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Jaburetox, Peptídeo Tóxico Derivado da Urease: Estudos de Estrutura e Função

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Lista de abreviaturas

A: absorbância

F: farad

I: corrente iônica

JBU: *jackbean urease*

CNTX: canatoxina

JBURE-II: jackbean urease II

kDa: quilodaltons

MW: massa molecular

DO: densidade óptica

pA: picoampere

PC: fosfatidilcolina

PE: fosfatidiletanolamina

Ch: colesterol

PLB: *planar lipid bilayer*

pS: picosiemens

V: volt

Resumo

Ureases (E.C. 3.5.1.5) são metaloenzimas dependentes de níquel, que estão envolvidas na biodisponibilidade de nitrogênio e em mecanismos de defesa em plantas. Nosso grupo descreveu a atividade inseticida da Canatoxina, uma isoforma da urease. Esta toxicidade envolve a liberação de um peptídeo interno de 10 kDa (pepcanatox) da proteína, por ação hidrólitica de catepsinas encontradas no sistema digestivo de insetos suscetíveis. Baseado na sequência N-terminal do pepcanatox, um fragmento de 270 pb correspondente (jaburetox-2Ec) foi clonado, a partir da sequência da JBURE-II, e expresso em *Escherichia coli*. Este peptídeo recombinante Jaburetox-2Ec (carregando epítopo V5 e cauda His) foi testado contra os insetos *Dysdercus peruvianus*, *Rhodnius prolixus* e *Spodoptera frugiperda* e 100% de mortalidade foi observado em todos os modelos após a ingestão de microgramas do peptídeo. Outros dados mostram que jaburetox-2Ec tem capacidade de interagir com bicamadas lipídicas acídicas, permeabilizando lipossomas, além de atividade antifúngica. Estudos de modelagem do peptídeo revelaram a presença de um grampo beta, que poderia estar envolvido nesta toxicidade. A fim de estudar os motivos envolvidos nestas atividades biológicas, escolhemos uma abordagem de mutagênese dirigida. Para isto, a sequência de DNA do jaburetox foi clonado em plasmídeo pET-23a, obtendo-se a expressão do jaburetox, contendo apenas cauda de histidina, e a partir deste, obtivemos diferentes mutantes: 1) deleção de todo o grampo beta (aminoácidos 61-75); 2) deleção da metade N-terminal do peptídeo, que corresponde a uma região ausente nas ureases bacterianas; 3) deleção da metade C-terminal do peptídeo. Ensaios de formação de canais iônicos em bicamadas lipídicas artificiais com o jaburetox e mutantes mostraram que todos formam canais iônicos, ainda que com diferentes características. Bioensaios com o percevejo *Oncopeltus fasciatus* (injeção na hemocele) e leveduras mostraram que a região do grampo-beta não está envolvida nos efeitos do peptídeo nesses organismos, sendo que é a região N-terminal responsável pelas atividades inseticida e antifúngica do Jaburetox.

Abstract

Urease (EC 3.5.1.5) are nickel dependent metalloenzymes, that are involved in nitrogen bioavailability and defense mechanisms in plants. Our group described the insecticidal activity of canatoxin, an isoform of urease. This toxicity involves the release of an internal peptide of 10 kDa (pepcanatox), released by the hydrolytic action of cathepsins found in the susceptible insects gut. Based on the N-terminal sequence of pepcanatox, a corresponding fragment of 270 bp (jaburetox-2Ec) was cloned from the JBURE-II and expressed in *Escherichia coli*. This recombinant peptide Jaburetox-2Ec (carrying the V5 epitope and His tag) was tested against the insect *Dysdercus peruvianus*, *Rhodnius prolixus* and *Spodoptera frugiperda*, and 100% mortality was observed in all the models after ingestion of micrograms of the peptide. Other data showed that Jaburetox-2Ec has the ability to interact with acidic lipid bilayers, liposomes leakage and also antifungal activity. Molecular modeling studies of the peptide revealed the presence of a β-hairpin motif, which could be involved in this toxicity. In order to identify structures involved in the biological activities, we chose a directed mutagenesis approach. For this, the jaburetox cDNA was cloned into pET-23a vector (to yield the expression of jaburetox containing only histidine tag), and from this, we obtained different mutants: 1) deletion of the entire β-hairpin motif (amino acids 61 -75); 2) deletion of the N-terminal half of the peptide, which corresponds to a region absent in bacterial ureases; 3) deletion of the C-terminal half of the peptide. Planar lipid bilayers experiments were performed with jaburetox and all mutants, and we observed channels activity in all cases. Bioassays with *Oncopeltus fasciatus and Rhodnius prolixus* showed that the region of β-hairpin is not involved in the effects of the peptide in these organisms, and the N-terminal region of the Jbtx carries the most important entomotoxic domain which is fully active in the absence of the β-hairpin motif.

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1. Introdução

Nos próximos anos o mundo poderá enfrentar uma crise de escassez de alimentos, o que representa um desafio para a agricultura para aumentar a produção de alimentos. Danos às culturas devido a insetos, fungos, bactérias e vírus podem representar até 35% do total de perdas na produção agrícola. Portanto, melhorias para os programas existentes de controle de pragas são urgentemente necessárias (Pardo-Lopez, *et al.*, 2012). De modo geral, os pesticidas químicos utilizados para controle de pragas são extremamente tóxicos para organismos não alvos e, em muitos casos, são prejudiciais para a saúde dos seres humanos e animais. Adicionalmente, inseticidas químicos são recalcitrantes, ou seja, demoram a ser degradados, lentamente levando à contaminação do solo e da água. Além disso, muitos insetos desenvolveram resistência aos diferentes pesticidas químicos, resultando em controle ineficiente de insetos pragas (Devine & Furlong 2007).

