de *C. ensiformis* e do peptídeo recombinante Jaburetox-2Ec sobre as contrações do intestino de *O. fasciatus,* foram utilizadas ninfas de quinto instar sem jejum prévio. O primeiro ventrículo foi retirado do inseto e colocado em uma gota de salina (100 μL) em uma placa de Petri com fundo preenchido com silicone. O intestino foi preso com alfinetes, de modo a ficar levemente estendido (sem afetar os movimentos peristálticos). A freqüência de contrações foi medida durante 5 minutos, para cada tratamento, em um conversor de impedância (UFI, modelo 2991), no modo AC (corrente alternada). A mesma preparação foi utilizada para ensaios dose-resposta, com lavagens com salina (3 vezes, 30 segundos cada) entre as doses. Para os ensaios da JBU ou do peptídeo em presença de outros agentes, os compostos foram misturados previamente antes da adição à preparação. Os resultados foram expressos como porcentagem em relação à freqüência de contrações em presença de solução salina (controle).

1.7. Análises estatísticas

Os dados foram expressos como média e erro padrão. A significância dos resultados foi determinada usando-se ANOVA de via única. Os dados foram considerados significantemente diferentes quando $p < 0,05$.

2. Resultados

2.1. Atividade tóxica de Jaburetox-2Ec em ninfas de quinto instar de *O. fasciatus*

O peptídeo recombinante Jaburetox-2Ec é tóxico tanto para insetos suscetíveis quanto resistentes a urease intacta (Mulinari *et al.*, 2007). Aqui testamos o efeito tóxico do peptídeo quando injetado na região ventral do abdômen de ninfas de quinto instar, numa dose de 0,02 μg por mg de peso corpóreo. Os insetos apresentaram uma taxa de mortalidade de 100% após 48 horas (Fig. 4). Os insetos injetados com Jaburetox-2Ec apresentam um padrão diferenciado de morte, em relação àqueles que morrem por injúria ou de forma natural. No primeiro caso observamos que há um perfil convulsivo, com descontrole e perda da mobilidade das patas e das antenas, e subseqüente morte, sempre com a parte ventral do corpo voltada para cima. No segundo caso, os insetos morrem de formas variadas, não necessariamente voltando a parte ventral do corpo para cima, nem apresentando descontrole de patas e antenas.

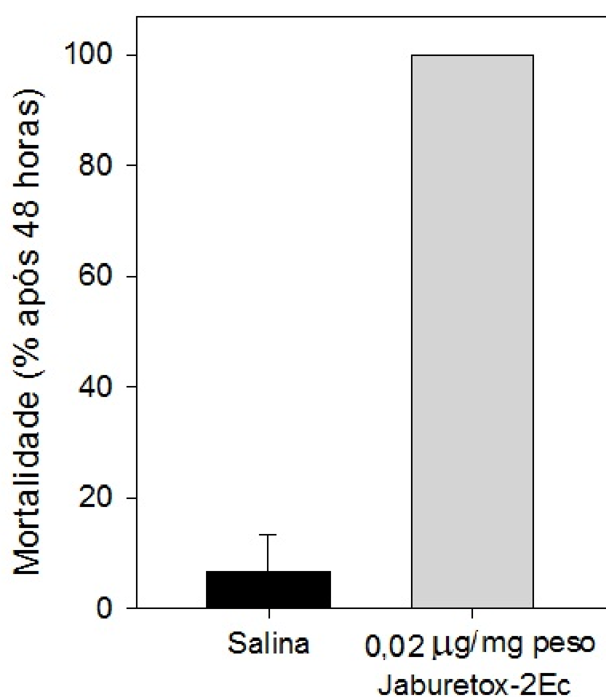


Figura 4. Efeito tóxico do peptídeo recombinante Jaburetox-2Ec sobre ninfas de quinto instar de *O. fasciatus*. Os insetos foram injetados com salina (controles) ou com solução salina contendo Jaburetox-2Ec, numa concentração de 0,02 μg/ mg de peso. Valores são médias e desvios padrão de triplicatas, com 15 indivíduos em cada replicata.

2.2. Imunolocalização de Jaburetox-2Ec no sistema nervoso central de *O. fasciatus*

Devido aos efeitos aparentemente neurotóxicos observados no ensaio biológico com o peptídeo Jaburetox-2Ec com ninfas de *O. fasciatus*, decidimos investigar uma possível interação com o sistema nervoso central. Para isso, ninfas de quinto instar foram injetadas com o peptídeo (0,04 μg por mg), e a distribuição foi avaliada por imunohistoquímica. Observamos marcações na Massa Gangliônica Mesotorácica, próximo aos nervos abdominais (Figs. 5 e 6) e no Gânglio Pró-torácico (Fig. 7). A figura 8 representa o Gânglio Pró-torácico de um inseto controle, sem marcações observadas pelo anticorpo Anti-Jaburetox-2Ec.

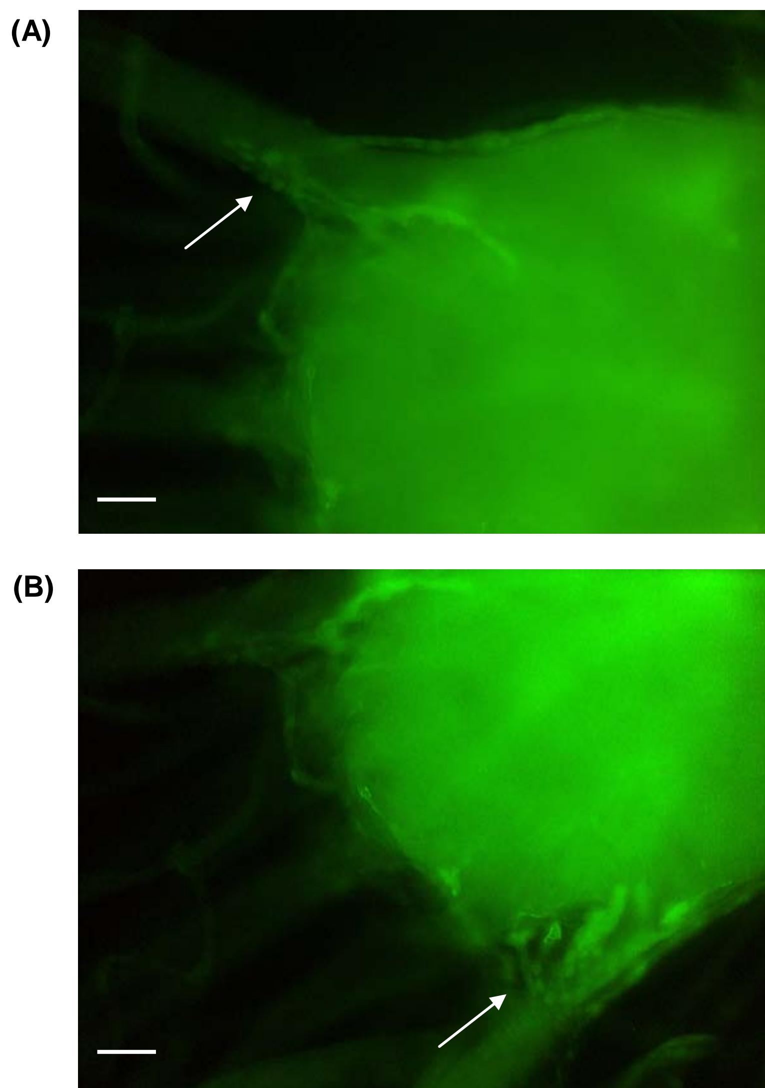


Figura 5. Microscopia de epifluorescência da Massa Gangliônica Mesotorácica de *O. fasciatus* marcada com anticorpo anti-Jaburetox-2Ec. O sistema nervoso central foi isolado de ninfas de quinto instar 12 horas após injeção com Jaburetox-2Ec, numa dose de 0,04 μg por mg de peso corpóreo. As setas indicam as regiões próximas aos nervos abdominais, onde se concentraram regiões de reconhecimento pelo anticorpo. (A) Lado direito do gânglio e (B) lado esquerdo do gânglio. Barra = 50 μm

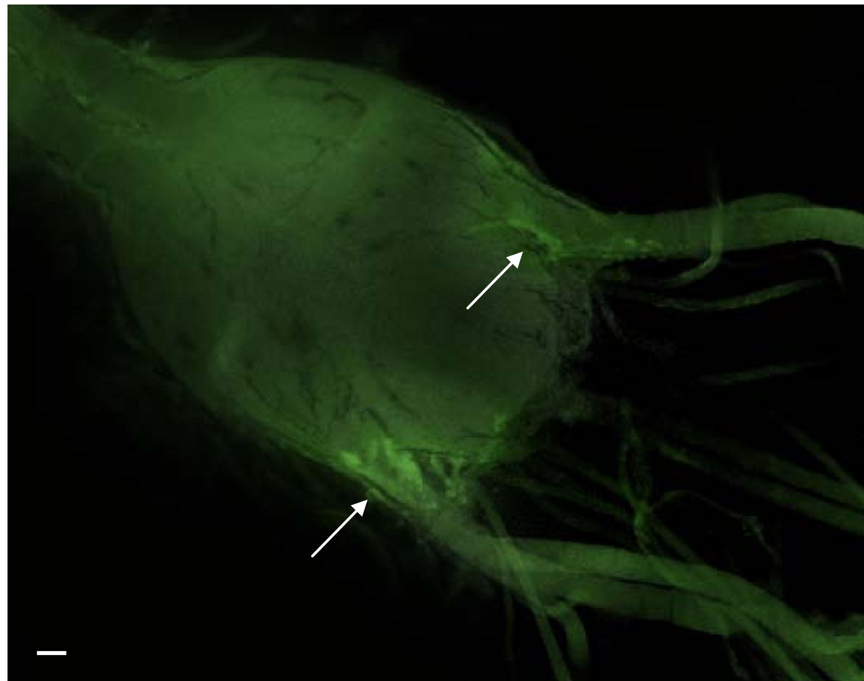


Figura 6. Microscopia confocal da Massa Gangliônica Mesotorácica de *O. fasciatus* marcada com anticorpo anti-Jaburetox-2Ec. O sistema nervoso central foi isolado de ninfas de quinto instar 12 horas após injeção com Jaburetox-2Ec, numa dose de 0,04 μg por mg de peso corpóreo. As setas indicam as regiões próximas aos nervos abdominais, onde se concentraram regiões de reconhecimento pelo anticorpo. Barra = 50 μm

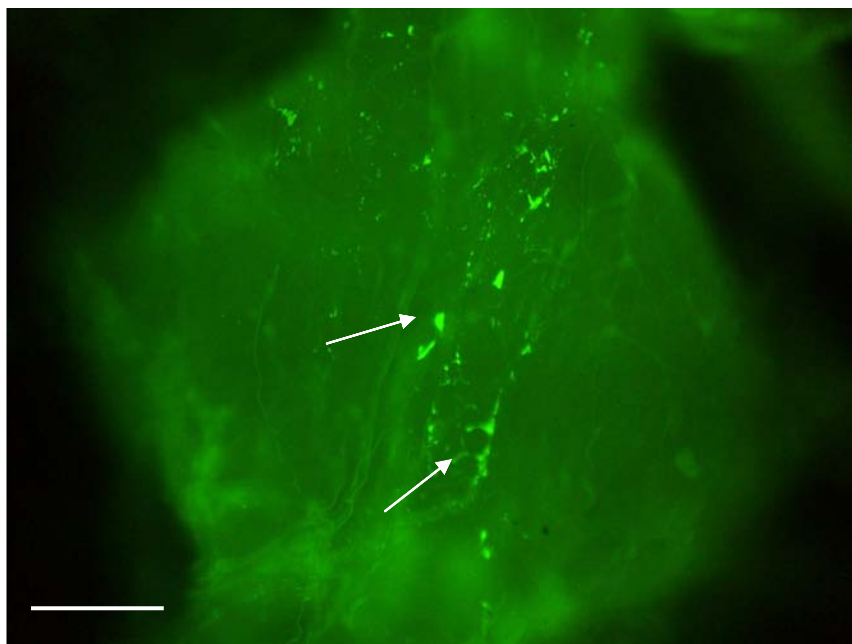


Figura 7. Microscopia de epifluorescência do Gânglio Pró-torácico de *O. fasciatus* marcado com anticorpo anti-Jaburetox-2Ec. O sistema nervoso central foi isolado de ninfas de quinto instar 12 horas após injeção com Jaburetox-2Ec, numa dose de 0,04 μg por mg de peso corpóreo. As setas indicam as regiões onde há reconhecimento pelo anticorpo. Barra = 50 μm

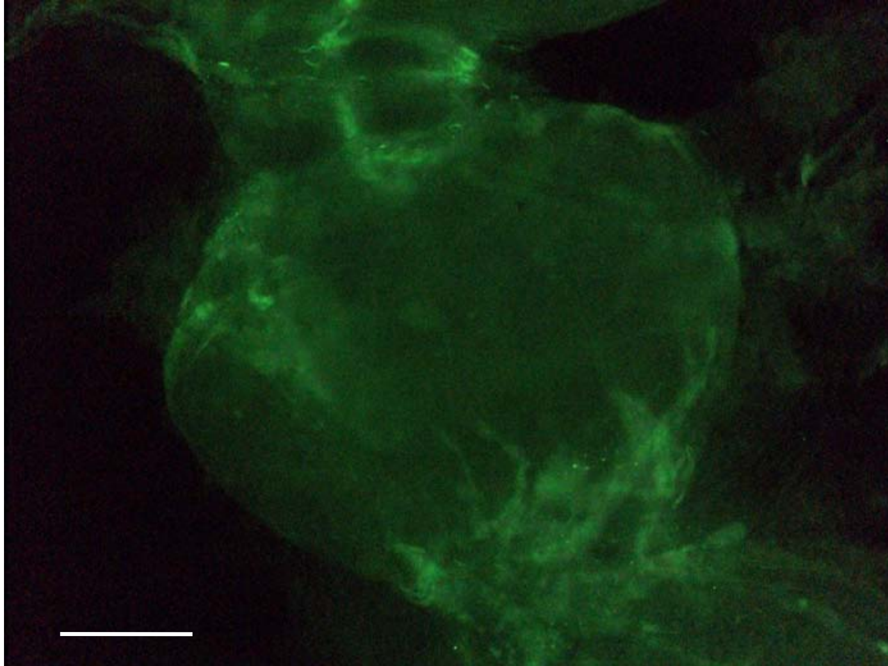


Figura 8. Microscopia de epifluorescência do Gânglio Pró-torácico de *O. fasciatus* marcado com anticorpo anti-Jaburetox-2Ec. O sistema nervoso central foi isolado de ninfas de quinto instar injetadas com solução salina, utilizadas como controle. Barra = 50 μm

2.3. Efeito da urease de *C. ensiformis* e do peptídeo recombinante Jaburetox-2Ec nas contrações de intestinos de *O. fasciatus*

Em estudo não publicado, Stanisçuaski *et al.* mostraram que a JBU é capaz de estimular contrações intestinais induzidas por serotonina em *Rhodnius prolixus*. Considerando-se este dado, fizemos ensaios *ex vivo* de monitoramento das contrações musculares de intestinos isolados de *O. fasciatus*, quantificando a freqüência dessas contrações. A presença de somente urease no meio altera a freqüência em uma única concentração (10^{-10} M) entre as que foram testadas, causando um aumento de cerca de 40% (Fig. 8). Quando adicionamos somente serotonina ao meio, a freqüência de contrações dos intestinos de *O. fasciatus* aumenta de forma dose-dependente (Fig. 9), e quando se adiciona urease à serotonina, o efeito é diminuído em algumas concentrações (Fig. 10). Todas concentrações de urease testadas sobre 10^{-8} M de serotonina têm o mesmo efeito inibitório, enquanto que nenhuma concentração de urease testada com 10^{-7} M de serotonina altera o efeito excitatório (Fig. 11).

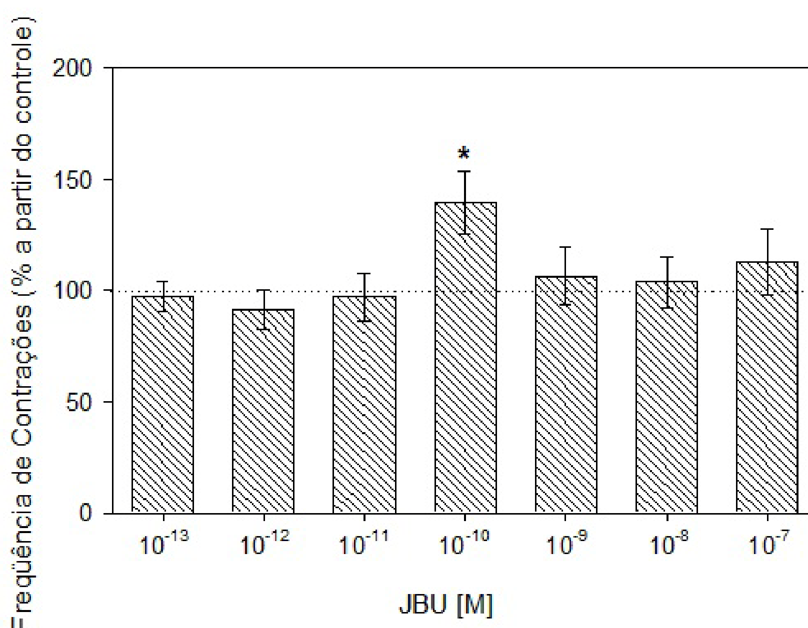


Figura 8. Efeito da JBU nas contrações de intestinos de *O. fasciatus*. O primeiro ventrículo do intestino anterior foi isolado de ninfas de quinto instar e colocado em uma gota de 100 μL de salina. Os eletrodos do conversor de impedância foram colocados paralelamente ao intestino dentro da gota. O tecido foi então tratado com JBU, como descrito nos métodos. Valores são expressos como média e erro padrão de 15 replicatas. * indica valores estatisticamente diferentes do tratamento controle, somente com salina ($p \leq 0,05$).

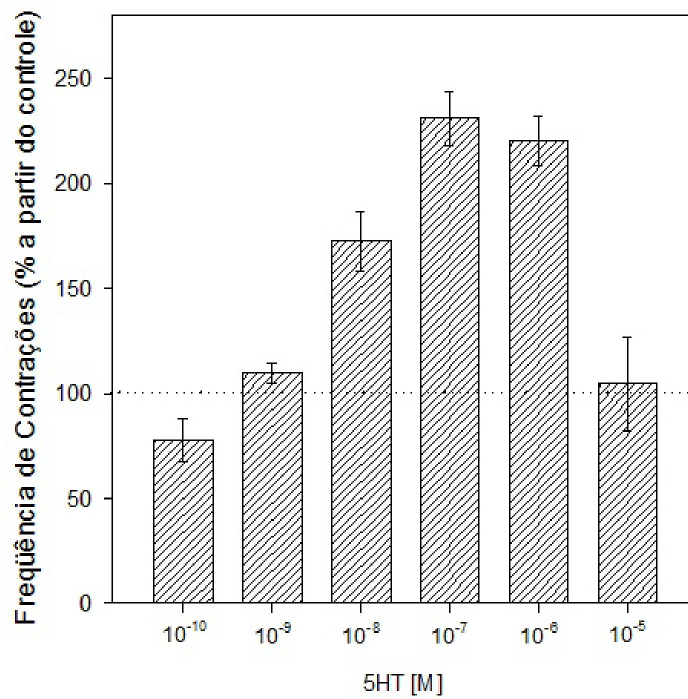


Figura 9. Efeito da serotonina nas contrações de intestinos de *O. fasciatus*. O primeiro ventrículo do intestino anterior foi isolado de ninfas de quinto instar e colocado em uma gota de 100 μL de salina. Os eletrodos do conversor de impedância foram colocados paralelamente ao intestino dentro da gota. O tecido foi então tratado com serotonina, como descrito nos métodos. Valores são expressos como média e erro padrão de 15 replicatas.

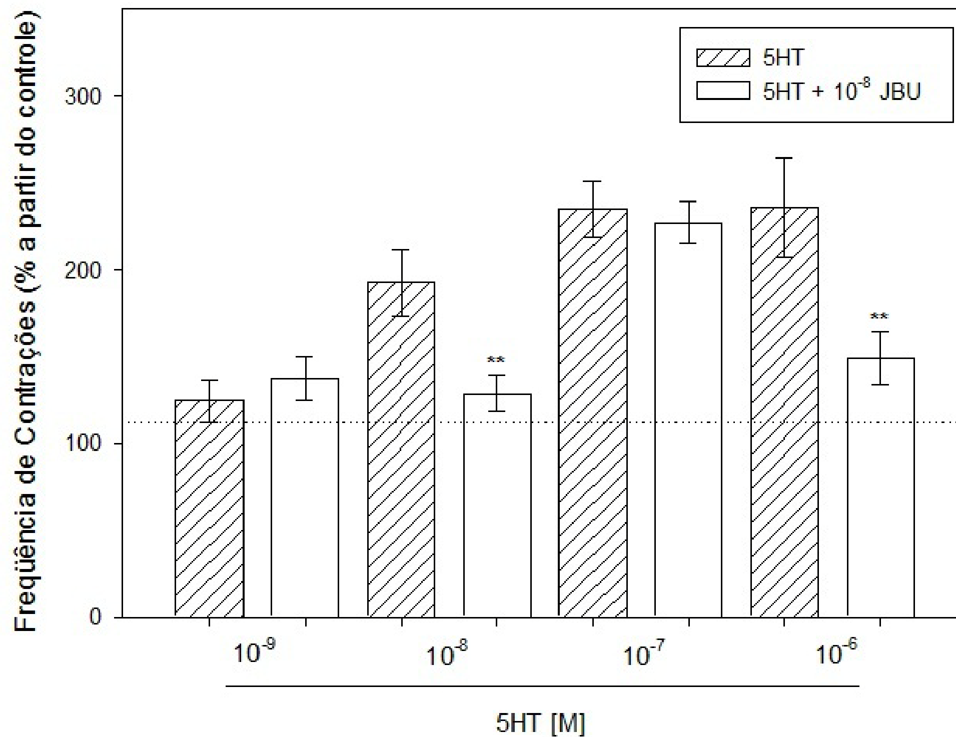


Figura 10. Efeito de JBU nas contrações estimuladas por serotonina em intestinos de *O. fasciatus*. O primeiro ventrículo do intestino anterior foi isolado de ninfas de quinto instar e colocado em uma gota de 100 μL de salina. Os eletrodos do conversor de impedância foram colocados paralelamente ao intestino dentro da gota. O tecido foi então tratado com somente serotonina ou com serotonina em presença de JBU, como descrito nos métodos. Valores são expressos como média e erro padrão de 15 replicatas. * indicam valores estatisticamente diferentes do tratamento somente com serotonina ($p \leq 0{,}05$).

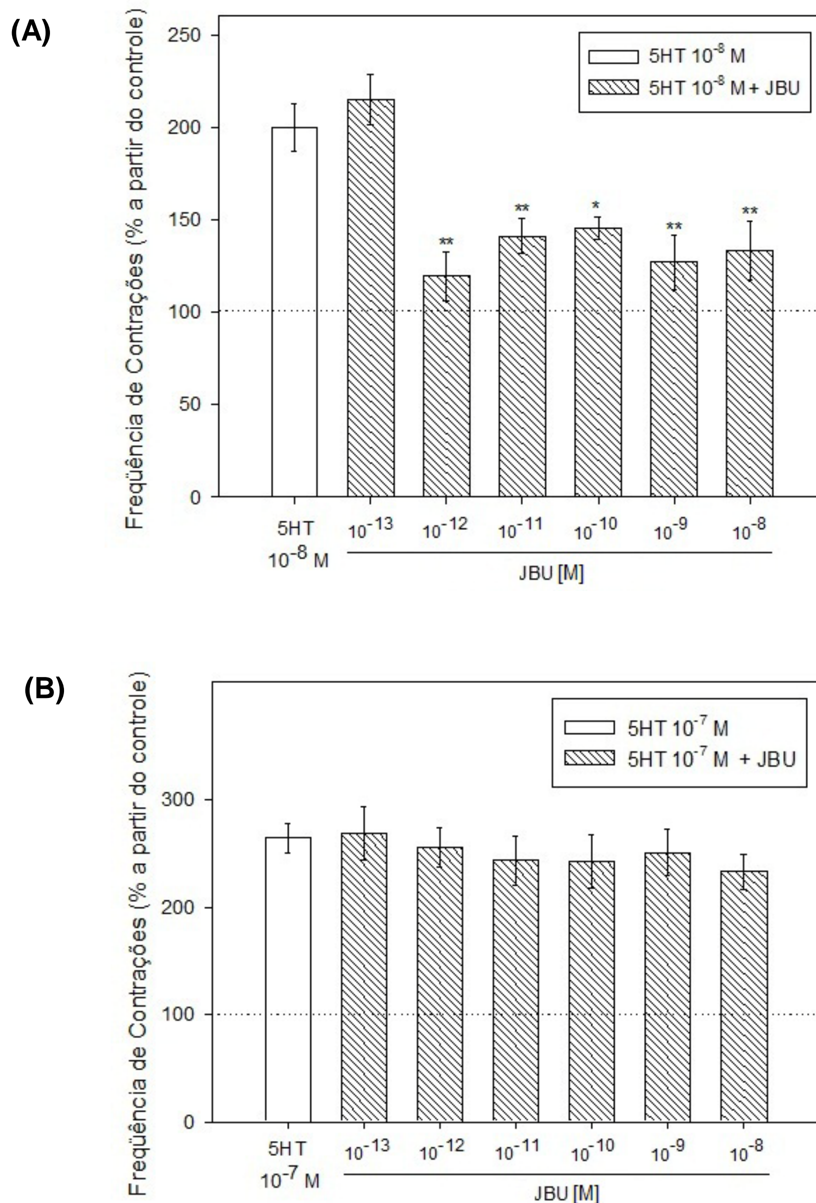


Figura 11. Efeito de JBU nas contrações estimuladas por serotonina em intestinos de *O. fasciatus*. O primeiro ventrículo do intestino anterior foi isolado de ninfas de quinto instar e colocado em uma gota de 100 μL de salina. Os eletrodos do conversor de impedância foram colocados paralelamente ao intestino dentro da gota. O tecido foi então tratado com somente serotonina ou com serotonina em presença de JBU, como descrito nos métodos. (A) Diferentes concentrações de JBU em presença de 10^{-8} M de serotonina e (B) diferentes concentrações de JBU em presença de 10^{-7} M de serotonina. Valores são expressos como média e erro padrão de 10 replicatas. * indicam valores estatisticamente diferentes do tratamento somente com serotonina ($p \leq 0,05$).

Já foi sugerido que o AMPc pudesse exercer um papel de segundo mensageiro de serotonina no intestino anterior de *R. prolixus* (Barret *et al.,* 1993). Para avaliar se esse efeito da urease na freqüência de contrações é dependente de serotonina, as contrações foram estimuladas com o análogo estável de AMPc, 8-bromo-AMPc. Observamos que AMPc aumenta a freqüência e que esse efeito não é alterado com a presença de urease (Fig. 12).

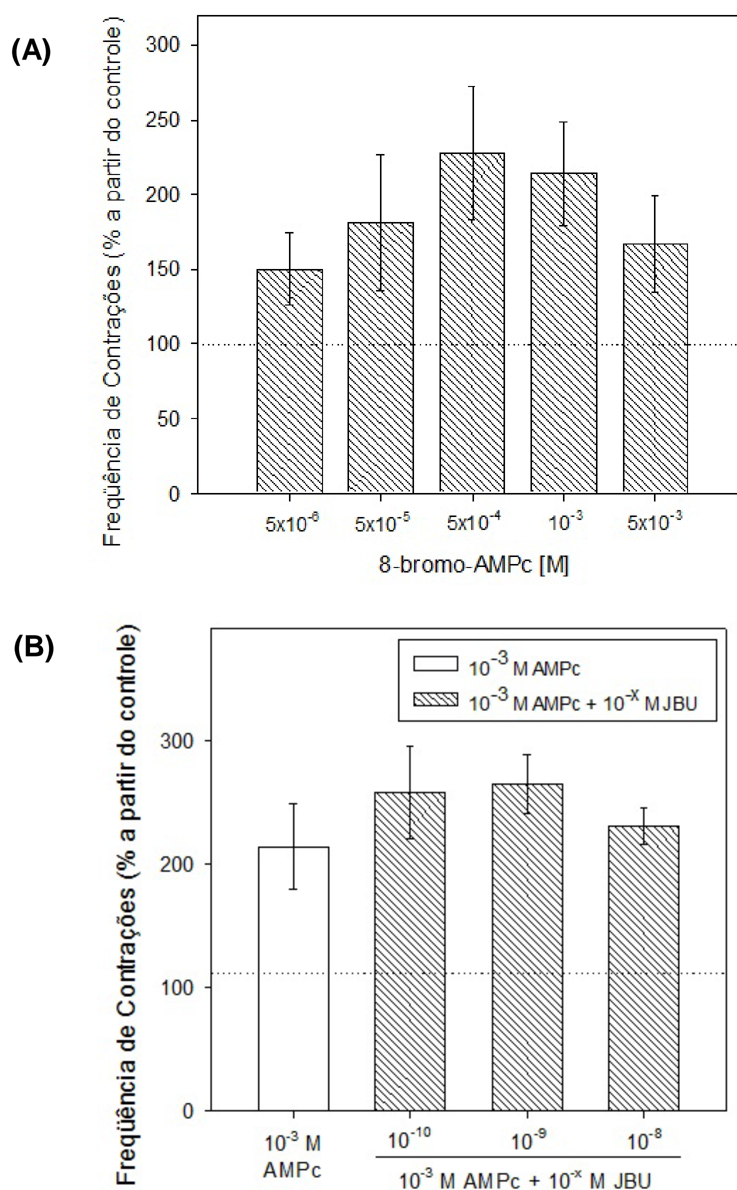


Figura 12. Efeito de somente AMPc (A) e AMPc em presença de JBU (B) nas contrações de intestinos de *O. fasciatus*. O primeiro ventrículo do intestino anterior foi isolado de ninfas de quinto instar e colocado em uma gota de 100 μL de salina. Os eletrodos do conversor de impedância foram colocados paralelamente ao intestino dentro da gota. O tecido foi então tratado com AMPc ou AMPc com JBU, como descrito nos métodos. Valores são expressos como média e erro padrão de 10 replicatas.

O peptídeo recombinante Jaburetox-2Ec não causou nenhum efeito sobre as contrações intestinais *ex vivo* de *Oncopeltus fasciatus* em nenhuma das concentrações testadas, tanto quando aplicado sozinho (Fig. 13) ou quando aplicado em presença de serotonina (dados não apresentados).

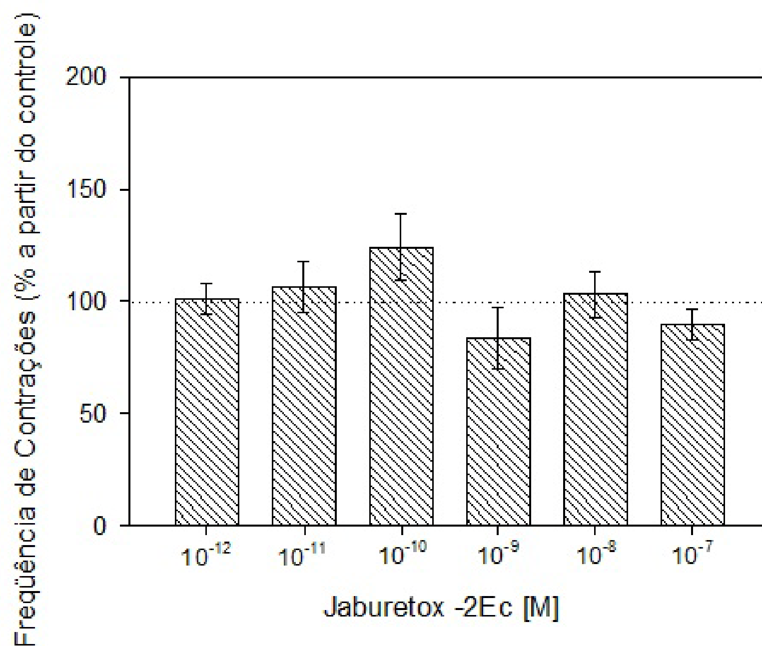


Figura 13. Efeito de Jaburetox-2Ec nas contrações de intestinos de *O. fasciatus*. O primeiro ventrículo do intestino anterior foi isolado de ninfas de quinto instar e colocado em uma gota de 100 μL de salina. Os eletrodos do conversor de impedância foram colocados paralelamente ao intestino dentro da gota. O tecido foi então tratado com o peptídeo, como descrito nos métodos. Valores são expressos como média e erro padrão de 10 replicatas.

III. Discussão

Este trabalho foi desenvolvido em duas partes: em um primeiro momento, estudamos a toxicidade da urease majoritária de *Canavalia ensiformis* sobre ninfas de *Oncopeltus fasciatus,* e identificamos e caracterizamos as enzimas digestivas envolvidas no processamento e ativação da proteína; em um segundo momento investigamos a distribuição tecidual *in vivo* e a toxicidade do peptídeo Jaburetox-2Ec, e a ação da urease sobre a musculatura de intestinos isolados de ninfas de *O. fasciatus*.