A utilização de insecticidas biológicos, como substitutos para produtos químicos, é uma alternativa para o controle de insetos em culturas agrícolas. Inseticidas biológicos preparados a partir de bactérias entomotóxicas são baseados principalmente em *Bacillus thuringiensis* (Bt), o qual acumula proteínas durante a fase vegetativa de desenvolvimento e que formam cristais, na fase de esporulação (Bravo et al., 2007). As toxinas Bt são utilizadas no controle de insetos lepidópteros, dípteros e alguns coleópteros, e têm como vantagem o fato de serem seletivas para algumas famílias de insetos e não gerar resíduos poluentes, além de não atingirem outros organismos como mamíferos e pássaros (Gatehouse, 2002). Outra alternativa para o controle biológico de pragas, são as bactérias *Xenorhabdus* spp. e *Photorhabdus* spp., pertencentes à família Enterobacteriaceae, que formam associações simbióticas com nematóides

entomopatogênicos. Estas bactérias produzem potentes toxinas inseticidas, que podem representar alternativa adicional para o controle de insetos (Ffrench-Constant & Bowen, 2000). Muitas classes de proteínas isoladas de plantas possuem atividade entomotóxica e podem ser utilizadas para o controle de pragas. Dentre elas, destacam-se as ureases vegetais, que apresentam potencial para uso no controle de insetos (Carlini & Grossi-de-Sá, 2002; Carlini & Polacco, 2008) e como agente antifúngico (Becker-Ritt *et al.*, 2007; Becker-Ritt & Carlini, 2012; Postal *et al.*, 2012).

1.1 Ureases

As ureases (EC 3.5.1.5) são enzimas dependentes de níquel que catalisam a hidrólise da ureia para formar amônia e dióxido de carbono (Dixon *et al.*, 1975). Ureases têm sido isoladas de uma ampla variedade de organismos incluindo plantas, fungos e bactérias (Mobley *et al.*, 1989; Sirko & Brodzik, 2000; Follmer, 2008). As ureases de plantas e fungos são proteínas homo-hexaméricas, ou seja, formadas por seis subunidades idênticas (Sirko & Brodzik, 2000), enquanto as ureases bacterianas são proteínas formadas por duas ou três subunidades distintas (Figura 1).

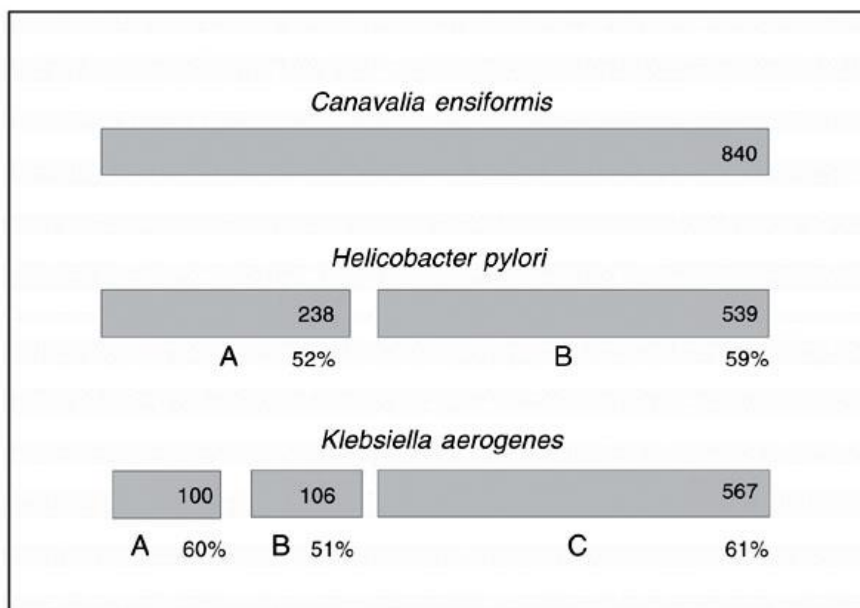

Figura 1. Esquema representativo da unidade estrutural das ureases de plantas e bactérias. Estão indicados o número de aminoácidos de cada subunidade e a porcentagem de identidade correspondente à região equivalente da urease de *Canavalia ensiformis*, mostrado abaixo das subunidades bacterianas (Olivera-Severo *et al.,* 2006).

Em bactérias, a urease é reconhecida como um fator de virulência em infecções humanas do trato urinário e gastrointestinal (Mobley *et al*., 1995), auxiliando a sobrevivência destes microrganismos em pH desfavorável. Ureases são sintetizadas por bactérias de solo e por bactérias anaeróbicas presentes em ruminantes, favorecendo a reciclagem de compostos nitrogenados. Em plantas, a urease ocorre em todos os tecidos, sendo particularmente abundante em sementes, apesar da ureia não ser um metabólito majoritário em plantas (Polacco & Holland, 1993; Sirko & Brodzic, 2000), o que despertou curiosidade para estudo das funções fisiológicas desta enzima nos vegetais.

1.2 Ureases da semente do feijão-de-porco (*Canavalia ensiformis*)

A semente de *Canavalia ensiformis* possui três isoformas de urease: a urease clássica (JBU ou JBURE-I), a canatoxina (CNTX), e a JBURE-II. A JBU é constituída por

uma cadeia peptídica de 840 aminoácidos e massa molecular de 90,77 kDa. A forma mínima da enzima ativa é trimérica, com 270 kDa, e a enzima é encontrada em sua forma nativa como um hexâmero de 540 kDa (Zerner, 1991). JBU possui dois átomos de níquel no sítio ativo, cada um coordenado por dois resíduos de histidina. Em 1981, Carlini & Guimarães isolaram a CNTX, uma proteína tóxica para insetos e mamíferos, que anos mais tarde, foi caracterizada como uma isoforma de urease (Follmer *et al.*, 2001). A CNTX apresenta massa molecular de 184 kDa quando analisada por gel-filtração sob pH 7,5, sendo uma metalo-proteína contendo zinco e níquel. Por SDS-PAGE, em meio redutor, sua massa molecular é de 95 kDa, sugerindo que a forma nativa da proteína seja um dímero, mantido por ligações não covalentes. A CNTX apresenta apenas 30 - 40% da sua atividade enzimática sobre ureia em relação à urease majoritária.