Observamos que ninfas de *O. fasciatus* apresentam uma taxa de mortalidade maior que 80%, após duas semanas, quando alimentadas com diferentes doses de urease, e que *in vitro* as enzimas do inseto são capazes de liberar peptídeos reconhecíveis pelo anticorpo anti-Jaburetox-2Ec. Quando injetadas com o peptídeo recombinante diretamente na hemolinfa, a taxa de mortalidade é de 100% após 48 horas. Esses dados reiteram que a ação do peptídeo recombinante é mais rápida que a ação da urease intacta, como já foi mostrado em outros estudos (Mulinari *et al.,* 2007), pois não há a necessidade de processamento da molécula para que ocorra toxicidade.

Para obtenção dos dados da primeira parte do trabalho, foram utilizados substratos fluorogênicos e protéicos associados a inibidores de peptidases classe-específicos. Assim, caracterizamos o extrato de enzimas digestivas de ninfas de quarto instar, o qual é composto por cisteíno e aspártico peptidases. Também isolamos uma catepsina-L (cisteíno endopeptidase) do extrato bruto, por um processo de purificação de duas etapas. A composição enzimática do trato digestório é bastante semelhante ao que já foi reportado para outras espécies de hemípteros (Bongers, 1970; Woodring *et al.*, 2007), como por exemplo para *Dysdercus peruvianus* (Silva & Terra, 1994; Piovesan *et al.*, 2008), e assim como Woodring *et al.* (2007) observamos a ausência de serino e metalo peptidases no extrato.

Utilizamos substratos sintéticos correspondentes às regiões flanqueadoras, N e C-terminal, do peptídeo entomotóxico dentro da urease (Mulinari *et al.*, 2007). O homogeneizado de intestinos foi capaz de clivar o peptídeo correspondente à região C-terminal, sendo que esta atividade foi completamente bloqueada por um inibidor de cisteíno peptidases (E-64). Em um estudo prévio, com *D. peruvianus*, observamos que este mesmo substrato é clivado entre resíduos de arginina e asparagina (RD) (Piovesan *et al.,* 2008), e a hidrólise deste tipo de ligações RD por catepsinas-L já foi relatada em outras espécies (Dahl *et al.*, 2001). Porém, o homogeneizado de intestinos de *O. fasciatus* não clivou o substrato correspondente à região N-terminal,

que, em contraste, é clivado em *D. peruvianus* por metalopeptidases (Piovesan *et al.*, 2008). Dessa forma, podemos inferir que o sítio de clivagem da região N-terminal em *O. fasciatus* é diferente daquele em *D. peruvianus*, mas mesmo assim não há alterações no perfil de toxicidade da urease entre as espécies. Mulinari *et al.* (2007) mostraram que há uma menor conservação da região da urease que corresponde ao Jaburetox-2Ec, do que em outras regiões da molécula. Como as ureases de *C. ensiformis* e de *Glycine max* (soja) (Follmer *et al.*, 2004a,b) e também do feijão guando (*Cajanus cajan*) (Balasubramanian *et al.*, 2010) são entomotóxicas, pode-se inferir que a atividade inseticida admita uma alta variação de seqüência. Esses dados sugerem que os sítios de clivagem dos peptídeos entomotóxicos podem ser variáveis, gerando regiões terminais diferenciadas mas com a manutenção da toxicidade.

Recentemente Barros *et al.* (2009) demonstraram que o peptídeo Jaburetox-2Ec é capaz de perturbar bicamadas lipídicas de membranas, adotando uma conformação de ‘β-hairpin’, como em peptídeos antimicrobianos formadores de poros. Essa informação reforça a idéia citada antes, de que as regiões terminais seriam menos importantes para a manutenção da toxicidade dos peptídeos, uma vez que a estrutura de 'β-hairpin’ se forma a partir de resíduos localizados mais perto da região C-terminal na molécula.

Após injetar Jaburetox-2Ec em ninfas de quinto instar de *O. fasciatus*, fizemos uma localização *in situ* da molécula, utilizando o anticorpo específico. Houve reconhecimento pelo anticorpo em regiões do sistema nervoso central, 12 horas após a injeção, próximas aos nervos abdominais, na Massa Gangliônica Mesotorácica, e no Gânglio Protorácico, de onde partem nervos que controlam asas, patas e musculatura abdominal (Tyrer & Gregory, 1982). Considerando-se o modo de ação proposto para Jaburetox-2Ec, de perturbação de membranas, e a localização no sistema nervoso central observada, podemos inferir que exista uma ação prejudicial na transmissão nervosa causada pelo peptídeo.

Em *Rhodnius prolixus* foi observado que a serotonina estimula as contrações intestinais *ex vivo* (Orchard, 2006) e que a urease aumenta esse efeito (Stanisçuaski *et al.*, submetido). No presente trabalho observamos que se aplicada sozinha ao intestino isolado, na dose de 10^{-10} M, a JBU aumenta significativamente a freqüência de contrações, efeito que não se observa em nenhuma outra das concentrações testadas. A JBU também interfere, *in vitro,* no efeito excitatório da serotonina sobre as contrações intestinais em *O. fasciatus*, porém neste caso, diminuindo-o. Esse efeito inibitório da urease se deu em duas das quatro concentrações de serotonina testadas (10^{-8} e 10^{-6} M), sendo que o efeito inibitório mostrou-se semelhante em todas concentrações, desaparecendo abaixo de 10^{-13} M.

Para avaliar se o efeito da JBU na freqüência de contrações é dependente de serotonina, testamos um análogo de AMPc (8-bromo-AMPc), o qual já foi identificado como segundo mensageiro de serotonina em processos no intestino e nas glândulas salivares de *O. fasciatus* (Miggiani *et al.,* 1999). O AMPc aumenta a freqüência de contrações, e a JBU não interfere nesse efeito. O peptídeo Jaburetox-2Ec não interfere na freqüência de contrações do intestino de *O. fasciatus*, seja em presença de serotonina ou sozinho.

Essas informações nos levam a crer que, no intestino de *O. fasciatus,* a interferência com as ações da serotonina ocorre exclusivamente para a urease, mas não para seus peptídeos, tipo Jaburetox-2Ec. Esse efeito da urease pode envolver ação direta na membrana ou via receptor na superfície celular, atuando como agonista ou antagonista, dependendo da concentração dos dois compostos no meio. Há pouca bibliografia a respeito de receptores em intestinos de insetos, porém no estômago de alguns insetos já foram identificados receptores de serotonina tipo $5HT_2$, com propriedades semelhantes aos receptores $5HT_2$ de mamíferos (Banner *et al.*, 1987; Barret *et al.*, 1993). Ainda que existam similaridades entre receptores de vertebrados e invertebrados, a comunicação celular é bastante especifica em cada grupo, bem como os receptores (Osborne *et. al.*, 1990), e como a maioria das drogas são feitas para sistemas de vertebrados, a utilização destas para caracterizar receptores de invertebrados é bastante imprecisa.

As ureases apresentam altos níveis de toxicidade nos insetos suscetíveis, sendo de 20 a 50 vezes mais potentes que outras proteínas vegetais inseticidas, como lectinas, inibidores de proteases e de α-amilases (Carlini & Grossi-de-Sá, 2002). Apesar de ser uma espécie adaptada para a detoxificação dos glicosídios nas plantas da família Asclepiadaceae (Feir, 1974), *O. fasciatus* é tão suscetível ao efeito da urease, a 0,01% ou a 0,2% (p/p), como os outros hemípteros já testados. Este fato evidencia o processo de co-evolução de insetos e plantas, já demonstrado em outras situações de adaptação de insetos aos mecanismos de defesa de suas plantas hospedeiras (Carlini & Grossi-de-Sá, 2002). Até recentemente acreditava-se que o efeito tóxico das ureases era somente devido à liberação dos peptídeos após clivagem proteolítica. Agora se analisa a possibilidade de a molécula intacta estar participando desse efeito, que se mostra bastante complexo, envolvendo vários sistemas e vias de sinalização, sendo de difícil contorno para os insetos. O dados aqui apresentados reforçam a potencialidade do uso das ureases como bioinseticidas e auxiliam no entendimento sobre os mecanismos de ação dessas proteínas.

IV. Conclusões

- ✓ A urease majoritária de *Canavalia ensiformis* e o peptídeo recombinante Jaburetox-2Ec são tóxicos para ninfas de *Oncopeltus fasciatus* como em outros modelos de insetos testados, reforçando a potencialidade do uso dessas proteínas como controladores de pragas.
- ✓ As peptidases presentes nos homogeneizados de intestinos são capazes de hidrolisar *in vitro* a urease majoritária de *C. ensiformis* e liberar peptídeos reconhecíveis pelo anticorpo anti-Jaburetox-2Ec, indicando que esses peptídeos resultantes são bastante semelhantes aos gerados pela hidrólise da urease por enzimas de outras espécies de insetos.
- ✓ Peptidases cisteínicas e aspárticas estão presentes nos intestinos de *O. fasciatus*, e entre as cisteínicas identificamos especificamente uma catepsina L, que ao que tudo indica é uma das enzimas responsáveis pela hidrólise da urease.
- ✓ A urease de *C. ensiformis* é capaz de interagir com serotonina, alterando o efeito *in vitro* sobre intestinos isolados de *O. fasciatus*, sugerindo que a proteína possa interferir na neurotransmissão e processos dependentes dessa molécula *in vivo*.
- ✓ Os ensaios de imunohistoquímica indicam que o peptídeo Jaburetox-2Ec, após injeção no inseto, localiza-se no sistema nervoso central em ninfas de *O. fasciatus*.
- ✓ Os dados obtidos sugerem que a ação da urease nos insetos não é somente devido aos peptídeos resultantes da clivagem proteolítica, havendo contribuição da molécula intacta, o que aumenta o leque de questões acerca dos mecanismos de ação a serem investigados em estudos futuros.

V. Referências Bibliográficas

Balasubramanian, A., Elumalai, S., Mathivanan, N., Ponnuraj, K. (2010). Insecticidal and antifungal activities of urease from pigeon pea (*Cajanus cajan*). Protein and Peptide Letters. *In press.*

Banner S.E., Osborne R.H. & Cattell K.J. (1987). The pharmacology of the isolated foregut of the locust *Schistocerca gregaria* – II. Characterization of a 5HT2-like receptor. Comp. Biochem. Physiol. 88:139-144.

Barcellos, G.B.S., Almeida, L.A., Moreira, R.A., Sousa-Cavada, B., Oliveira, J.T.A., Carlini, C.R. (1993). Canatoxin-cross reactive, concanavalin A-cross reactive, and canavalin-cross reactive materials during maturation of *Canavalia brasiliensis* seeds. Planta, 189(3): 397-402.

Barja-Fidalgo, T.C., Guimarães, J.A., Carlini, C.R. (1991a). Lipoxygenase-mediated secretory effect of canatoxin, the toxic protein from *Canavalia ensiformis* seeds. Toxicon, 29(4-5): 453-459.

Barja-Fidalgo, T.C., Guimarães, J.A., Carlini; C.R. (1991b). Canatoxin, a plant protein, induces insulin secretion from isolated pancreatic islets. Endocrinology, 128(2): 675-679.

Barret, F.M., Orchard, I., Te Brugge, V. (1993). Characteritics of serotonin-induced cyclic AMP elevation in the integument and anterior midgut of the blood-feeding bug, *Rhodnius prolixus*. J. Insect Physiol. 39:581-587.

Barret, A.J., Rawlings, N.D., Woessner, J.F. (1998). Handbook of Proteolytic Enzymes. Academic Press, San Diego, USA, 1666 p.

Barros, R.P., Stassen, H., Freitas, M.S., Carlini, C.R., Nascimento, M.A.C., Follmer, C. (2009). Membrane-disruptive properties of the bioinsecticide Jaburetox-2Ec: Implications to the mechanism of the action of insecticidal peptides derived from ureases. Biochimica *et* Biophysica Acta. 1794 (12): 1848-1854.

Becker-Ritt, A.B., Martinelli, A.H., Mitidieri, S., Feder, V., Wassermann, G.E., Santi, L., Vainstein, M.H., Oliveira, J.T., Fiuza, L.M., Pasquali, G., Carlini, C.R. (2007) Antifungal activity of plant and bacterial ureases. Toxicon, 50(7): 971-983.

Bolter, C., Jongsma, M.A. (1997). The adaptation of insects to plant protease inhibitors. Journal of Insect Physiology, 43(10): 885-895.

Bongers, J. 1970. Die Carbohydrasen und Esterasen in Speicheldrüsen und Mitteldarm von *Oncopeltus fasciatus* (Lygaeidae). Zeitschrift für Vergleichende Physiologie. 70, 382–400.

Broadway, R.M., Duffey, S.S. (1986). Plant peptidase-inhibitors - Mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. Journal of Insect Physiology, 32(10): 827-833.

Broadway, R.M. (1995). Are insects resistant to plant peptidase inhibitors? Journal of Insect Physiology, 41(2): 107-116.

Broadway, R.M. (1996) Resistance of plants to herbivorous insects: Can this resistance fail? Canadian Journal of Plant Pathology, 18(4): 476-481.

Buzzi, Z.J., Miyazaki, R.D. (1999). Entomologia didática. Editora da Universidade Federal do Paraná, Curitiba, 262 p.

Carlini, C. R., Guimarães, J. A. (1981). Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jack bean) seeds, distinct from concanavalin A. Toxicon, 19(5): 667-675.

Carlini, C. R., Gomes, C., Guimarães, J.A., Markus, R.P., Sato, H., Trolin, G. (1984). Central nervous effects of the convulsivant protein canatoxin. Acta Pharmacological et Toxicologica, 54(3): 161-166.

Carlini, C.R., Guimarães, J.A., Ribeiro, J.M.C. (1985). Platelet relase reaction and aggregation induced by canatoxin, a convulsant protein - Evidences for the involvement of the platelet lipoxygenase pathway. British Journal of Pharmacology, 84 (2): 551-560.

Carlini, C.R., Barcellos, G.B.S., Baeta-Neves, A.D.V., Guimarães, J.A. (1988). Immunoreactivity for canatoxin and concanavalin-A among proteins from leguminous seeds. Phytochemistry, 27(1): 25-30.

Carlini, C.R., Guimarães, J.A. (1991). Plant and microbial toxic proteins as hemilectins: emphasis on canatoxin. Toxicon, 29(7): 791-806.

Carlini, C.R., Oliveira, A.E.A., Azambuja, P., Xavier-Filho, J., Wells, M.A. (1997). Biological effects of canatoxin in different insect models: Evidence for a proteolytic activation of the toxin by insect cathepsin-like enzymes. Journal of Economic Entomology, (90)2: 340-348.

Carlini, C.R., Grossi-de-Sá, M. F. (2002). Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. Toxicon, 40(11): 1515-1539.

Carlini, C.R., Polacco, J.C. (2008) Toxic properties of urease. Crop Science. 48:1665-1672.

Chapman, R.F. (1998). The insects: Structure and Function. Cambridge University Press, Cambridge, 770 p.

Dahl, S.W., Halkier, T., Lauritzen, C., Dolenc, I., Pedersen, J., Turk, V., Turk, B. (2001). Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. Biochemistry 40, 1671–1678.

Dixon, N.E., Gazzola, C., Blakeley, R. L., Zerner, B. (1975). Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? Journal of The American Chemical Society. 97(14): 4131-4133.

Eidels, L., Proia, R.L., Art, D.A. (1983). Membrane receptors for bacterial toxins. Microbiological Reviews. 47(4): 596-620.

Feir, D. (1974). Oncopeltus fasciatus: a research animal. Annual Review of Entomology 19, 81–96.

Felton, G.W. (1996). Nutritive quality of plant protein: Sources of variation and insect herbivore responses. Archives of Insect Biochemistry and Physiology, 32(1): 107-130.

Ferreira-DaSilva, C.T., Gombarovits, M.E.C., Masuda, H., Oliveira, C.M., Carlini, C.R. (2000). Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. Archives of Insect Biochemistry and Physiology, 44(4): 162-171.

Follmer, C., Barcellos, G.B.S., Zingali, R.B., Machado, O.L.T., Alves, E.W., Barja-Fidalgo, C., Guimarães, J.A., Carlini, C.R. (2001). Canatoxin, a toxic protein of Jack beans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.15). Biological effects of urease independent of its ureolytic activity. Biochemical Journal, 360(1): 217-224.

Follmer, C., Wassermann, G.E., Carlini, C.R. (2004a). Separation of jackbean (*Canavalia ensiformis*) urease isoforms by immobilized metal affinity chromatography and characterization of insecticidal properties unrelated to ureolytic activity. Plant Science, 167(2): 241-246.

Follmer, C., Real-Guerra, R., Wasserman, G.E., Oliveira-Severo, D., Carlini, C.R. (2004b). Jackbean, soybean and *Bacillus pasteurii* ureases: Biological effects unrelated to ureolytic activity. European Journal of Biochemistry, 271(7): 1357-1363.

Follmer, C. (2008). Insights into the role and structure of plant ureases. Phytochemistry 69:18–28.

Hirayama C., Sugimura M., Saito H., Nakamura M. (2000). Host plant urease in the hemolymph of the silkworm, *Bombyx mori*. J. Insect Physiol. 46:1415-1421.

Hill S.R. & Orchard I. (2003). FMRFamide-related peptides in the gut of *Locusta migratoria* L.: a comprehensive map and developmental profile. Peptides 24:1511-1524.

Gallo, D., Nakano, O., Silveira-Neto, S., Carvalho, R.P.L., Baptista, G.C., Berti-Filho, E. Parra, J.R., Zucchi, R.A., Alves, S.B., Vendramin, J.D., Marchini, L.C., Lopes, J.R.S., Omoto, C. (2002). Entomologia agrícola. Piracicaba, 920 p.

Ghazaleh, F.A., Araujo C.F., Barja-Fidalgo C., Carlini, C.R. (1992). Canatoxin induces activation on mice peritoneal macrophages. Brazilian Journal of Medical and Biological Research, 25(10):1033-1035.

Ghazaleh, F.A., Francischetti, I.M., Gombarovitz, M.E., Carlini, C.R. (1997). Stimulation of calcium influx and platelet activation by canatoxin: Methoxyverapamil inhibition and downregulation by cGMP. Archives of Biochemistry and Biophysics, 339(2): 362-367.

Greenberg M.J. & Price D.A. (1992). Relationships among the FMRFamide-like peptides. Prog. Brain Res. 92:25-37. Review.

Gombarovits, M.E.C. (1999). Peptídeos entomotóxicos da canatoxina: obtenção, isolamento, propriedades biológicas e caracterização físico-química. Dissertação de Mestrado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Keller, M., Sneh, B., Strizhov, N., Prudovsky, E., Regev, A., Konez, C., Schell, J., Zilberstein, A. (1996). Digestion of delta-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC. Insect Biochemistry and Molecular Biology, 26(4): 365-373.

Koiwa, H., Kato, H., Nakatsu, T., Oda, J., Yamada, Y., Sato, F. (1997). Purification and characterization of tobacco pathogenesis-related protein PR-5d, an antifungal thaumatin-like protein. Plant and Cell Physiology, 38(7): 783-791.

Koiwa, H., Shade, R.E., Zhu-Salzman, K., Subramanian, L., Murdock, L.L., Nielsen, S.S., Bressan, R.A., Hasegawa, P.M. (1998). Phage display selection can differentiate insecticidal activity of soybean cystatins. Plant Journal, 14(3): 371-379.

Kurahashi H., Atiwetin P., Nagaoka S., Miyata S., Kitajima S., Sugimura Y. (2005). Absorption of mulberry root urease to the hemolymph of the silkworm, *Bombyx mori.* J. Insect Physiol. 51:1055-1061.

Meredith, J., Moore, L. & Scudder, G.G.E. (1984). Excretion by ouabain by Malpighian tubules of *Oncopeltus fasciatus*. American Journal of Physiology. 246: R705-R715.

Miggiani, L., Orchard, I. & TeBrugge, V. (1999). The distribution and function of serotonin in the large milkweed bug, *Oncopeltus fasciatus*. A comparative study with the blood-feeding bug, *Rhodnius prolixus*. Journal of Insect Physiology. 45:1029-1036.

Mulinari, F. (2004). Jaburetox 2-Ec: um peptídeo recombinante derivado de urease de *Canavalia ensiformis* com potencial bioinseticida. Dissertação de Mestrado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Mulinari, F., Stanisçuaski, F., Bertholdo-Vargas, L.R., Postal, M., Oliveira-Neto, O.B., Rigden, D.J., Grossi-de-Sá, M.F., Carlini, C.R. (2007) Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the plant *Canavalia ensiformis*. Peptides, 28(10): 2042-2050.

Murdock, L.L. ,Shade, R.E. (2002). Lectins and protease inhibitors as plant defenses against insects. Journal of Agricultural and Food Chemistry, 50(22): 6605-6611.

Oliveira, A.E.A., Gomes, V.M., Sales, M.P., Fernandes, K.V.S., Carlini, C.R., Xavier-Filho, J. (1999). The toxicity of of jack bean [*Canavalia ensiformis* (L.) Dc] canatoxin to plant pathogenic fungi. Revista Brasileira de Biologia, 59: 59-62.

Orchard, I. (2006). Serotonin: A coordinator of feeding-related physiological events in the blood-gorging bug, *Rhodnius prolixus*. Comp. Biochem. Physiol. Part A 144:316 – 324.

Osborne R.H., Banner S.E. & Wood S.J. (1990). The pharmacology of the gut of the desert locust, *Schistocerca gregaria* and other insects. Comp. Biochem. Physiol. C 96:1–9.

Piovesan, A.R., Stanisçuaski, F., Marco-Salvadori, J., Real-Guerra, R., Defferrari, M.S. & Carlini, C.R. (2008). Stage-specific gut peptidases of the cotton stainer bug *Dysdercus peruvianus*: Role in the release of entomotoxic peptides from *Canavalia ensiformis* urease. Insect Biochemistry and Molecular Biology. 38: 1023–1032.

Pires-Alves, M. (2002). Genes de ureases na *Canavalia ensiformis*: caracterização de um novo membro (JBURE-2). Potencial como bioinseticida. Tese de Doutorado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Polacco, J.C., Holland, M.A. (1993) Role of urease in plant-cells. International Review of Cytology – A Survey of Cell Biology. 145: 65-103.

Real-Guerra, R. (2007) Aspectos biológicos e estruturais das ureases de Canavalia ensiformis. Dissertação de Mestrado. Universidade Federal do Rio Grande do Sul, Porto Alegre.

Romeiro, A., Solé-Cava, A., Sousa, M.A., De Souza, W., Attias, M. (2000) Ultrastructural and biochemical characterization of promastigotes and cystic form of *Leptomonas wallacei* n. sp. isolated from the intestine of its natural host *Oncopeltus fasciatus* (Hemiptera: Lygaeidae). J. Eukaryot. Microbiol. 47, 208–220.

Ruppert, E.E. & Barnes, R.D. (1996). Zoologia dos Invertebrados. Editora Roca, São Paulo. 6a Ed.

Ryan, C. A. (1990). Portease inhibitors in plants: genes for improving defenses against insects and pathogens. Annual Review of Phytopathology, 28: 425-449.

Settembrini B.P. & Villar M.J. (2004). Distribution of serotonin in the central nervous system of the blood-feeding Heteropteran, *Triatoma infestans* (Heteroptera: Reduviidae). J. Morphol. 260:21-32.

Settembrini B.P. & Villar M.J. (2005). FMRFamide-like immunocytochemistry in the brain and subesophageal ganglion of *Triatoma infestans* (Insecta: Heteroptera). Coexpression with beta-pigment-dispersing hormone and small cardioactive peptide B. Cell Tissue Res. 321:299-310.

Silva, C.P., Terra, W.R. (1994). Digestive and absorptive sites along the midgut of the cotton seed sucker bug Dysdercus peruvianus (Hemiptera: Pyrrhocoridae). Insect Biochem. Mol. Biol. 24, 493–505.

Stanisçuaski, F., Ferreira-DaSilva, C.T., Mulinari, F., Pires-Alves, M., Carlini, C.R. (2005). Insecticidal effects of canatoxin on the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Toxicon, 45(6): 753-760.

Stanisçuaski, F., TeBrugge, V., Carlini, C.R., Orchard, I. (2009). In vitro effect of *Canavalia ensiformis* urease and the derived peptide Jaburetox-2Ec on *Rhodnius prolixus* Malpighian tubules. Journal of Insect Physiology. 55: 255–263.

Stotz, H.U., Kroymann, J., Mitchell-Olds, T. (1999). Plant-insect interactions. Current Opinion in Plant Biology, 2(4): 268-272.

Sumner, J.B. (1926). The isolation and crystallization of the enzyme urease. Journal of Biological Chemistry, 69: 435-441.

Sumner, J.B., Howell, S.F. (1936). The identification of the hemaglutinin of the jackbean with concanavalin A. Journal of Bacteriology, 32: 227-237.

TeBrugge, V.A., Orchard, I. (2007). Distribution and activity of aDippu DH31-like peptide in the large milkweed bug *Oncopeltus fasciatus.* Peptides. 29: 206–213.

Terra W.R. (1988). Physiology and Biochemistry of insect digestion: an evolutionary perspective. Brazilian J. Med. Biol. Res. 21:675-734.

Terra, W.R., Ferreira, C. (1994). Insect digestive enzymes: properties, compartmentalization and function. Comparative Biochemistry and Physiology, 109B(1): 1–62.

Tyrer, N.M., Gregory, G.E. (1982). A guide to the neuroanatomy of locust subesophageal and thoracic ganglia. Philosophical Transactions of the Royal Society of London. B 297: 91-123.

Ubatuba, F.B. (1955). Occurrence of a trypsin inhibiting factor in the seeds of *Canavalia ensiformis*. Revista Brasileira de Biologia, 15: 1-8.

Woodring, J., Hoffman, K.H., Lorenz, M.W. (2007). Feeding, nutrient flow, and digestive enzyme release in the giant milkweed bug, *Oncopeltus fasciatus.* Physiological Entomology. 32: 328–335.

Zerner, B. (1991). Recent advances in the chemistry of an old enzyme, Urease. Bioorganic Chemistry, 19(1): 116-131.

Zhu-Salzman, K., Koiwa, H., Salzman, R.A., Shade, R.E., Ahn, J.-E. (2003). Cowpea bruchid *Callosobruchus maculatus* uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. Insect Molecular Biology, 12(2): 135-145.

ANEXO I

Marina Schumacher Defferrari
Curriculum Vitae

Fevereiro/ 2010

Marina Schumacher Defferrari

Curriculum Vitae

Dados Pessoais

Nome Marina Schumacher Defferrari
Filiação Mauro Defferrari e Eloísa Mattos Schumacher
Nascimento 24/06/1984 - Porto Alegre/RS - Brasil

Endereço profissional Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Departamento de Biofísica
Rua Bento Gonçalves, 9500 Prédio 43422 Sala 204
Agronomia - Porto Alegre
91501-970, RS - Brasil
Telefone: 051 33087600

Endereço eletrônico msdeff@cbiot.ufrgs.br

Formação Acadêmica/Titulação

2008 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Insecticidal effects of Canavalia ensiformis major urease on nymphs of the milkweed bug Oncopeltus fasciatus and digestive peptidases profile characterization
Orientador: Célia Regina Ribeiro da Silva Carlini
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2003 - 2007 Graduação em Ciências Biológicas.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Identificação, caracterização e purificação parcial de peptidases digestivas de Oncopeltus fasciatus (Hemiptera: Lygaeidae): papel no processamento da urease de Canavalia ensiformis
Orientador: Célia Regina Ribeiro da Silva Carlini
Bolsista do(a): UFRGS

Formação complementar

2008 - 2009 Intercâmbio em nível de mestrado.
University of Toronto at Mississauga, UTM, Canadá

2009 - 2009 Intercâmbio em nível de mestrado.
Universidad Austral, ACES, Argentina

Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2008 - Atual Vínculo: Estudante de pós-graduação
Enquadramento funcional: Mestrado
Regime: Dedicação Exclusiva
2006 - 2008 Vínculo: Iniciação Científica

Enquadramento funcional: Bolsista (BIC/ UFRGS)
Carga horária: 20,Regime: Dedicação Exclusiva

2004 - 2004 Vínculo: Estágio Voluntário
Enquadramento funcional: Estagiária
Carga horária: 15,Regime: Parcial

Atividades

03/2008 - Atual Pesquisa e Desenvolvimento, Centro de Biotecnologia, Departamento de Biofísica
Linhas de Pesquisa:
Mecanismo de ação de proteínas tóxicas em insetos; purificação de proteínas e enzimologia

10/2006 - 02/2008 Estágio, Instituto de Biociências, Departamento de Biofísica
Estágio:
Iniciação científica no laboratório de proteínas tóxicas (LAPROTOX)

04/2004 - 07/2004 Estágio, Instituto de Biociências, Departamento de Genética
Estágio:
Estudos em genética do comportamento humano

2. Pontifícia Universidade Católica do Rio Grande do Sul - PUC RS

Vínculo institucional

2004 - 2006 Vínculo: Estágio voluntário
Enquadramento funcional: Estagiária
Carga horária: 15, Regime: Parcial

Atividades

10/2004 - 09/2006 Estágio, Faculdade de Biociências, Centro de Biologia Genômica e Molecular
Estágio:
Estudos em filogenia molecular

Idiomas

Inglês Compreende Bem , Fala Bem, Escreve Bem, Lê Bem

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

Produção em C, T& A

Produção bibliográfica

Artigos completos publicados em periódicos

1. PIOVESAN, A, STANISCUASKI, F, MARCOSALVADORI, J, REALGUERRA, R, DEFFERRARI, M. S., CARLINI, C. R. Stage-specific gut peptidases of the cotton stainer bug Dysdercus peruvianus: Role in the release of entomotoxic peptides from Canavalia ensiformis urease. Insect Biochemistry and Molecular Biology. , v.38, p.1023 - 1032, 2008.

Apresentação de Trabalho

1. MARCELINO, T. B., DEFFERRARI, M. S., CARLINI, C. R. **CARACTERIZAÇÃO DE PEPTIDASES DIGESTIVAS DE ADULTOS DE ONCOPELTUS FASCIATUS (HEMIPTERA:LYGAEIDAE) E SEU PAPEL NA HIDRÓLISE DE UREASES DE CANAVALIA ENSIFORMIS**, 2009. (Seminário,Apresentação de Trabalho)

2. DEFFERRARI, M. S., MARCELINO, T. B., CARLINI, C. R. **DIGESTIVE PEPTIDASES PROFILE CHARACTERIZATION OF ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE): ROLE ON PROCESSING OF CANAVALIA ENSIFORMIS UREASE**, 2009. (Outra,Apresentação de Trabalho)

3. DEFFERRARI, M. S., TEBRUGGE, V. A., Orchard, I, CARLINI, C. R. **TOXIC EFFECTS OF CANAVALIA ENSIFORMIS UREASE ON ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAIDAE)**, 2009. (Congresso,Apresentação de Trabalho)

4. DEFFERRARI, M. S., CARLINI, C. R. **CHARACTERIZATION OF DIGESTIVE PEPTIDASES FROM ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE): ROLE ON PROCESSING OF CANAVALIA ENSIFORMIS UREASE**, 2008. (Congresso,Apresentação de Trabalho)

5. DEFFERRARI, M. S., SALVADORI, J. M., CARLINI, C. R. **CARACTERIZAÇÃO E PURIFICAÇÃO DE PEPTIDASES DIGESTIVAS DE ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE).**, 2007. (Seminário,Apresentação de Trabalho)

6. DEFFERRARI, M. S., SALVADORI, J. M., CARLINI, C. R. **CARACTERIZAÇÃO E PURIFICAÇÃO DE PEPTIDASES DIGESTIVAS DE ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE): PAPEL NO PROCESSAMENTO DA UREASE DE CANAVALIA ENSIFORMIS.**, 2007. (Congresso,Apresentação de Trabalho)

7. DEFFERRARI, M. S., SALVADORI, J. M., CARLINI, C. R. **IDENTIFICAÇÃO, CARACTERIZAÇÃO E PURIFICAÇÃO PARCIAL DE PEPTIDASES DIGESTIVAS DE ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE)**, 2007. (Congresso,Apresentação de Trabalho)

8. STRAPASSON, A. C. P., PEREIRA, L.O., NABINGER, P. M., SIQUEIRA, I. R., DEFFERRARI, M. S., NETTO, C. A. **EFEITOS DA HIPÓXIA-ISQUEMIA CEREBRAL NEONATAL E DO ENRIQUECIMENTO AMBIENTAL SOBRE O ESTRESSE OXIDATIVO NO HIPOCAMPO DE RATOS.**, 2006. (Seminário,Apresentação de Trabalho)

9. DEFFERRARI, M. S., SILVA, F. B., WALDOW, V. A., BAZ, F. C., BONATTO, S. L. **FILOGENIA MOLECULAR DE PLANÁRIAS TERRESTRES NEOTROPICAIS (PLATYHELMINTHES, TRICLADIDA, TERRICOLA, GEOPLANIDAE) DA SUBFAMÍLIA GEOPLANINAE UTILIZANDO-SE O GENE RIBOSSOMAL 18S RDNA E O GENE MITOCONDRIAL CITOCROMO OXIDASE I**, 2006. (Seminário,Apresentação de Trabalho)

10. DEFFERRARI, M. S., WALDOW, V. A., BAZ, F. C., BONATTO, S. L. **ANÁLISE FILOGENÉTICA MOLECULAR DE PLANÁRIAS TERRESTRES NEOTROPICAIS (PLATYHELMINTHES, TRICLADIDA, TERRICOLA, GEOPLANIDAE) DA SUBFAMÍLIA GEOPLANINAE UTILIZANDO O GENE RIBOSSOMAL 18S RDNA**, 2005. (Congresso,Apresentação de Trabalho)

Eventos

Participação em eventos

1. Apresentação de Poster / Painel no(a) **XXI Salão de Iniciação Científica**, 2009. (Seminário)
CARACTERIZAÇÃO DE PEPTIDASES DIGESTIVAS DE ADULTOS DE ONCOPELTUS FASCIATUS (HEMIPTERA:LYGAEIDAE) E SEU PAPEL NA HIDRÓLISE DE UREASES DE CANAVALIA ENSIFORMIS.