Pires-Alves *et al.*, (2003) realizaram a clonagem do cDNA parcial que codifica a isoforma JBURE-II de urease, predita como tendo uma cadeia polipeptídica de 78 kDa, um pouco mais curta em relação as demais isoformas. Recentemente, Mulinari *et al.*, (2011) realizou a clonagem do cDNA de *C. ensiformis* que codifica uma sequência mais longa da JBURE-II (agora chamada de JBURE-II b),capaz de codificar uma terceira isoforma de urease da *C. ensiformis*, tendo o mesmo tamanho esperado para as demais isoformas. Ela difere da JBURE-II na sua região 5’ e não possui a terminação traducional prematura sugerida por Pires-Alves *et al.*(2003). A expressão em *E.coli* da proteína JBURE-IIb recombinante foi obtida por Mulinari *et al.*(2011), e a sua presença na planta foi confirmada em Demartini *et al.*(2011).

Quando injetada por via intraperitoneal, a CNTX induz convulsão e morte de ratos e camundongos, com uma dose letal média (DL_{50}) de 2 mg/kg. Porém, esta proteína é inócua quando administrada por via oral, provavelmente devido à sua instabilidade em meio ácido (Carlini *et al.*, 1984; Carlini & Guimarães, 1991). Estudos do mecanismo de

ação da CNTX em modelos mamíferos mostraram que a proteína induz exocitose em diversos tipos celulares, tais como plaquetas, afetando os fluxos de cálcio e ativando a cascata dos eicosanóides, além de interagir com glicoconjugados (para uma revisão, ver Olivera-Severo *et al.*, 2006). A CNTX apresenta propriedades antifúngicas (Oliveira et al., 1999) e potente atividade inseticida (Carlini *et al.*,1997; Ferreira-DaSilva *et al.*,2001; Carlini & Grossi-de-Sá, 2002), que depende em grande parte da clivagem da proteína por enzimas digestivas dos insetos, gerando um peptídeo entomotóxico de 10 kDa. Em Follmer et al. (2001), demonstrou-se que a urease clássica (JBU) possui atividades biológicas descritas para a CNTX, como indução de ativação plaquetária e interação com gangliosídeos, mas não é letal por via intraperitoneal em camundongos. Esse estudo ainda mostrou que quando a CNTX ou a JBU são tratadas com um inibidor irreversível de urease, *p*-hidroxi-mercuribenzoato, elas perdem a atividade ureolítica, mas suas outras atividades biológicas permanecem inalteradas, sugerindo que estas propriedades são independentes da atividade enzimática (Follmer *et al.*, 2001). A urease de sementes de soja (SBU) e a da bactéria do solo *Bacillus pasteurii* (Follmer *et al.*, 2004a), bem como a urease da bactéria patogênica *Helicobacter pylori* (HPU) (Wasserman et al., 2010) também apresentam propriedades biológicas independentes da atividade ureolítica. A JBU, e também a SBU, compartilham com a CNTX propriedades inseticidas (Follmer et al., 2004) e antifúngicas (Becker-Ritt *et al.*, 2007), ambas independentes da atividade ureolítica das proteínas. Esses dados indicam a existência de domínios proteicos distintos, responsáveis por atividades biológicas diferentes: um domínio com atividade hidrolítica sobre a ureia, suscetível de inibição por agentes oxidantes; e pelo menos mais um domínio responsável pelas outras propriedades farmacológicas das ureases.

1.3 Ureases: proteínas de defesa em plantas?

Vários artigos do nosso grupo descreveram a atividade inseticida da CNTX e da JBU contra insetos de diferentes ordens, como pode ser visto na tabela I. Nesses trabalhos, ficou claro que a CNTX atua em alvos específicos: apenas insetos que possuem enzimas digestivas do tipo catepsinas (cisteíno e aspártico proteases) são suscetíveis á toxina, como coleópteros bruquídeos e percevejos. Já os insetos com enzimas digestivas do tipo tripsinas (serino proteases) como, por exemplo, lepidópteros e dípteros não mostraram suscetibilidade (Carlini *et al*., 1997, Stanisçuaski & Carlini, 2012). Estudos mostraram que o efeito inseticida não está associado à atividade ureolítica da toxina, pois depende da formação de peptídeos entomotóxicos a partir da sua hidrólise da por enzimas digestivas dos insetos. A fim de investigar o efeito destes peptídeos, realizou-se a digestão *in vitro* da CNTX com enzimas obtidas da larva de *Callosobruchus maculatus*. Obteve-se, desta forma, um conjunto de peptídeos tóxicos com cerca de 10 a 15 kDa, os quais foram administrados por via oral e por via intraperitonial no barbeiro *R. prolixus.* O fragmento mais tóxico apresentou massa de 10 kDa e foi chamado de pepcanatox. Este peptídeo foi sequenciado em sua região N-terminal e a partir desta sequência, utilizando-se como molde a sequência do cDNA codificador da urease JBURE-II de *C. ensiformis*, foi obtido por expressão heteróloga em *Escherichia coli* um peptídeo recombinante, chamado de jaburetox-2Ec (Mulinari *et al*., 2007), que apresentou atividade inseticida e outros efeitos descritos no item 1.4.

Oliveira *et al*. (1999) descreveram a atividade antifúngica da CNTX sobre alguns fungos filamentosos. Becker-Ritt *et al*. (2007), descreveram a ação fungitóxica das ureases vegetais SBU (embrião-específica da soja) e JBU e também da urease bacteriana de *H. pylori*, sobre fungos filamentosos fitopatogênicos. Foram realizados ensaios de inibição de crescimento de colônias fúngicas por difusão em disco de papel e

por método de turbidimetria, além do emprego da microscopia eletrônica de varredura de fungos afetados. As três ureases estudadas foram tóxicas aos fungos, sendo as ureases vegetais mais potentes do que a bacteriana, causando inibição de crescimento e permeabilização das hifas na faixa de 10^{-7} M (Becker-Ritt *et al.*, 2007). Foi descrita também a atividade antifúngica da urease de *Gossypium hirsutum* (Menegassi *et al.*, 2008) e da JBURE-IIb recombinante (Mulinari *et al.*, 2011).