2. Apresentação de Poster / Painel no(a) **XI Encontro Anual do Programa de Pós-Graduação em Biologia Celular e Molecular**, 2009. (Encontro)
DIGESTIVE PEPTIDASES PROFILE CHARACTERIZATION OF ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE): ROLE ON PROCESSING OF CANAVALIA ENSIFORMIS UREASE.

3. Apresentação de Poster / Painel no(a) **XVI World Congress of the International Society on Toxinology e X Congresso da Sociedade Brasileira de Toxinologia**, 2009. (Congresso)
TOXIC EFFECTS OF CANAVALIA ENSIFORMIS UREASE ON ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAIDAE).

4. Apresentação de Poster / Painel no(a) **XXXVII Reunião Anual da SBBq**, 2008. (Congresso)
CHARACTERIZATION OF DIGESTIVE PEPTIDASES FROM ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE): ROLE ON PROCESSING OF CANAVALIA ENSIFORMIS UREASE.

5. Apresentação Oral no(a) **XIX Salão de Iniciação Científica**, 2007. (Seminário)
CARACTERIZAÇÃO E PURIFICAÇÃO DE PEPTIDASES DIGESTIVAS DE ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE).

6. Apresentação de Poster / Painel no(a) **XXII Reunião Anual da FeSBE**, 2007. (Congresso)
CARACTERIZAÇÃO E PURIFICAÇÃO DE PEPTIDASES DIGESTIVAS DE ONCOPELTUS FASCIATUS (HEMIPTERA: LYGAEIDAE): PAPEL NO PROCESSAMENTO DA UREASE DE CANAVALIA ENSIFORMIS..

7. Apresentação de Poster / Painel no(a) **IX Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular**, 2007. (Congresso)
Identificação, caracterização e purificação parcial de peptidases digestivas de Oncopeltus fasciatus (Hemiptera: Lygaeidae).

8. Apresentação Oral no(a) **VXIII Salão de Iniciação Científica**, 2006. (Outra)
FILOGENIA MOLECULAR DE PLANÁRIAS TERRESTRES NEOTROPICAIS (PLATYHELMINTHES, TRICLADIDA, TERRICOLA, GEOPLANIDAE) DA SUBFAMÍLIA GEOPLANINAE UTILIZANDO-SE O GENE RIBOSSOMAL 18S RDNA E O GENE MITOCONDRIAL CITOCROMO OXIDASE I.

9. Apresentação de Poster / Painel no(a) **Congresso Brasileiro de Genética**, 2005. (Congresso)
ANÁLISE FILOGENÉTICA MOLECULAR DE PLANÁRIAS TERRESTRES NEOTROPICAIS (PLATYHELMINTHES, TRICLADIDA, TERRICOLA, GEOPLANIDAE) DA SUBFAMÍLIA GEOPLANINAE UTILIZANDO O GENE RIBOSSOMAL 18S RDNA.

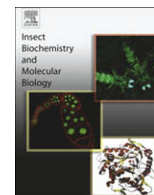
ANEXO II

Insect Biochemistry and Molecular Biology 38 (2008) 1023–1032

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb



Stage-specific gut proteinases of the cotton stainer bug *Dysdercus peruvianus*: Role in the release of entomotoxic peptides from *Canavalia ensiformis* urease

Angela R. Piovesan [a,1], Fernanda Stanisçuaski [b,*,1], Juliana Marco-Salvadori [a], Rafael Real-Guerra [a], Marina S. Defferrari [a], Célia R. Carlini [a,b]

[a] Graduate Program in Cellular and Molecular Biology, Center of Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Building 43421, CEP 91501-970 Porto Alegre, Rio Grande do Sul, Brazil

[b] Department of Biophysics and Center of Biotechnology, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Building 43422, CEP 91501-970 Porto Alegre, Rio Grande do Sul, Brazil

ARTICLE INFO

Article history:
Received 12 May 2008
Received in revised form 24 August 2008
Accepted 11 September 2008

Keywords:
Dysdercus peruvianus
Urease
Proteolytic enzyme
Insecticidal peptide
Enzyme inhibitor

ABSTRACT

Canavalia ensiformis ureases are toxic to insects of different orders. The entomotoxicity of urease is due to a 10 kDa internal peptide released by proteinases in the insect digestive tract. We previously observed that, given orally, urease is toxic to nymphs of *Dysdercus peruvianus*, but does not affect adults. Here we characterized the major proteolytic activities of *D. peruvianus* midgut homogenates and investigated their *in vitro*-catalyzed release of the 10 kDa entomotoxic peptide from urease. Cysteine, aspartic and metalloproteinases are present in both homogenates. Variations in optimal pH and susceptibility to inhibitors indicated differences in the enzyme profiles in the two developmental stages. Only nymph homogenates released ~10 kDa fragment(s) from urease, recognized by antibodies against the entomotoxic peptide. Fluorogenic substrates containing urease partial sequences flanking the N-terminal or the C-terminal portion of the entomotoxic peptide were efficiently cleaved by homogenates from nymphs, but much more slowly by the adult homogenate. Different classes of enzymes in the homogenates cleaved both substrates suggesting that *in vivo* the release of the entomotoxic peptide results from the concerted action of at least two different proteinases. Our findings support the view that a differential processing of ingested urease by the insects explains at least in part the lack of toxicity in adults.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Ureases (urea amidohydrolase; EC 3.5.1.5), nickel-dependent enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide, are synthesized by plants, fungi and bacteria (Mobley et al., 1995; Follmer, 2008). In plants, ureases are homotrimers or hexamers of a ~90 kDa subunit and participate in the use of urea as nitrogen source (Polacco and Holland, 1993; Sirko and Brodzik, 2000; Follmer, 2008). Canatoxin, a toxic protein isolated from *Canavalia ensiformis* seeds (Carlini and Guimarães, 1981) and more recently identified as a minor isoform of urease (Follmer et al., 2001), displays insecticidal properties (Carlini et al., 1997). Canatoxin and the major isoform of urease of *C. ensiformis* seeds (herein designated "urease") are toxic to *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae), and this toxicity is independent of their ureolytic activity (Follmer et al., 2004a; Stanisçuaski et al., 2005; Follmer et al., 2004b).

Insects with cathepsin-like digestive enzymes are susceptible to the toxic effects of urease, while insects with trypsin-like digestive enzymes are not (Carlini et al., 1997), possibly due to differences in the proteolytic processing of ureases in the insects. The entomotoxic activity is due to a 10 kDa internal peptide released from ureases by insect digestive enzymes. This peptide, called pepcanatox, was isolated and characterized (Ferreira-DaSilva et al., 2000). A recombinant peptide, Jaburetox-2Ec equivalent to pepcanatox, obtained by heterologous expression in *Escherichia coli* (Mulinari et al., 2004, 2007), is highly insecticidal and did not affect mice or neonate rats when administered by oral or intraperitoneal routes (Mulinari et al., 2007). Interestingly, Jaburetox-2Ec kills insects that are resistant to intact ureases, such as *Spodoptera frugiperda* (Mulinari et al., 2007) or adults of *Triatoma infestans* (Tomazzeto et al., 2007).

The cotton stainer bug *D. peruvianus* feeds on cotton seeds (*Gossypium hirsutum*), staining the cotton fibers, damaging the seeds, besides being a vector for phytopathogenic microorganisms, and thus can cause heavy losses in cotton production (Gallo, 1988).

* Corresponding author. Tel.: +55 51 3308 7606; fax: +55 51 3308 7003.
E-mail address: fernanda.staniscuaski@ufrgs.br (F. Stanisçuaski).
URL: http://www.ufrgs.br/laprotox

[1] These authors have contributed equally to this work.

0965-1748/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2008.09.004

Nymphs are susceptible to canatoxin or urease, while adults are not. In a previous work, we showed that adults and nymphs have distinct repertoires of gut enzymes, and differences in the processing of ureases by stage-specific enzymes were proposed to explain the lack of susceptibility of adults (Stanisçuaski et al., 2005).

Digestive proteinases play two critical roles in insect physiology: breaking down proteins into amino acids essential for growth and development, and inactivating protein toxins ingested as a consequence of feeding (Terra et al., 1996). Endoproteinases belong to one of six groups based on the active site amino acid residue, or metal ion, involved in peptide bond hydrolysis (Barret et al., 1998): serine, cysteine, aspartic acid, glutamic acid, threonine, and metalloproteases. Insects having midguts with pH in the neutral or alkaline range usually rely on serine proteinases. In contrast, insects having acidic or mildly acidic midguts generally utilize cysteine and aspartic proteinases (Terra et al., 1996; Terra and Ferreira, 1994; Silva et al., 1996; Wilhite et al., 2000).

In this study, we aimed to investigate further the processing of ureases by nymphs and adults of *D. peruvianus*. Because the release of the entomotoxic peptide could be due to the concerted or sequential action of more than one enzyme, we decided to study the enzymatic profile of whole midguts' homogenates. We characterized the enzymatic activities present in these homogenates and performed *in vitro* hydrolysis of urease with gut homogenates. We show that homogenates from nymphs and adults generate different fragments from urease and that a 10 kDa peptide recognized by anti-Jaburetox-2Ec antibodies is formed only by nymph homogenates.

2. Materials and methods

2.1. Reagents

Azocasein, *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64), pepstatin-A, phenylmethanesulphonylfluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), methylcoumarin amide (MCA)-coupled substrates, anti-rabbit IgG conjugated with alkaline phosphatase, nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma Chemicals Company (Saint Louis, USA). Hybond-P membrane (PVDF, 0.45 μm) was from Amersham Biosciences (Little Chalfont, England). Molecular mass markers were from Invitrogen Life Technologies (California, USA). The synthetic peptide substrates coupled to Abz-EDDnp were a kind gift from Dr. Luis Juliano Neto and Dr. Maria Aparecida Juliano (Dept. Biophysics, Universidade Federal de São Paulo, São Paulo, Brazil).

2.2. Insects

A colony of *D. peruvianus*, established and operated in our laboratory for five years, is maintained as described (Stanisçuaski et al., 2005). The insects develop from eggs through five nymphal stages in about 20–25 days. The insects were fed with cotton seeds (*G. hirsutum*) and had free access to water.

2.3. Urease and Jaburetox-2Ec

C. ensiformis major urease was purified as previously described (Follmer et al., 2001, 2004b). Briefly, seed meal (100 g) was extracted with buffer A (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 2 mM 2-mercaptoetanol) for 1 h at 4 °C. The meal was removed by centrifugation and 28% (v/v) ice-cold acetone (final concentration) was added to the supernatant. After removing the precipitate, the concentration of acetone in the supernatant was increased to 31.6% (v/v). The new precipitate was discarded, the supernatant was dialyzed against buffer B (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 5 mM 2-mercaptoetanol) and then mixed with 40 mL of Q-Sepharose in buffer B. The urease-enriched fraction was eluted by adding 300 mM NaCl in buffer B. This material was concentrated and gel-filtered on a Superose 6 HR 10/30 column in buffer B. The active peak was dialyzed against 20 mM sodium phosphate, pH 7.0, 0.5 M NaCl (buffer C) and submitted to immobilized metal affinity chromatography using 10 mL of Co^{2+} loaded iminodiacetic acid–Sepharose (IDA–Co^{2+}), equilibrated in buffer C. This step separates the isoforms of jack bean urease, the major urease being recovered in the non-retained fraction (Follmer et al., 2004b).

The recombinant urease-derived entomotoxic peptide Jaburetox-2Ec, produced and purified as described in Mulinari et al. (2007), was used to immunize rabbits and prepare anti-Jaburetox-2Ec polyclonal antibodies (Tomazzeto et al., 2007).

2.4. Bioassay of urease insecticidal activity

The bioassay was carried out essentially as described in Stanisçuaski et al. (2005). Third instars or adults of *D. peruvianus* were fed on artificial cotton seeds, consisting of gelatin capsules (size 2, Elli Lilly Co.) containing cotton seed flour and 0.01–0.04% (w/w) freeze-dried urease. Survival rates were recorded daily until day 16.

2.5. Gut homogenates

Homogenates of whole intestines of *D. peruvianus* were used for determination of proteolytic activities. For that, non-starved fourth instars or adult males were anaesthetized in ice and the whole intestines were removed and stored at −20 °C in 50% v/v glycerol in water, in a proportion of 20 intestines per milliliter. To prepare the homogenates, the material was thawed, homogenized manually with an ice-cold Potter pestle, centrifuged at 4 °C at 4000*g* (twice, 10 min each) and then at 12,000*g* for 5 min. The final supernatants were kept frozen at −20 °C until used. Homogenates of nymphs' guts and adults' guts are referred from now on as NH and AH, respectively.

2.6. Enzymatic assays

For determination of azocaseinolytic activity, the homogenates (2.5 mg protein/mL) were incubated with azocasein (final concentration 0.5%) in sodium acetate 0.05 M, pH 5.6, at 37 °C, in 300 μL, with or without 1 mM PMSF. After 1 h, 250 μL of 10% trichloroacetic acid (TCA) was added, followed by centrifugation (5 min, 18,000g). The supernatant (450 μL) was mixed with 250 μL of NaOH (2 M) and read at 420 nm. One unit of azocaseinolytic activity was defined as the amount of enzyme releasing 1.0 A_{420} of acid-soluble azopeptides per hour at 37 °C.

Different fluorogenic synthetic substrates were employed to characterize the enzymatic activities in the homogenates. Aliquots of the homogenates were incubated in microplates at 37 °C, with 20 μM of substrates Abz-AIAFFSRQ-EDDnp or N-Cbz-Phe-Arg-MCA, in 100 μL final volume. Synthetic substrates corresponding to the N- and C-terminal regions flanking the entomotoxic peptide within urease sequence (Mulinari et al., 2007) were also tested, at 20 μM final concentration. The substrates were Abz-NAIADGPVQ-EDDnp (equivalent to the N-terminal region) and Abz-KVIRDGMGQ-EDDnp (equivalent to the C-terminal region). The pH was varied using 50 mM citrate–phosphate buffer (pH 2.0–6.0) or Tris–HCl (pH 7.0–9.0). Reactions were monitored for 1 h in an F-Max fluorometer (Molecular Devices Inc.), using 320 nm excitation – 420 nm emission filters for EDDnp substrates and 370 nm excitation – 460 nm emission filters for the MCA substrate. Results are shown as relative fluorescence in milliunits (mRFU) per min per mg of homogenate.

To investigate the classes of the proteinases present in the homogenates, aliquots were pre-incubated 30 min at room temperature with either 10 μM E-64, 10 μM pepstatin-A, 1 mM EDTA or 1 mM PMSF, and then tested for proteolytic activity (Barret et al., 1998). Results are shown as percentage of the activity in the absence of the inhibitor.

2.7. In vitro hydrolysis of urease

Digestion of urease with *D. peruvianus* proteinases was performed as described in Ferreira-DaSilva et al. (2000) using a ratio of 0.5 mU azocaseinolytic activity of homogenates per microgram urease, in 5 mM ammonium formate, pH 5.6, at 37 °C, under continuous stirring. The reaction was stopped by freeze-drying the samples.

2.8. Western blot

The hydrolysis of urease was followed by Western blot (Towbin et al., 1979) after SDS-PAGE (Weber and Osborn, 1969) in 10–18% polyacrylamide gels. After being transferred to 0.2 μm PVDF filters, the membranes were incubated for 2 h with anti-Jaburetox-2Ec antibodies (1:25,000) and then exposed for 2 h to anti-rabbit IgG coupled to alkaline phosphatase (1:30,000). The color reaction was developed with NBT and BCIP in buffer containing $MgCl_2$, pH 9.6.

2.9. Determination of cleavage site by LC–ESI-MS

The synthetic substrates (2 nmol) were incubated with the homogenates (100 μg protein) at pH 4.0, 37 °C, overnight. The reaction was stopped by sample lyophilization. Digestion analysis was performed on a Q-TOF Micro instrument (Waters Corporation) with a nanoflow electrospray probe, capillary voltage at 3500 V and a cone voltage of 30 V, calibrated in the m/z region 50–2000 using phosphoric acid. A lock spray interface with phosphoric acid was employed, the lock mass data being averaged over 5 scans for correction. Chromatographic separations for LC–MS were performed in a Nano Acquity Ultra Performance LC (Waters Corporation), equipped with columns Symmetry C18 and BEH 130-C18 (Waters Corporation), and a linear gradient of 0–90% acetonitrile in 0.1% formic acid. The mass spectrometric data were collected in a full scan mode from m/z 50 to 1400 in positive mode. Data were processed and analyzed using Mass Lynx software.

2.10. Statistical analysis

Results are expressed as mean ± standard deviation. Significance of differences between means was determined using one-way ANOVA. Data were considered statistically different when $p < 0.05$.

3. Results

3.1. Urease insecticidal effect

Fig. 1 shows the insecticidal effect of *C. ensiformis* urease fed to third instar *D. peruvianus* reaching 100% of mortality after 11 days. In contrast, adults survived 14 days ingesting a concentration of urease 4-fold higher. The adult insects, however, were as susceptible to sodium azide as the nymphs. These results are very similar to our previous observation for canatoxin, a minor isoform of urease present in the same seed (Stanisçuaski et al., 2005).

3.2. Enzymatic activity upon azocasein

Azocasein is a nonspecific protease substrate. Both homogenates have azocaseinolytic activity at pH 5.6. This activity is strongly inhibited by PMSF (a serine protease inhibitor) in adult's homogenate (AH) (~65% inhibition). In contrast, no inhibition by PMSF of nymph's homogenate (NH) activity was observed (Fig. 2).

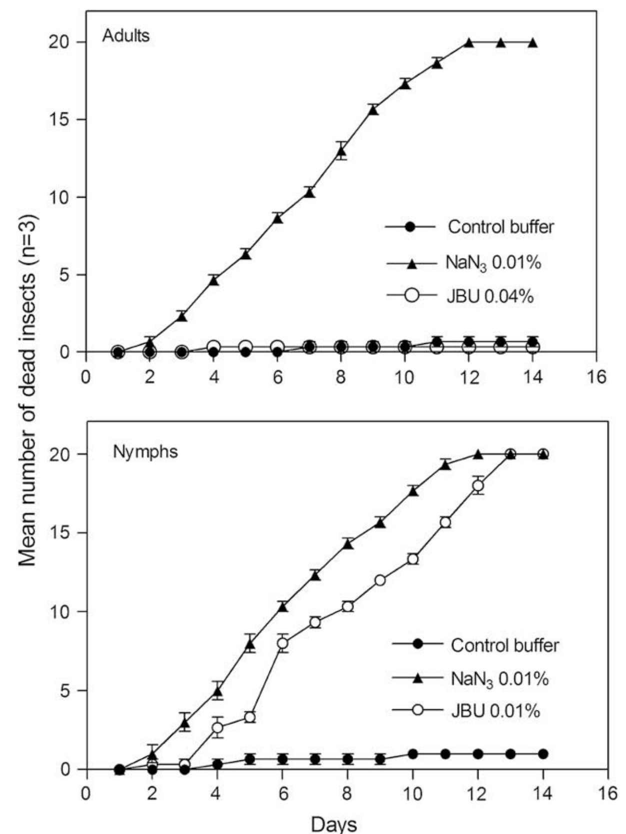


Fig. 1. Insecticidal effect of urease on *Dysdercus peruvianus*. Adults (upper panel) or nymphs (third instar) (lower panel) fed on artificial seeds containing urease, buffer alone (controls) or 0.01% w/v sodium azide. Surviving insects were counted daily up to 16 days. Results are expressed as mean and standard deviations of triplicated points ($N = 20$ per point).

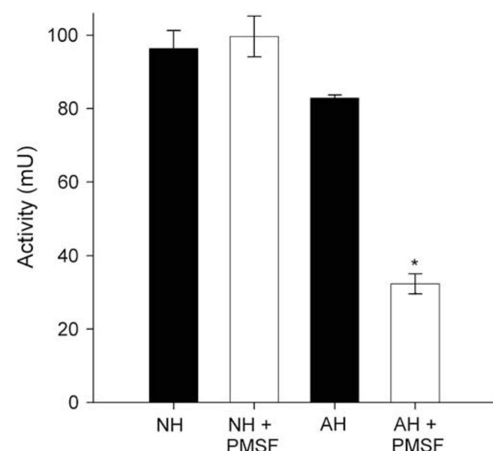


Fig. 2. Hydrolysis of azocasein by NH and AH and effect of PMSF. The homogenates (2.5 mg protein/mL) were incubated with 0.5% w/v azocasein at 37 °C, pH 5.6. For the PMSF treatment, homogenates were pre-incubated with the inhibitor (1 mM) for 30 min before the addition of the substrate. The release of soluble azopeptides derived from azocasein was quantified colorimetrically. Activity is expressed as mU (described in Section 2). Values are means and standard deviation ($N = 4$). * Indicates a statistically significant difference between the control and the PMSF treatment ($p < 0.05$).

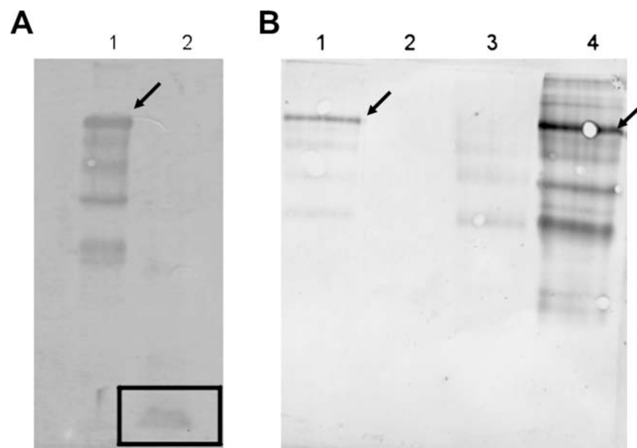


Fig. 3. Western blot of urease in vitro hydrolysis developed with anti-jaburetox antibodies. (A) Urease hydrolysis by NH. Lane 1: urease incubated at 37 °C, pH 5.6 in the absence of NH; lane 2: urease incubated at 37 °C, pH 5.6 in the presence of NH. (B) Urease hydrolysis by AH. Lane 1: urease incubated at 37 °C, pH 5.6 in the absence of AH; lane 2: AH; lane 3: urease incubated at 37 °C, pH 5.6 in the presence of AH. Lane 4: urease incubated at 37 °C, pH 5.6 in the presence of AH and PMSF (1°mM). The arrows indicate intact urease. The square highlights the ~10 kDa fragment recognized by anti-jaburetox antibodies.

3.3. *In vitro hydrolysis of urease*

Because the release of the entomotoxic peptide could be due to the concerted or sequential action of more than one enzyme, we decided to analyze homogenates of whole midguts. The fragmentation patterns of urease produced by NH and AH were clearly distinct. After 24 h of incubation at 37 °C, pH 5.6, urease was extensively hydrolyzed by NH, and formed a ~10 kDa fragment, recognized by anti-Jaburetox-2Ec antibodies (Fig. 3A). By size and antigenicity criteria this peptide is equivalent to the entomotoxic peptide characterized by Ferreira-DaSilva et al. (2000). Under the same reactions conditions, AH also hydrolyzed urease, but no 10 kDa fragment recognizable by anti-Jaburetox-2Ec antibodies was formed (Fig. 3B, lane 3). When the hydrolysis was performed in the presence of PMSF (1 mM), the fragmentation of urease by AH was reduced (Fig. 3B, lane 4), but no inhibition was observed in hydrolysis produced by NH (data not shown).

3.4. *Enzymatic activity upon synthetic substrates*

Four synthetic substrates were used to characterize the proteinases present in the homogenates (data summarized in Table 1). Abz-AIAFFSRQ-EDDnp was developed for cathepsin-D like aspartic proteinases (Sorgine et al., 2000). Both homogenates have similar activity upon this substrate, but each with a different pH optimum. For NH (Fig. 4, left panels), the optimal pH was 3.0, while AH (Fig. 4, right panels) showed maximal activity at pH 5.0. The activity of NH was completely inhibited by pepstatin-A between pH 2.0 and 6.0 (Fig. 4B). On the other hand, inhibition of AH activity by pepstatin-A was less significant, with a residual activity of 36% at pH 5.0 (Fig. 4F). Although both adult and nymph digestive tracts have aspartic proteinases, the differential susceptibility to inhibitors and the different pH optima suggest that different members of this proteinase family are present according to insect stage. EDTA was also effective in inhibiting AH activity upon this substrate, mainly at pH 6.0 (Fig. 4H), but no inhibition was observed for NH (Fig. 4D).

N-Cbz-Phe-Arg-MCA is a substrate suitable for the cysteine proteases cathepsins B and L (Sorgine et al., 2000; Renard et al., 2000). NH showed two distinct peaks of activity with this substrate (Fig. 5, left panels), one at pH 4.0 and a second at pH 6.0. AH activity was relatively equally distributed over pH 3.0–6.0 (Fig. 5, right panels). The activity of NH upon this substrate was about 2.5-fold higher than AH at pH 4.0 and 2-fold at pH 6.0 (Table 1). E-64 completely abolished the proteolytic activity of both homogenates in the pH 3.0–4.0 range, inhibiting to a lower extent the activity at pH 5.0–7.0 (Fig. 5C and F). Again, the data suggest the presence of different members of the cysteine protease family according to stage of *D. peruvianus*.

We assayed NH and AH protease action on synthetic substrates that correspond to the N- and C-terminal regions flanking the entomotoxic peptide within urease to gain further insights into gut processing of urease. NH has a higher activity upon the substrate Abz-NAIADGPVQ-EDDnp (equivalent to the N-terminal region), with an optimal pH at 4.0. For AH, the pH optimum is 3.0 (Fig. 6A). While AH activity upon this substrate is inhibited by E-64 (Fig. 6B), the activity of NH is not (Fig. 6E). Pepstatin-A failed to inhibit either AH or NH activities (Fig. 6C and F), while EDTA was a strong inhibitor of both (Fig. 6D and G).

Both homogenates showed similar activities upon the C-terminal substrate, Abz-KVIRDGMGQ-EDDnp, with optimal pH at 4.0–4.5 (Fig. 7A). As observed for the N-terminal substrate, inhibition by E-64 was higher for AH than NH activity (Fig. 7E and B). At pH 4.0, E-64 inhibited about 50% of NH activity and almost 100% AH activity. Pepstatin-A had no effect (Fig. 7C and F) on either of the gut homogenates. Contrasting with the results with the N-terminal substrate, NH and AH activities were not significantly inhibited by EDTA (Fig. 7D and G).

Table 1
Major proteolytic activities of midgut homogenates of nymphs (third instars) or adult *Dysdercus peruvianus*.

Substrate	Nymph's midgut homogenates			Adult's midgut homogenates		
	Optimal pH	mU/min/mg or A_{280}/3 h/mg[a]	Inhibitor(s)[b]	Optimal pH	mU/min/mg or A_{280}/3 h/mg[a]	Inhibitor(s)[b]
Azocasein, pH 5.6	n.d.	95.0	Not inhibited by PMSF	n.d	82.0	PMSF
Hemoglobin**	3.5	0.26	E-64, EDTA, pepstatin-A	5.0	0.20	Pepstatin-A
				8.5	0.11	PMSF
Abz-AIAFFSRQ-EDDnp (substrate designed for cathepsin-D)	3.0	820	Pepstatin-A	3.0	400	No inhibition
				5.0	800	PMSF, EDTA, Pepstatin-A
N-Cbz-Phe-Arg-MCA (substrate designed for cathepsin-B)	4.0	3700	E-64	3–5	1600	E-64
	6.0	2100	E-64			
Abz-NAIADGPVQ-EDDnp (N-terminal flanking sequence)	4.5	22,000	EDTA	3.0	9000	E-64, EDTA
Abz-KVIRDGMGQ-EDDnp (C-terminal flanking sequence)	4.5	25,000	E-64	4.0	30,000	E-64

n.d. – Not determined.
[a] Hemoglobinolytic activity was measured as A_{280}/3 h/mg (**data taken from Stanisçuaski et al. (2005)).
[b] Inhibition greater than 40–50%. Inhibitors tested were 10 μM pepstatin-A, 10 μM E-64, 1 mM PMSF and 1 mM EDTA.

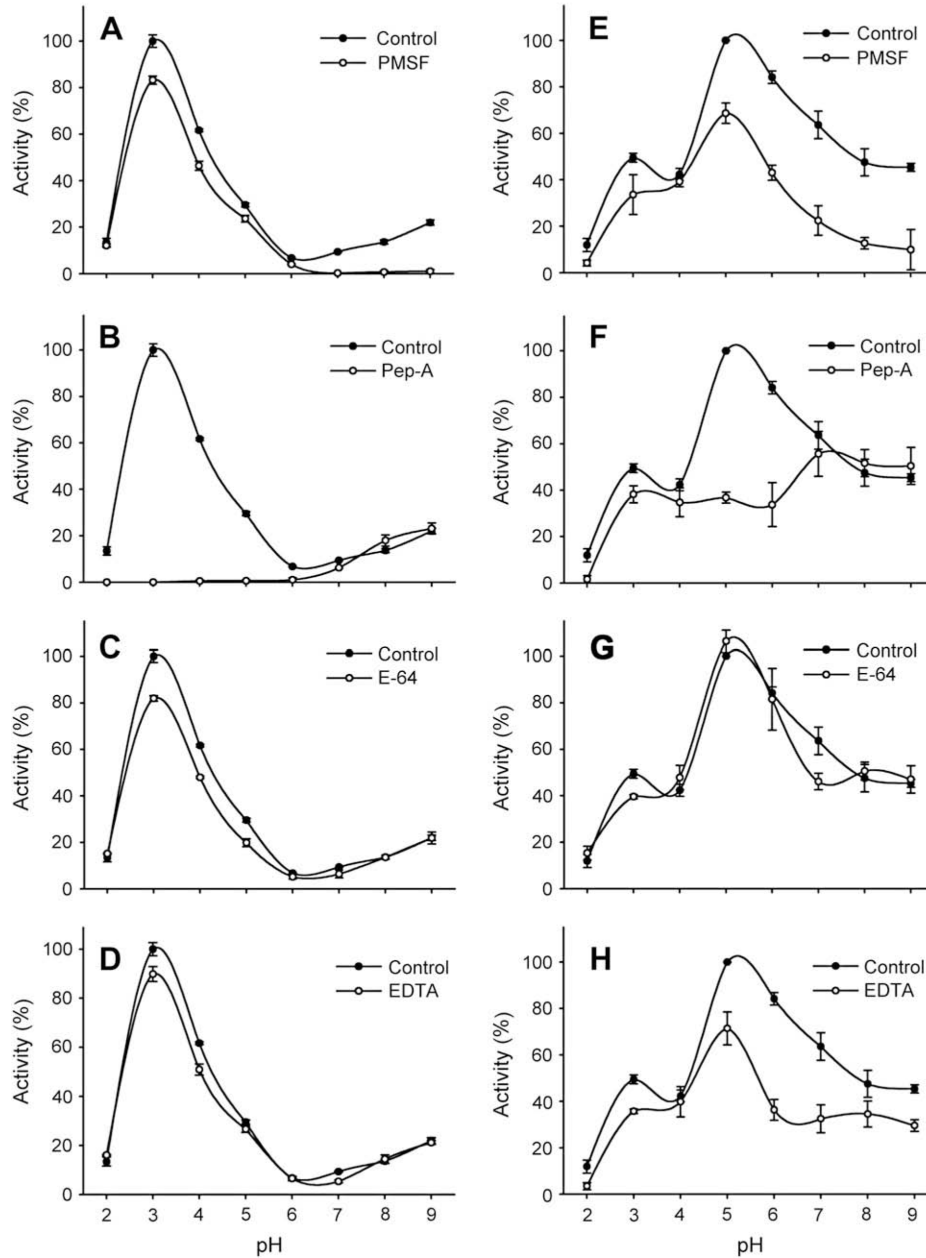


Fig. 4. Effect of inhibitors on Abz-AIAFFSRQ-EDDnp hydrolysis by NH (A–D) and AH (E–H). Final concentration of the substrate was 20 μM. Activity was assayed at 37 °C for 1 h in the presence of 1 mM PMSF (A, E), 10 μM pepstatin-A (B, F), 10 μM E-64 (C, G) or 1 mM EDTA (D, H). Results are expressed as mean and standard deviation ($N = 4$).