Tabela I. Efeito inseticida da CNTX e JBU em diferentes ordens de insetos. Adaptado de (Stanisçuaski & Carlini, 2012).

Ordem	Insetos	Via de administração	Efeito	Urease
Lepidoptera	*M. sexta*	oral	Nenhum	CNTX
Orthoptera	*S.americana*	oral	Nenhum	CNTX
Diptera	*D. melanogaster*	oral	Nenhum	CNTX
Diptera	*A. aegypt*	oral	Nenhum	
Coleoptera	*C. maculatus*	oral	Mortalidade Atraso no desenvolvimento	CNTX
Hemiptera	*R. prolixus*	intraperitoneal	Mortalidade (depende do instar do inseto)	CNTX/JBU
Hemiptera	*R. prolixus*	oral	Mortalidade antidiurético (depende do instar do inseto)	CNTX/JBU
Hemiptera	*D. peruvianus*	oral	Mortalidade Atraso no desenvolvimento (depende do instar do inseto)	CNTX/JBU
Hemiptera	*N. viridula*	oral	Mortalidade	CNTX
Hemiptera	*O. fasciatus*	oral	Mortalidade	JBU

Mais recentemente, Postal *et al.* (2012), demonstrou a atividade fungicida de JBU contra leveduras patogênicas, acompanhado de alterações no metabolismo energético e permeabilização das células. Ainda nesse trabalho, foram obtidos peptídeos antifúngicos derivados da urease por hidrólise enzimática com papaína, sendo que um desses peptídeos contem parte da sequência N-terminal do jaburetox. Ensaios com o peptídeo recombinante em leveduras confirmaram o seu efeito antifúngico, ainda que em doses 10 vezes maiores do as verificadas para a JBU (Postal *et al.*, 2012).

1.4. Efeito tóxico do peptídeo jaburetox 2-Ec

O peptídeo tóxico pepcanatox, produzido pela hidrólise *in vitro* da CNTX com enzimas digestivas das larvas de *C. maculatus*, foi purificado e caracterizado (Ferreira-DaSilva *et al.*, 2000). Conforme descrito anteriormente, um peptídeo análogo derivado da JBURE-II foi expresso em *E. coli e* denominado jaburetox-2Ec. Este peptídeo possui 93 aminoácidos, e em virtude do vetor plasmideal de clonagem e expressão (pET 101/D-TOPO) utilizado, possui o epitopo V5 (18 aminoácidos) para reconhecimento de anticorpo e uma cauda de seis histidinas (Mulinari *et al.*, 2007). O Jaburetox-2Ec tem um espectro mais amplo de ação inseticida, atuando sobre insetos insensíveis à urease intacta, como a lagarta *S. frugiperda*, além de ser inseticida contra *R. prolixus* e *Triatoma infestans*. O Jaburetox-2Ec também apresentou atividade tóxica contra ninfas de *D. peruvianus*, quando alimentados em sementes artificiais contendo 0,01% do peptídeo purificado (m/m). O efeito mostrou-se tempo dependente, com fase de latência de 3-4 dias, alcançando 100% de mortalidade após 11 dias de ensaio, uma toxicidade maior que a observada para CNTX (Mulinari *et al.,* 2007).

É importante destacar que apesar de ser letal para insetos, o peptídeo jaburetox-2Ec mostrou-se inócuo para ratos neonatos e camundongos, quer administrado por via oral ou intraperitoneal, em doses 5 vezes maiores do que aquela em que se registra toxicidade para a CNTX (Mulinari *et al.*, 2007). Follmer *et al.*(2004), observaram que a urease de *Bacillus pasteurii* não tem atividade inseticida. Este fato deve-se, provavelmente, à falta de parte da sequência do jaburetox nessa urease, já que o peptídeo localiza-se em uma região "linker" entre as cadeias B e C, ausente nas proteínas bacterianas, como pode ser visto na Figura 2.

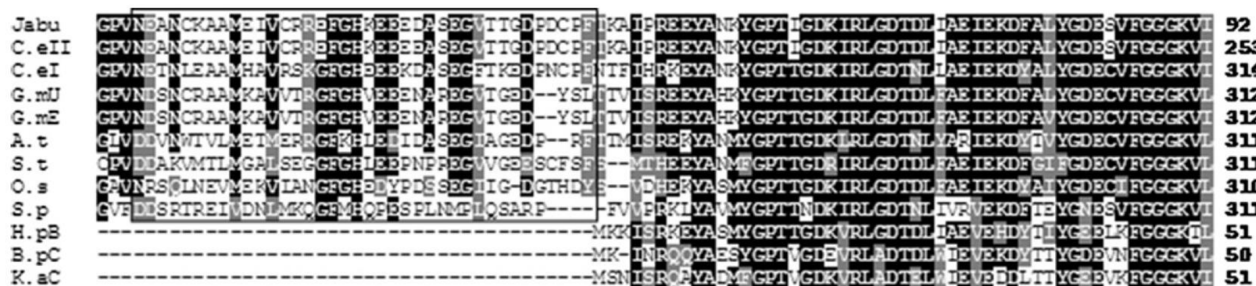

Figura 2. Sequência de aminoácidos de jaburetox-2Ec (Jabu), alinhada com as sequências homólogas de outras ureases. C.eII, C.eI, C.eII – ureases de *C.ensiformis*; G.mU, G.mE – ureases ubíqua (U) e embrião-específica (E) de soja (*Glycine max)*; A.t. A.t. - *Arabidopsis thaliana*; S.t. – *Solanum tuberosum*; O.s. – *Oryza sativa*; S.p. – *Schizosaccharomyces pombe*; H.pB – *Helicobacter pylori*, cadeia B; B.pC – *Bacillus pasteurii*, cadeia C; K.aC. – *Klebsiella aerogenes*, cadeia C. (Retirado de Mulinari et al., 2007).