3.5. Determination of cleavage site by LC–ESI-MS

The cleavage site(s) of Abz-NAIADGPVQ-EDDnp and Abz-KVIRDGMGQ-EDDnp by the homogenates were determined by LC–ESI-MS (Table 2). For the hydrolysis of the N-terminal substrate Abz-NAIADGPVQ-EDDnp at pH 4.0 by either of the homogenates, the fragment DGPVQ-EDDnp (*m*/*z* 723,302) was the most abundant. The fragment GPVQ-EDDnp was also present, but less abundant. These results indicate that both homogenates preferentially cleave between Ala and Asp residues in this substrate, but they are also effective in cleaving the bond between Asp and Gly residues. When the hydrolysis was performed at pH 5.6, which resembles the condition of the midgut of *D. peruvianus*, the preferred cleavage site in Abz-NAIADGPVQ-EDDnp was Asp–Gly for both homogenates (data not shown). In the case of the hydrolysis of the C-terminal substrate Abz-KVIRDGMGQ-EDDnp by the homogenates at pH 4.0, the most abundant fragment was DGMGQ-EDDnp (*m*/*z* 317,703), indicating that AH and NH preferentially cleave between Arg and Asp residues. For the hydrolysis with AH, a second fragment, Abz-KVIRD (*m*/*z* 374,204), was observed with a lower intensity. This indicates that AH, but not NH, also cleaves the peptide bond between Asp and Gly residues in this substrate. At pH 5.6, most of Abz-KVIRDGMGQ-EDDnp was cleaved between the Ile and Arg residues by both homogenates (data not shown).

4. Discussion

The primary aim of this work was to investigate the major digestive proteolytic activities of nymphs and adults of *D. peruvianus* and to establish a possible relation between these activities and the stage-dependent resistance to toxic effects of ingested ureases. Because the release of the entomotoxic peptide could be

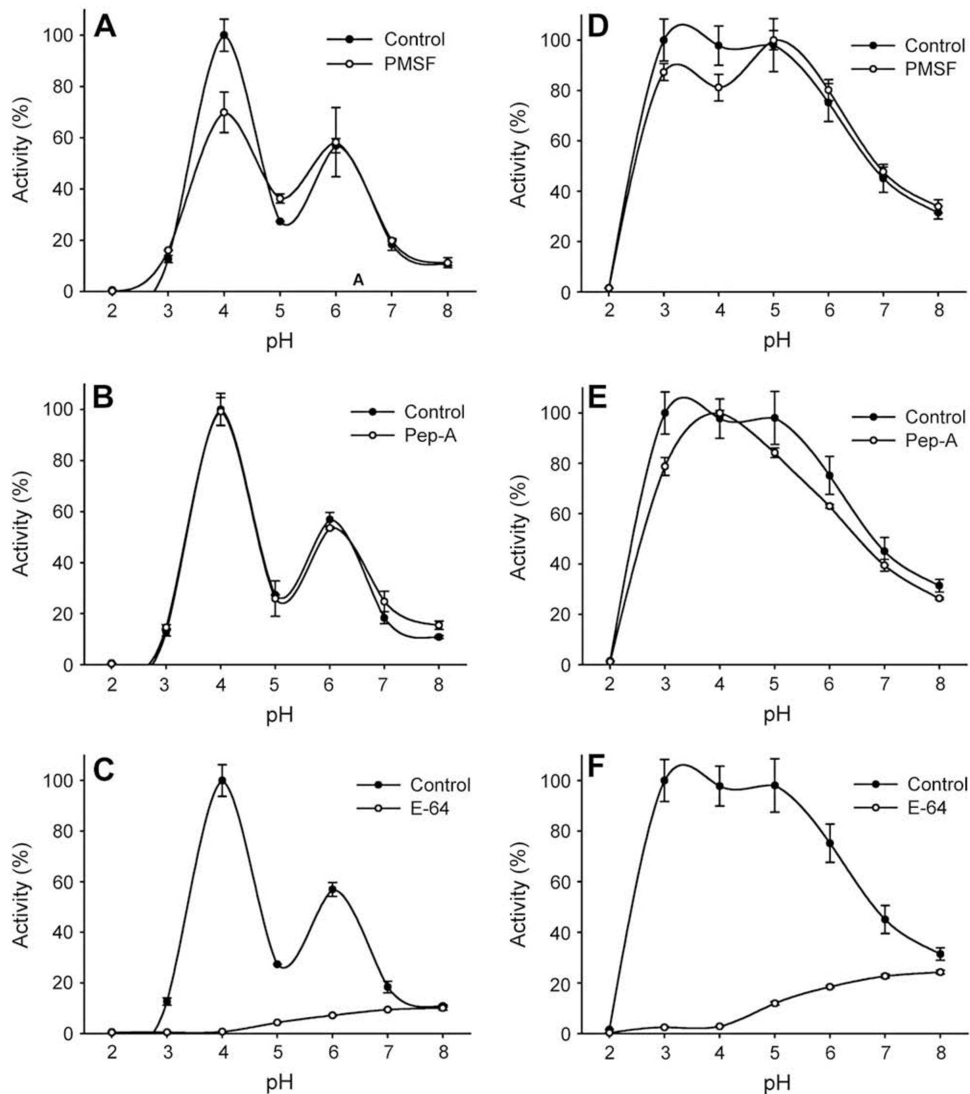


Fig. 5. Effect of inhibitors on N-Cbz-Phe-Arg-MCA hydrolysis by NH (A–C) and AH (D–F). Final concentration of the substrate was 20 μM. Activity was assayed at 37 °C for 1 h in the presence of 1 mM PMSF (A, D), 10 μM pepstatin-A (B, E) or 1 mM EDTA (C, F). Results are expressed as mean and standard deviation ($N = 4$).

due to the concerted or sequential action of more than one enzyme, we decided to work with homogenates of whole midguts.

Ferreira-DaSilva et al. (2000) confirmed the proteolytic activation of canatoxin by enzymes from *Callosobruchus maculatus* larvae, and the release of a toxic peptide of 10 kDa. Here, we showed that *C. ensiformis* major urease is hydrolyzed by *D. peruvianus* NH and AH, but only NH is able to release a ~10 kDa peptide, recognized by anti-Jaburetox-2Ec antibodies. Although we cannot exclude at this point differences regarding midgut permeability, receptor(s), transport across the membranes of epithelial cells, or signal-transduction, this finding strongly suggests that adults of *D. peruvianus* are resistant to urease toxic effects due to differences in the proteolytic processing of the protein. Differences in the response to toxins during the life cycle of insects have been reported. Increased resistance to *Bacillus thuringiensis* Cry1C toxin in the late larval stages of *Spodoptera littoralis* as compared to young larvae was attributed to different enzymatic processing of the toxin (Keller et al., 1996).

Silva and Terra (1994) described digestive enzymes, absorptive sites and internal pH along the midgut, divided into three sections (V1–V3), of female adults *D. peruvianus*. They showed a luminal pH variation from 6.2 to 5.6 and that a soluble cysteine proteinase with a pH 5.5 optimum and produced in the V2 region was the most important proteolytic enzyme. However, this type of information is not available for young forms of any hemipteran. It is reasonable to expect that more acidic microenvironments probably exist in which the enzymes we described here (particularly in nymphs) would approach their maximal activities.

Comparing the enzyme activities in homogenates of fed and starved *D. peruvianus*, Silva and Terra (1994) concluded that cotton meal contributes with an aminopeptidase activity found in the midgut contents. It is not known if symbionts present in hemipteran midgut contribute to the repertoire of enzymes found (Terra and Ferreira, 1994; Silva and Terra, 1994). Since in the present study we used non-starved insects, both stages fed on the same lot of cotton seeds, the differences we found in the repertoire of proteolytic enzymes are likely to be stage-specific. Thus, although there is no change in the feeding habits of young forms and adult *D. peruvianus*, our data point to important differences in the digestive physiology of the insect during its life cycle.

We showed that NH azocaseinolytic activity was not affected by the serine protease inhibitor PMSF, while AH activity was reduced almost 70% (Fig. 2), confirming similar observations in a previous study (Stanisçuaski et al., 2005). More interestingly, urease hydrolysis by AH is inhibited by PMSF, while NH is still able to fully degrade urease in the presence of this inhibitor (data not shown). The data indicate the presence of serine proteinases in adults, but

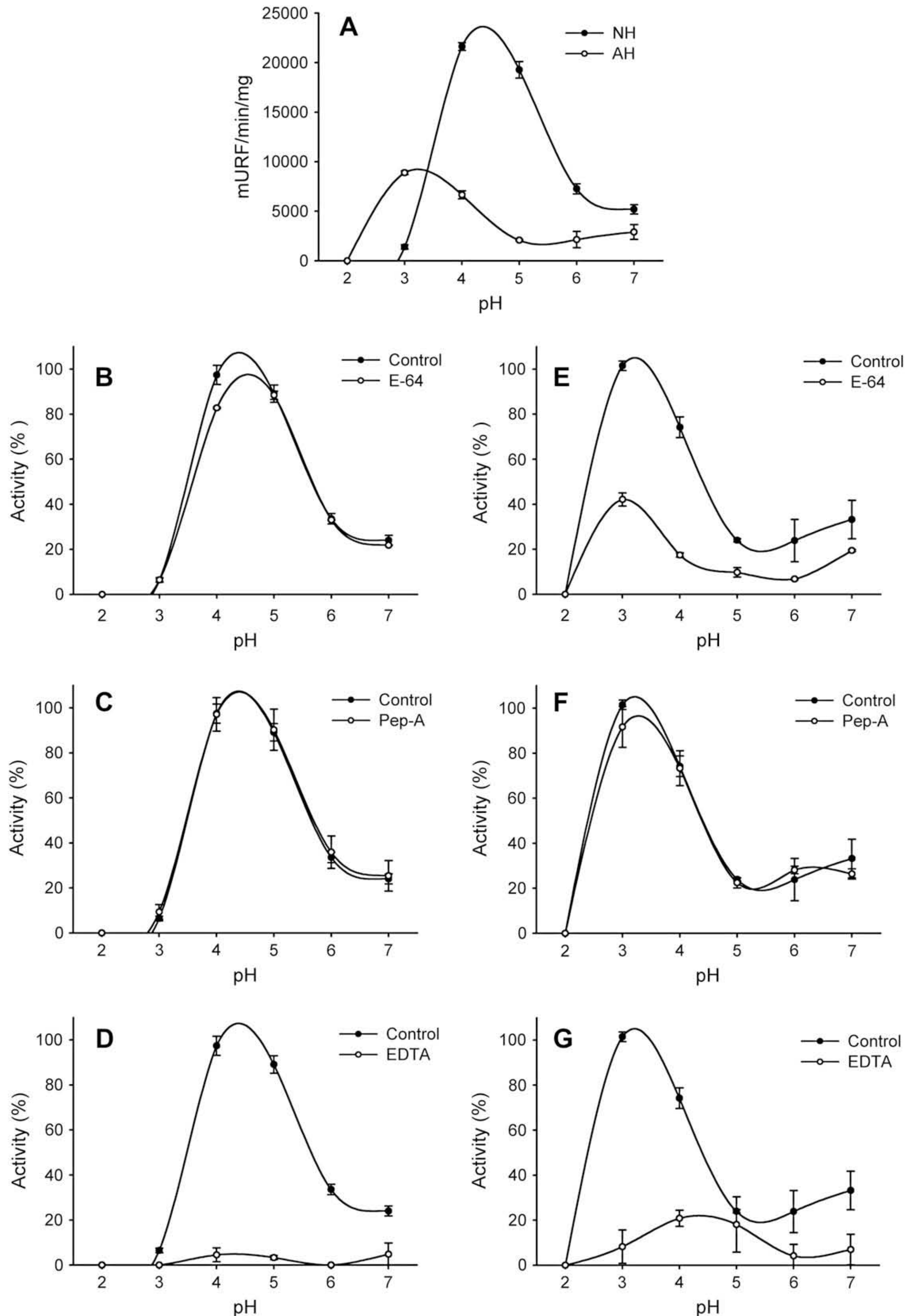


Fig. 6. Effect of pH and inhibitors on the hydrolysis of Abz-NAIADGPVQ-EDDnp (final concentration 20 μM) by NH and AH. Activity was assayed at 37 °C for 1 h in the absence (A) or presence of 10 μM E-64 (B, E), 10 μM pepstatin-A (C, F) or 1 mM EDTA (D, G). Results are expressed as specific activity (*A*) or mean ± s.d. (B–G) ($N = 4$).

not in nymphs, of *D. peruvianus*. Hemipteran insects are said to rely on cathepsin-like digestive enzymes (Terra and Ferreira, 1994; Cristofoletti et al., 2003). To our knowledge, there are no published reports on the identification of serine trypsin-like digestive enzymes in any hemipteran insect. One alternative explanation would be that PMSF is nonspecifically inhibiting cysteine proteases (Whitaker and Perez-Villasenor, 1968).

Midgut homogenates of both insect stages were able to cleave several substrates in acidic media up to pH 6.0. The activities upon N-Cbz-Phe-Arg-MCA and Abz-AIAFFSRQ-EDDnp indicate the

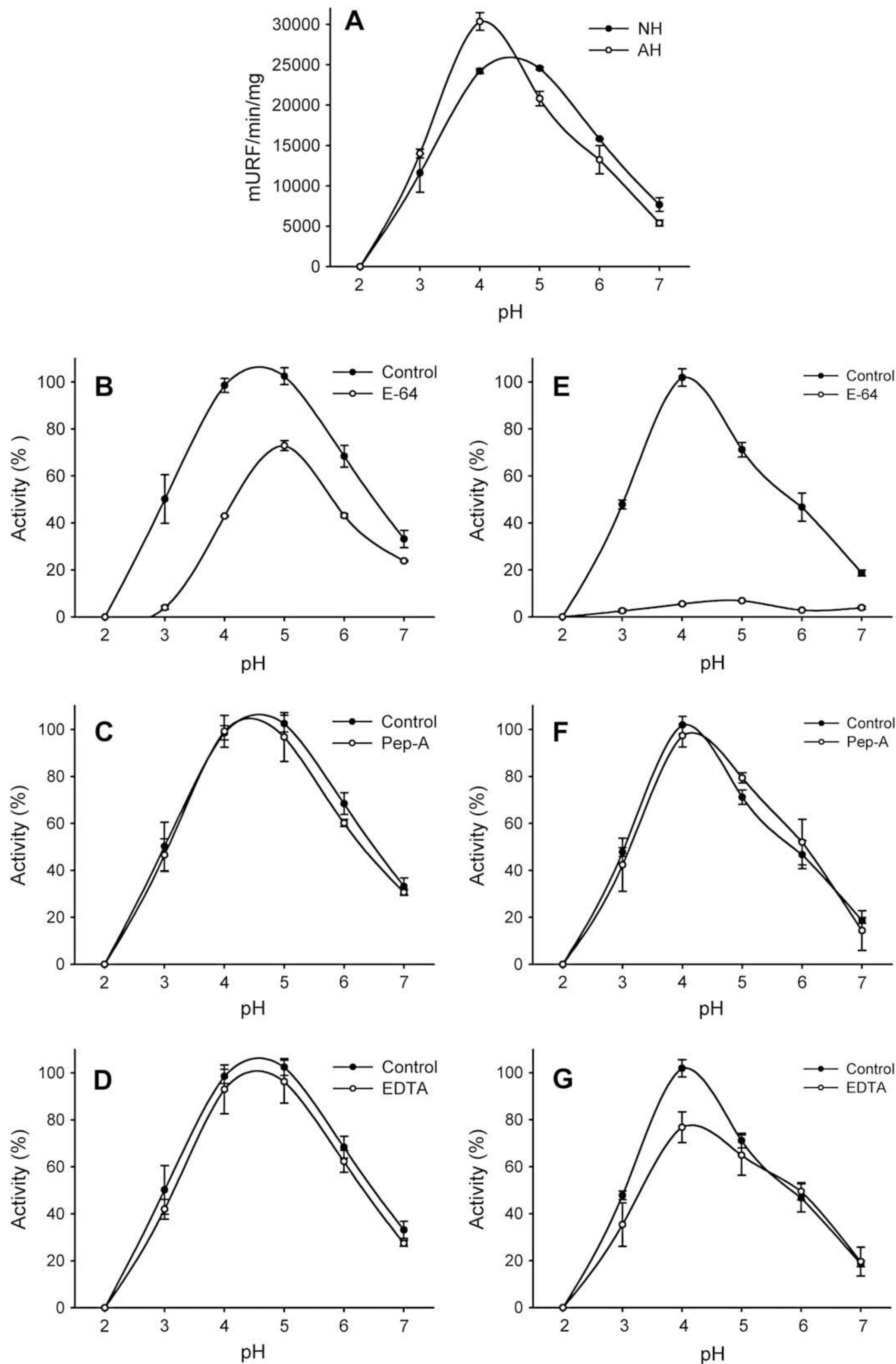


Fig. 7. Effect of pH and inhibitors on the hydrolysis of Abz-KVIRDGMGQ-EDDnp (final concentration 20 μM) by NH and AH. Activity was assayed at 37 °C for 1 h in the absence (A) or presence of 10 μM E-64 (B, E), 10 μM pepstatin-A (C, F) or 1 mM EDTA (D, G). Results are expressed as specific activity (*A*) or mean ± s.d. (B–G) ($N = 4$).

presence of cysteine and aspartic proteinases in both homogenates. NH activity upon Abz-AIAFFSRQ-EDDnp was completely inhibited by pepstatin-A (Fig. 4B), while AH was still partially active in the presence of pepstatin-A (Fig. 4F). The data suggest the presence of a different isoform of aspartic proteinase in adults, less sensitive to pepstatin-A, or alternatively, adults have another proteinase able to cleave this substrate, not susceptible to the inhibitor. Inhibition by E-64 of the cleavage of N-Cbz-Phe-Arg-MCA indicated the presence of cysteine proteinases in both AH and NH (Fig. 5), the activities differing in their pH optima and specific activities.