O mecanismo de ação inseticida das ureases JBU, CNTX e JBURE-II e do peptídeo derivado jaburetox-2Ec ainda não está completamente elucidado. Foi demonstrado que JBU e CNTX afetavam a perda de peso *R. prolixus* após a alimentação, sugerindo um efeito na diurese dos insetos, possivelmente alterando o balanço de água e

íons na célula. A fim de investigar se estas proteínas de fato afetam o balanço de água e íons, ensaios de secreção em tubulos de Malpighi foram realizados. Os ensaios comprovaram o efeito antidiurético da JBU e também do jbtx e mostraram que o efeito induzido por JBU envolve eicosanóides e canais de cálcio na sua ação antidiurética, enquanto jaburetox-2Ec age alterando os níveis de GMP cíclico e o potencial transmembrana das células dos túbulos de Malpighi (Stanisçuaski *et a*l., 2009). Nem a CNTX e tampouco o(s) peptídeo(s) derivados inibem as enzimas digestivas de insetos (Carlini *et al*., 1997). Após ingestão do Jaburetox-2Ec, os insetos ficam momentaneamente paralisados e mostram movimentos descoordenados de antenas que precede a morte, sugerindo que o peptídeo possa agir como uma neurotoxina (Mulinari *et al*., 2007; Stanisçuaski *et al*., 2009).

O Jaburetox-2Ec tem capacidade de afetar membranas de bicamadas lipídicas acídicas, permeabilizando lipossomas, sendo este efeito fortemente influenciado pelo estado de agregação do peptídeo (Barros *et a*l., 2009). A permeabilização de membranas de células de leveduras acompanhou o efeito antifúngico produzido pelo peptídeo (Postal *et al*., 2012). Mulinari *et al*., (2007) e posteriormente Barros *et al*., (2009), identificaram, por modelagem molecular, na estrutura do peptídeo um motivo β-*hairpin* (Figura 3) similar à estrutura de alguns peptídeos neurotóxicos formadores de poro/canal, como protegrina-1, taquiplesina-1, polifemusina PV5 (Barros *et al*., 2009) ou inibidores de canal de sódio, como caribdotoxina*.* A existência desse β-*hairpin* na urease de *C. ensiformis* (JBU) foi recentemente confirmada com a resolução da estrutura da proteína por cristalografia de raios-X (Balasubramanian & Ponnuraj, 2010).

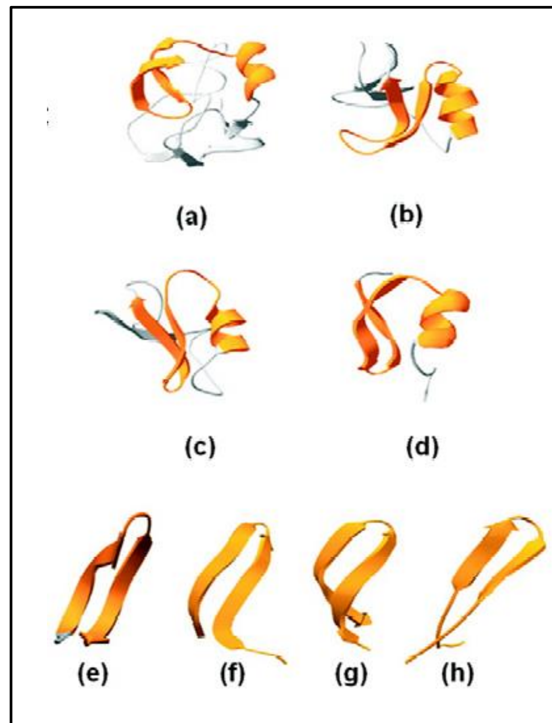

Figura 3. Modelo estrutural de jaburetox-2Ec (a); estruturas das neuroxinas de escorpião, neurotoxina α-*like* BMK M1, inibidor de canal de sódio (PDB ID: 1djt) (b); β-neurotoxina (PDB ID: 1b3c) (c), BMKK4 (PDB ID: 1s8 k) (d). Para comparação, os domínios β-grampo são mostrados para: jaburetox-2Ec (e),e peptídeos envolvidos na formação de poros/canais: protegrina-1 (f), taquiplesina-1 (g) e polifemusina PV5 (h). Os dois últimos peptídeos são reconhecidos, inibidores de canais de potássio. Para a modelagem não foram considerados o epitopo V5 e a cauda His, somente os 93 aminoácidos derivados da urease. (Retirado de Barros *et al.*, 2009)

1.5. Mutagênese-sítio-dirigida

Um dos objetivos dos estudos de Biologia Molecular é entender as relações estrutura-função de proteínas. A estratégia mais comumente utilizada é a de introduzir mutações em diferentes sítios do gene alvo e observar os efeitos na função da proteína (Qi & Scholthof, 2008). Logo, a mutagênese-sítio-dirigida tornou-se uma ferramenta indispensável para pesquisas em Biologia Molecular e em genética. Um método muito utilizado é o da substituição de resíduos de aminoácidos por alanina (“alanine scanning”),

a fim de encontrar resíduos importantes para a função de proteína ou peptídeo. Este método foi empregado na identificação de um cluster de resíduos, importantes para a atividade do ciclotídeo kalata B1 (Simonsen *et al.*, 2008).

Outros métodos de mutagênese, são os métodos baseados na reação de polimerase em cadeia (PCR), incluindo "overlap" PCR (Ho *et al.*, 1989) e "Megaprimer" PCR (Landt et al., 1990). Entretanto esses métodos requerem o uso de muitos "primers" e/ou tratamentos enzimáticos e purificação de DNA molde (Qi & Scholthof, 2008). A introdução da técnica de PCR por recombinação minimizou o número de primers e o número de passos necessários para realizar a mutagênese (Jones, 1994; Jones & Winistorfer, 1992). Essa técnica utiliza uma rodada única de amplificação por PCR com o uso de um par de primers complementares que carregam a mutação desejada em sua sequência. Posteriormente o produto de PCR é transfectado em *E. coli* e subsequentemente circularizado *in vivo* por recombinação entre as extremidades 3' e 5'. Baseado neste método kits comerciais como o QuikChange Site-Directed Mutagenesis (Stratagene) foram desenvolvidos. Para a para utilização deste kit é necessária a clonagem prévia do gene alvo em um vetor plasmideal, que servirá de DNA molde para a reação de mutagênese. A figura 4 mostra o esquema de mutagênese utilizando-se este kit comercial.

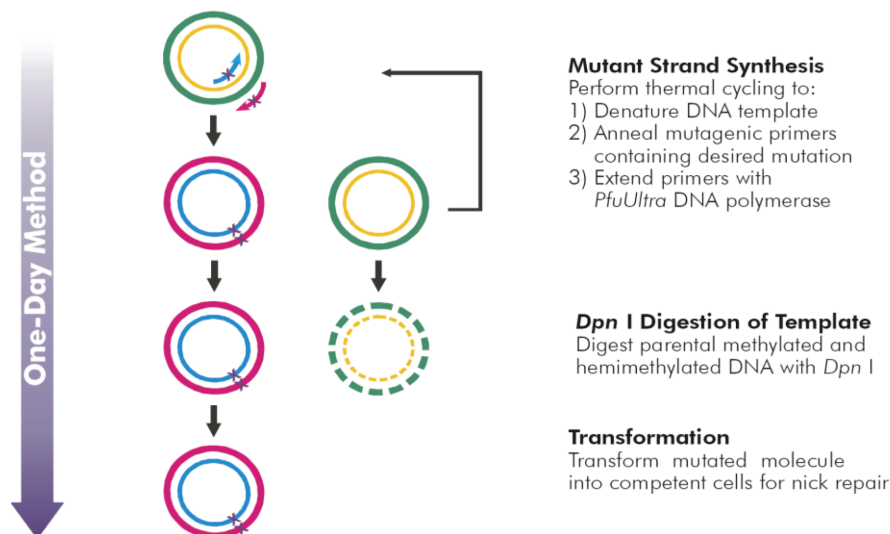

Figura 4. Esquema representativo do método do kit QuikChange site-directed-mutagenesis II. O esquema foi retirado do manual do fabricante (Stratagene).

1.6 Estudos de eletrofisiologia

Canais iônicos são proteínas integrais de membrana que formam poros hidrofílicos, que permitem a passagem de fluxo de íons sob controle de um gradiente eletroquímico (Hille, 2001). O mecanismo molecular através do qual uma proteína, com sua enorme cadeia de aminoácidos com cargas e hidrofílica, atravessa uma bicamada hidrofóbica de fosfolipídeo tem sido alvo de muitos debates e estudos (Harsman *et al*., 2011). O estudo eletrofisiológico de um canal iônico implica em caracterizar o estímulo que abre ou fecha o canal (probabilidade de abertura do canal *versus* voltagem, concentração de ligante ou tensão da membrana), a condutância do canal sob condições iônicas específicas e a especificidade de um canal (quais íons podem passar através deste). No geral, as atividades de canais iônicos podem ser estudadas na membrana plasmática nativa de células, pela técnica de *patch-clamp e/*ou por técnicas de microeletrodos, ou, após a purificação da proteína por reconstituição em bicamada lipídica planar (do inglês, *planar*

lipid bilayer ou PLB). A reconstituição de canais iônicos é a montagem de um canal iônico, retirado de uma membrana biológica, em uma membrana artificial ou purificação da proteína de interesse e sua inserção em membrana lipídica (Morera *et al*., 2007). Um receptor complexo, GP1, isolado de células epiteliais de intestino de *Manduca sexta* foi purificado e reconstituído em bicamada lipídicas planares. Nesta bicamada reconstituída, foram caracterizados os canais iônicos formados pelas toxinas Cry1Aa, Cry1Ac e Cry1C (Schwartz *et al*., 1999). Recentemente os canais de potássio ativados por canais de cálcio de grande condutância (BK) do parasita *Fasciola hepatica* foi incorporado e caracterizado em bicamadas lipídicas (Jang, *et al*., 2012).

Formato e organização da tese

Além da presente sessão de introdução geral, esta tese é constituída por dois capítulos de resultados experimentais. Cada capítulo consta da apresentação de uma justificativa, objetivos específicos, materiais e métodos, resultados e discussão. Ao final da tese uma sessão de conclusões gerais do trabalho e anexos são apresentados.

Capítulo 1: Structure and function studies of Jaburetox, a recombinant peptide derived from jack bean (*Canavalia ensiformis*) urease.

Capítulo 2. Jaburetox e mutantes derivados: estudo de atividade formadora de canais iônicos em bicamadas lipídicas planares.

CAPITULO I

Structure-function studies on Jaburetox, a recombinant insecticidal and antifungal peptide derived from jack bean (*Canavalia ensiformis*) urease.

Manuscrito publicado na revista BBA General Subjects

Justificativa:

As ureases são proteínas multifuncionais que apresentam potencial biotecnológico como agentes inseticidas ou antifúngicos. Estudos mostram que peptídeos representando sequências internas de ureases mantêm essas duas propriedades biológicas. Para o desenvolvimento de protótipos de compostos inseticidas ou antifúngicos a partir de urease é necessário conhecer as relações estrutura *versus* função desses peptídeos, visando futuramente otimizar as moléculas para essas aplicações. Nesse capítulo, estudos de mutagênese foram aplicados ao peptídeo jaburetox para estabelecer se, e quais, partes da molécula mantem suas propriedades biológicas.

Objetivos específicos

- Adaptar o cDNA codificador de jaburetox-2Ec ao plasmídeo pET23-a, eliminando-se a região do epitopo V5 de pET101/D-TOPO, e expressar o peptídeo resultante (chamado jaburetox ou jbtx) em *E. coli* contendo apenas uma cauda de histidinas para facilitar sua purificação;
- A partir do cDNA codificador de jbtx gerar diferentes formas variantes do peptídeo: 1) deleção do grampo beta (jbtxΔ-β), correspondente aos aminoácidos 61-75; 2) expressão da metade N-terminal do peptídeo (jbtx N-ter), que corresponde a uma região ausente nas ureases bacterianas (aminoácidos 1-43); 3) expressão da metade C- terminal do peptídeo (jbtx C-ter, aminoácidos 44-93).
- Com as diferentes versões de jbtx, realizar ensaios de extravasamento de lipossomos e atividade inseticida, visando identificar motivos estruturais importantes para estas atividades.