Table 2
Determination by LC–ESI-MS of cleavage sites of substrates by homogenates at pH 4.0.

Sequence	Mass (predicted)	Mass (observed)	
		Cleaved by AH	Cleaved by NH
N-terminal			
Abz-NAIADGPVQ-EDDnp	506,247	506,246	506,246
Abz-NAIA**DGPVQ-EDDnp**	722,295	722,294[a]	722,295[a]
Abz-NAIADGPVQ-EDDnp	621,277	621,280	621,281
Abz-NAIAD**GPVQ-EDDnp**	607,275	607,271	607,267
C-terminal			
Abz-KVIRDGMGQ-EDDnp	633,397	633,383[a]	633,391[a]
Abz-KVIR**DGMGQ-EDDnp**	714,235	714,238	n.o.
Abz-KVIRDGMGQ-EDDnp	748,417	748,409	n.o.
Abz-KVIRD**GMGQ-EDDnp**	599,215	n.o.	n.o.

The fragments observed are shown in bold. n.o. – not observed.
[a] The fragments that were more abundant in the spectrum.

Important differences were observed between NH and AH regarding their activity upon the substrates representing the regions flanking the entomotoxic peptide within the intact urease. We have previously shown that *in vitro* hydrolysis of canatoxin by cathepsins from *C. maculatus* larvae produced a family of entomotoxic peptides, the largest of which, pepcanatox, was the most toxic, the smaller ones probably representing further cleavage of the latter (Ferreira-DaSilva et al., 2000). The N-terminal sequence of pepcanatox was determined by Edman sequencing to be GPVQ- and based on its molecular mass its C-terminal was deduced to be –KVIRD (Mulinari et al., 2007). Here we observed that both homogenates cleaved the N-terminal substrate, and that this hydrolysis is not catalyzed by pepstatin-A-sensitive enzymes. Only AH was inhibited by E-64 when assayed with this substrate. Since EDTA was the only effective inhibitor of the hydrolysis of the N-terminal substrate catalyzed by NH, we suggest that a metalloprotease active at pH 4.0–5.0 is probably involved in the limited proteolysis of urease that releases the entomotoxic peptide in young forms of *D. peruvianus*. The LC–ESI-MS data (Table 2) indicated that both AH and NH at pH 4.0, in which all the activities studied are at or near to their maximal levels, cleave the substrate Abz-NAIADGPVQ-EDDnp preferentially between the residues Ala and Asp. Metalloproteases, as the peptidyl-Asp metallopeptidase, hydrolyzes bonds at the N-terminal side of aspartic acid (Drapeau, 1980; Hagmann, 2004), reinforcing our hypothesis that an enzyme of this class releases the N-terminal side of the entomotoxic peptide from within urease.

The cleavage of C-terminal substrate Abz-KVIRDGMGQ-EDDnp by AH and NH at pH 4.0 occurred between the residues Arg and Asp. The activity of both homogenates upon this substrate was inhibited significantly only by E-64, indicating the participation of cysteine proteinases. Cleavage of RD bonds is described for human cathepsin L (Dahl et al., 2001) and also for trypsin-1 (Nemoto et al., 1997). The data on azocasein hydrolysis suggested that adults have trypsin-like enzymes, while PMSF-sensitive enzymes were not found in nymphs. Dıaz-Mendoza et al. (2005) reported that E-64 nonspecifically inhibited trypsin-1 enzymes from insects. The more pronounced effect of E-64 on AH activity upon the C-terminal substrate could thus represent the inhibition of cysteine proteinases plus nonspecific inhibition of trypsin-like enzymes. This may be an important difference in the limited proteolysis of urease by the two stages of *D. peruvianus*. Within the entomotoxic peptide sequence (Mulinari et al., 2007) there are 11 potential cleavage sites for trypsin. If adults in fact have a trypsin-like enzyme(s), it is possible that the entomotoxic peptide is released from urease, but then degraded into smaller, not toxic, fragments. Another fact that supports the hypothesis that the entomotoxic peptide is degraded is that Jaburetox-2Ec is not toxic to *D. peruvianus* adults when administered orally (Stanisçuaski et al., 2005).

A second cleavage site, between Asp and Gly, was observed for both homogenates with Abz-NAIADGPVQ-EDDnp and for AH with Abz-KVIRDGMGQ-EDDnp. When the hydrolysis was performed at pH 5.6, Asp–Gly was the only cleavage site identified in Abz-NAIADGPVQ-EDDnp (data not show). The hydrolytic activities upon these substrates at pH 5.0–6.0 are comparatively very low (Figs. 6A and 7A). Asp–Gly is not a consensus cleavage site for cathepsins either from the aspartic or cysteine protease families. The Peptidase Databank MEROPS (http://merops.sanger.ac.uk) provides literature showing that the bond between Asp–Gly can be cleaved by cysteine proteases such as caspases (Hawkins et al., 2000), metalloproteases as falcilysin (Eggleson et al., 1999), serine proteases as dipeptidase E (Miller, 2004) and also by aspartyl-peptidases (Wilk, 2004). Brinkworth et al. (2001) have shown that cathepsin-D from blood-feeding parasites has unusual cleavage sites, when compared to mammalian cathepsin-D. These differences in substrate cleavage preference reflected subtle but significant differences in the enzyme binding pockets. It is possible that, in the course of evolution, insects had some of their enzymes adapted to their feeding habits and hence diverged their substrates preferences from the expected.

Although we cannot exclude that a single enzyme in *D. peruvianus* nymphs is responsible for the limited proteolysis that releases the entomotoxic peptide from urease, our data indicate that more than one class of enzyme in their midguts is able to hydrolyze the synthetic peptides containing the potential cleavage points, particularly at the C-terminal portion of the peptide. Indeed, in a previous study (Carlini et al., 1997) we demonstrated that simultaneous administration of E-64 or pepstatin-A and canatoxin to third instars *Rhodnius prolixus* significantly reduced the lethality rate, suggesting that both cysteine and aspartic proteinases are involved in the proteolytic activation of the protein. Another important point concerning these results is that the whole native urease is actually not the only substrate to be considered as a partially cleaved protein could be a better substrate for the enzyme(s) releasing the internal entomotoxic peptide.

Table 1 summarizes our data showing the presence of multiple, stage-specific proteinases in the digestive system of *D. peruvianus*. Purification and physicochemical characterization of some of the enzymatic activities detected in this work will be dealt with elsewhere. Insect digestive proteolytic activity is dynamic and depends on the interactions among tissue type, pH, proteinase class and phase of life cycle (Wright et al., 2006). Such complexity could be directly involved in the processing and inactivation of toxic defense proteins produced by plants (Peumans and Van Damme, 1995). The difference in susceptibility to ureases of adults and nymphs of *D. peruvianus* is probably a complex event, with the contribution of multiple factors. Here we have demonstrated that stage-specific differences in the release of the entomotoxic peptide from the intact protein might contribute to the resistance of adult *D. peruvianus* to urease entomotoxic effects. The data also emphasize the need for adequate understanding of insect physiology when considering the potential biotechnological use of insecticidal proteins in protecting crops against insect pests.

Acknowledgments

The authors are thankful to Antonio Polleti from Núcleo de Produção de Sementes de Aguai, SP, Brazil, for the cotton seeds supply, to Roseli Garcia, for maintaining the insect colony, and to Dr. Joseph C. Polacco, Univ. Missouri, Columbia, for critical reading of the manuscript. This project was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and FAPERGS (Fundação de Amparo a Pesquisa do Rio Grande do Sul).

References

Barret, A.J., Rawlings, N.D., Woessner, J.F., 1998. Handbook of Proteolytic Enzymes. Academic Press, San Diego, 1666 pp.

Brinkworth, R.I., Provic, P., Loukas, A., Brindley, P.J., 2001. Hemoglobin-degrading, aspartic proteases of blood-feeding parasites. J. Biol. Chem. 276, 38844–38851.

Carlini, C.R., Guimarães, J.A., 1981. Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jack bean) seeds, distinct from concanavalin A. Toxicon 19, 667–676.

Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J., Wells, M.A., 1997. Biological effects of canatoxin in different insect models. Evidence for a proteolytic activation of the toxin by insect cathepsinlike enzymes. J. Econ. Entomol. 90, 340–348.

Cristofoletti, P.T., Ribeiro, A.F., Deraison, C., Rahbé, Y., Terra, W.R., 2003. Midgut adaptation and digestive enzyme distribution in a phloem feeding insect, the pea aphid *Acyrthosiphon pisum*. J. Insect Physiol. 49, 11–24.

Dahl, S.W., Halkier, T., Lauritzen, C., Dolenc, I., Pedersen, J., Turk, V., Turk, B., 2001. Human recombinant pro-dipeptidyl peptidase I (cathepsin C) can be activated by cathepsins L and S but not by autocatalytic processing. Biochemistry 40, 1671–1678.

Dıaz-Mendoza, M., Ortego, F., Garcıa de Lacoba, M., Magana, C., de la Poza, M., Farinos, G.P., Castanera, P., Hernadez-Crespo, P., 2005. Diversity of trypsins in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification. Insect Biochem. Mol. Biol. 35, 1005–1020.

Drapeau, G.R., 1980. Substrate specificity of a proteolytic enzyme isolated from a mutant of *Pseudomonas fragi*. J. Biol. Chem. 255, 839–840.

Eggleson, K.K., Duffin, K.L., Goldberg, D.E., 1999. Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. J. Biol. Chem. 274, 32411–32417.

Ferreira-DaSilva, C.T., Gombarovits, M.E., Masuda, H., Oliveira, C.M., Carlini, C.R., 2000. Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. Arch. Insect Biochem. Physiol. 44, 162–171.

Follmer, C., 2008. Insights into the role and structure of plant ureases. Phytochemistry 69, 18–28.

Follmer, C., Barcellos, G.B.S., Zingali, R.B., Machado, O.L.T., Alves, E.W., Barja-Fidalgo, C., Guimarães, J.A., Carlini, C.R., 2001. Canatoxin, a toxic protein from jack beans (*Canavalia ensiformis*), is a variant from of urease (EC. 3.5.1.5): biological effects of urease independent of its ureolytic activity. Biochem. J. 360, 217–224.

Follmer, C., Real-Guerra, R., Wassermann, G.E., Olivera-Severo, D., Carlini, C.R., 2004a. Jackbean, soybean and *Bacillus pasteurii* ureases: biological effects unrelated to ureolytic activity. Eur. J. Biochem. 271, 1357–1363.

Follmer, C., Wassermann, G., Carlini, C.R., 2004b. Separation of Jack bean (*Canavalia ensiformis*) urease isoforms by immobilized metal affinity chromatography and characterization of insecticidal properties unrelated to ureolytic activity. Plant Sci. 167, 241–246.

Gallo, D., 1988. Manual de Entomologia Agrícola, second ed. CERES, São Paulo, 649 pp.

Hagmann, M.L., 2004. Peptidyl-Asp Metalloendopeptidase. In: Handbook of Proteolytic Enzymes, second ed. Elsevier, London, pp. 1037–1039.

Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y., Hay, B.A., 2000. The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. J. Biol. Chem. 275, 27084–27093.

Keller, M., Sneh, B., Strizhov, N., Prudovsky, E., Regev, A., Koncz, C., Schell, J., Zilberstein, A., 1996. Digestion of delta-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC. Insect Biochem. Mol. Biol. 26, 365–373.

MEROPS, http://merops.sanger.ac.uk/.

Miller, C.G., 2004. Dipeptidase E. In: Handbook of Proteolytic Enzymes, second ed. Elsevier, London, pp. 2033–2035.

Mobley, H.L., Island, M.D., Hausinger, R.P., 1995. Molecular biology of microbial ureases. Microbiol. Rev. 59, 451–480.

Mulinari, F., Freitas-Silva, M.A., Grossi-De-Sa, M.F., Moraes, M.G., Kurtenbach, E., Carlini, C.R., 2004. Toxina Praguicida, Construção Gênica e Método de Controle de Pragas. Patent registered at National Institute for Intellectual Property (INPI). Brazil; 08/04/2004 [protocol BRPI0403435A].

Mulinari, F., Stanisçuaski, F., Bertholdo-Vargas, L.R., Postal, M., Oliveira-Neto, O.B., Ridgen, D.J., Grossi-de-Sá, M.F., Carlini, C.R., 2007. Jaburetox-2Ec: an insecticidal peptide derived from an isoform of urease from the plant *Canavalia ensiformis*. Peptides 28, 2042–2050.

Nemoto, T., Sato, N., Iwanari, H., Yamashita, H., Takagii, T., 1997. Domain structures and immunogenic regions of the 90-kDa heat-shock protein (HSP90). J. Biol. Chem. 272, 26179–26187.

Peumans, W.J., Van Damme, E.J., 1995. Lectin as plant defense proteins. Plant Physiol. 109, 347–352.

Polacco, J.C., Holland, M.A., 1993. Roles of urease in plant cells. Int. Rev. Cytol. 145, 65–103.

Renard, G., Garcia, J.F., Cardoso, F.C., Richter, M.F., Sakanari, J.A., Ozaki, L.S., Termignoni, C., Masuda, A., 2000. Cloning and functional expression of a *Boophilus microplus* cathepsin L-like enzyme. Insect Biochem. Mol. Biol. 30, 1017–1026.

Silva, C.P., Terra, W.R., 1994. Digestive and absorptive sites along the midgut of the cotton seed sucker bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Insect Biochem. Mol. Biol. 24, 493–505.

Silva, C.P., Ribeiro, A.F., Terra, W.R., 1996. Enzyme markers and isolation of the microvillar and perimicrovillar membranes of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) midgut cells. Insect Biochem. Mol. Biol. 26, 1011–1018.

Sirko, A., Brodzik, R., 2000. Plant ureases: role and regulation. Acta Biochim. Pol. 47, 1189–1195.

Sorgine, M.H., Logullo, C., Zingali, R.B., Paiva-Silva, G.O., Juliano, L., Oliveira, P.L., 2000. A heme-binding aspartic proteinase from the eggs of the hard tick *Boophilus microplus*. J. Biol. Chem. 275, 28659–28665.

Stanisçuaski, F., Ferreira-Dasilva, C.T., Mulinari, F., Pires-Alves, M., Carlini, C.R., 2005. Insecticidal effects of canatoxin on the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Toxicon 45, 753–760.

Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109, 1–62.

Terra, W.R., Ferreira, C., Jordão, B.P., Dillon, R.J., 1996. Digestive Enzymes. In: Biology of the Insect Midgut. Chapman & Hall, London, pp. 153–194.

Tomazzeto, G., Mulinari, F., Staniscuaski, F., Settembrini, B.P., Carlini, C.R., Ayub, M.A.Z., 2007. Expression kinetics and plasmid stability of recombinant *E. coli* encoding urease-derived peptide with bioinsecticide activity. Enzyme Microb. Technol. 41, 821–827.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.

Weber, K., Osborn, M., 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406–4412.

Whitaker, J.R., Perez-Villasenor, J., 1968. Chemical modification of papain. 1. Reaction with the chloromethyl ketones of phenylalanine and lysine and with phenylmethylsulphonyl fluoride. Arch. Biochem. Biophys. 124, 70–78.

Wilhite, S.E., Elden, T.C., Brzin, J., Smigocki, A.C., 2000. Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. Insect Biochem. Mol. Biol. 30, 1181–1188.

Wilk, S., 2004. Beta-aspartyl peptidase. In: Handbook of Proteolytic Enzymes, second ed. Elsevier, London, pp. 2115–2116.

Wright, M.K., Brandt, S.L., Coudron, T.A., Wagner, R.M., Habibi, J., Backus, E.A., Huesing, J.E., 2006. Characterization of digestive proteolytic activity in *Lygus hesperus* knight (Hemiptera: Miridae). J. Insect Physiol. 52, 717–728.