Structure-function studies on Jaburetox, a recombinant insecticidal and antifungal peptide derived from jack bean (*Canavalia ensiformis*) urease.

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Short title: Structure-functions studies on the urease-derived peptide jaburetox

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Abstract

Background**:** Ureases are metalloenzymes involved in defense mechanisms in plants. The insecticidal activity of *Canavalia ensiformis* (jack bean) ureases relies partially on an internal 10 kDa peptide generated by enzymatic hydrolysis of the protein within susceptible insects. A recombinant version of this peptide, Jaburetox, exhibits insecticidal, antifungal and membrane-disruptive properties. Molecular modeling of Jaburetox revealed a prominent β-hairpin motif consistent with either neurotoxicity or pore formation. ***Methods*****:** Aiming to identify structural motifs involved in its effects, mutated versions of Jaburetox were built: 1) a peptide lacking the β-hairpin motif (residues 61-74), JbtxΔ-β; 2) a peptide corresponding the N-terminal half (residues 1-44), Jbtx N-ter, and 3) a peptide corresponding the C-terminal half (residues 45-93), Jbtx C-ter. ***Results*****:** 1) JbtxΔ-β disrupts liposomes, and exhibited entomotoxic effects similar to the whole peptide, suggesting that the β-hairpin motif is not a determinant of these biological activities; 2) both Jbtx C-ter and Jbtx N-ter disrupted liposomes, being the C-terminal peptide the most active; 3) while Jbtx N-ter persisted biologically active, Jbtx C-ter was less active when tested on different insect preparations. Molecular modeling and dynamics were applied to the urease-derived peptides to complement the structure-function analysis. ***Major Conclusions*****:** The N-terminal portion of the Jbtx carries the most important entomotoxic domain which is fully active in the absence of the β-hairpin motif. Although the β-hairpin contributes to same extent, probably by interaction with insect membranes, it is not essential for the entomotoxic properties of Jbtx.

General Significance**:** Jbtx represents a new type of insecticidal and membrane-active peptide.

1. Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase), nickel dependent enzymes that catalyze urea hydrolysis into ammonia and carbon dioxide, have been isolated from a wide variety of organisms including plants, fungi and bacteria. In plants these proteins contribute to the bioavailability of nitrogen and in defense mechanisms [1, 2]). Ureases represent an unexplored group of plant proteins with potential use for insect control [2, 3] and as antifungal agents [4]. Studies have shown that ureases from *Canavalia ensiformis* (jack bean) and *Glycine max* (soybean) display insecticidal activity (reviewed in [5]) and antifungal properties, inhibiting growth and affecting membrane integrity of filamentous fungi [6] as well as of yeasts [7] in the 10^{-7} M range. The urease from pigeon pea (*Cajanus cajan*) was recently described to exhibit insecticidal and antifungal properties at similar dose ranges [8].

The molecular basis of the insecticidal mechanism of action of plant ureases is not yet completely understood [5]. It has been demonstrated that the entomotoxic effect of canatoxin [9], an isoform of *Canavalia ensiformis* (jack bean) urease [10], is partially due to an internal 10 kDa peptide (pepcanatox), that is released from the protein upon hydrolysis by insect cathepsin-like digestive enzymes [11-14]. Jaburetox-2Ec (Jbtx-2Ec), a recombinant peptide analog to pepcanatox, exhibited a potent insecticidal effect on two economically important crop pests: *Spodoptera frugiperda* (fall armyworm) and *Dysdercus peruvianus* (cotton stainer bug) [15, 16]. Jbtx-2Ec was also shown to both permeabilize large unilamellar liposomes (LUVs) [17] and to affect transmembrane potential of insect Malpighian tubules, causing inhibition of diuresis [18]. A β-hairpin motif in the modeled structure of Jbtx-2Ec has been proposed [15, 17] and its presence has been confirmed in the crystallographic structures of jack bean [19] and pigeon pea [8] ureases. This motif is present also in one class of pore-forming peptides and neurotoxic peptides [20] such as charybdotoxin, which affect ion channels [21]. A variant form of Jbtx-2Ec lacking the fused V5-antigen, here called simply Jbtx, also exhibited antifungal activity [7].

Aiming to identify motifs possibly involved in the different biological activities of Jbtx, here we described the cloning and expression of mutated versions of the Jbtx-encoding cDNA. Truncated versions of the peptide, with deletions of the regions of the β-hairpin motif, the N-terminal or the C-terminal halves of the molecule, were tested on LUVs permeabilization, for insecticidal and other entomotoxic effects. Structural analyses of the truncated peptides were also carried out.

2. Materials and Methods

2.1. Jbtx cDNA constructs

Jaburetox-2Ec, the first version of the recombinant urease-derived peptide cloned in [15], harbored a V5-antigen with 18 amino acids derived from the pET101/D-TOPO plasmid. In order to eliminate this foreign sequence, the jack bean urease truncated cDNA encoding 93 amino acids, called simply jaburetox (Jbtx), was cloned and expressed in *Escherichia coli* via pET-23a vector (Novagen), as described in [7]. This sequence was used as template for site-directed mutagenesis and PCR amplifications of the mutant forms as described below.

2.2. Jbtx lacking the internal β-hairpin (JbtxΔ-β)

In order to delete the β-hairpin motif (residues 61-74) of the Jbtx peptide, site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene). As this method is often used to generate a few nucleotide deletions, some modifications in the primers design were made, as described by [22]. Pairs of complementary primers were designed (Table I), and site-directed mutagenesis was performed according to the kit manufacturer´s instructions. The deleted gene version was confirmed by sequencing on an ABI Prism 3100 automated sequencer (Applied Biosystems) plataform (ACTGene Ltd, Center of Biotechnology, UFRGS). Sequence comparisons were performed using the BLASTx software [23], available at (http://www.ncbi.nlm.nih.gov). The resulting peptide was called JbtxΔ-β.

2.3. Jbtx N-terminal (Jbtx N-ter) and C-terminal (Jbtx C-ter) domain versions

The Jbtx gene regions corresponding to the N-terminal (residues 1-44) and C-terminal (residues 45-93) halves of the peptide were amplified by PCR with specifically designed primers (Table I) and products were cloned into pET23a (Novagen). PCRs were performed in a final volume of 50 µL containing 50 ng of the template plasmid DNA, 200 ng of each primer, 200 µM each dNTPs, 2.5 U *Pfu* taq DNA polymerase (Fermentas) and 1x *Pfu* reaction buffer. Amplification was carried out under the following conditions: denaturation at 95°C for 3 min, annealing at 55°C for 30 s and elongation at 72°C for 2 min. After a total of 35 cycles, the final products were digested with *Nde*I and *Xho*I (Fermentas), dephosphorylated with thermosensitive alkaline phosphatase (Promega) and ligated into the expression vector pET23a (Novagen). The inserts of the recombinant plasmids were fully sequenced in order to confirm their sequences essentially as described above. The resulting peptides were called Jbtx N-terminal (Jbtx N-ter) and Jbtx C-terminal (Jbtx C-ter). A schematic representation of all Jbtx-related peptides is shown in Figure 1.

2.4. Expression and purification of Jbtx recombinant peptides

Recombinant pET23a plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) for Jbtx gene expressions following provider's instructions. For the purification of the original Jbtx peptide and its mutated forms, 200 mL of Luria B Bertrand medium containing 100 µg/mL ampicillin and 40 µg/mL chloramphenicol were separately inoculated with 2 mL overnight cultures of each *E. coli* strain. Cells were grown for approximately 2h at 37 ^{o}C under shaking until an optical density of 0.7 was reached. At this point, IPTG was added to cultures to a final concentration of 0.5 mM. After 3 h of additional culture, cells were harvested by centrifugation and suspended in 10 mL of lysis buffer (50 mM Tris buffer, pH 7.5, 500 mM NaCl, 5 mM imidazole), sonicated and centrifuged (14,000 *g*, 30 min). The supernatant was loaded onto a 5 mL Ni^{+2} loaded

Chelating Sepharose (GE Healthcare) column, previously equilibrated with the lysis buffer. After 30 min, the column was washed with 50 mL of the same buffer containing 50 mM imidazole. Bound protein was eluted with 200 mM imidazole in the lysis buffer. Samples were then dialyzed against buffer A (50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol) in order to remove the imidazole. Protein concentration was measured by Bradford assay [24]. Predicted molecular mass of the peptides was obtained by submitting the deduced sequences to the ProtScale tool [25] available at the Expasy site (http//web.expasy.org/protscale). Molar concentrations of peptides were calculated assuming a monomeric form in solution. Hydropathicity analysis of the peptides was carried out according to [26].

2.5. Tandem Mass Spectrometry (MS/MS)

In gel digestion of Jbtx peptides was performed for all samples analyzed, except for the positive control (C+), which was submitted to in solution digestion. For in gel digestion the protocol in [27] was followed, using 50 mM ammonium bicarbonate (AB) unless otherwise explained. Briefly, the bands on the gel were distained with 50 mM AB in 40 % acetonitrile (ACN). Following, gel pieces were dehydrated with 100 % ACN and lyophilized. Reduction was performed with 50 mM dithiothreitol (DTT) in 50 mM AB, for 30 min at 56 ^{o}C, in the dark, followed by the alkylation performed with 50 mM iodoacetamide (IAA) in 50 mM AB for 30 min, at room temperature, also in the dark. Gel pieces were washed with 50 mM AB + 40 % ACN for 15 min, and a second dehydration step was done. Proteins in the gel were digested for 3 h (Jbtx N-ter and Jbtx C-ter) or 16 h (Jbtx and JbtxΔ-β) in 500 μL of 100 mM AB solution containing 100 ng of sequencing grade trypsin (Promega) at 37 ^{o}C. After digestion, supernatants were transferred to microcentrifuge tubes and gel pieces were washed with 1 % formic acid in 60 % ACN, and the supernatants were combined accordingly. Digested peptides were lyophilized and submitted to tandem mass spectrometry analyses. In the case of in solution digestion, sample of Jbtx peptide (30 μg in 20 mM

sodium phosphate, 5 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5) was reduced with 50 mM DTT and alkylated with 50 mM IAA (30 min each, at room temperature). Then, DTT to a final concentration of 10 mM was added for 15 min at room temperature. Digestion was performed in this case with 0.6 mg of trypsin (Promega) for 3 h at 37°C. After the digestion process, the samples were desalted with ZipTip™ (Millipore®) according to the manufacturer´s instructions. The eluted peptides were lyophilized and analyzed by tandem mass spectrometry.

The lyophilized digested peptides were suspended in 0.1% formic acid (10 μL) and 5 μL of each solution was subjected to reversed phase chromatography (NanoAcquity UltraPerformance LC-UPLC® chromatograph (Waters) using a Nanoease C18, 75 μm ID at 35°C. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and the peptides were eluted in a 20 min gradient, ramping from 0 to 60% acetonitrile in 0.1% TFA at 0.6 nL/min constant flow. Eluted peptides were subjected to electrospray ionization and analyzed by mass spectrometry using a Q-TOF Micro™ spectrometer (Micromass). The voltage applied to the cone for the ionization step was 35 V. The three most intense ions in the range of m/z 200–2000 and +2 or +3 charges were selected for fragmentation. The acquired MS/MS spectra were processed using the Proteinlynx v.2.0 software (Waters) and the generated .mgf files were used to perform database searches using the MASCOT software version 2.4.00 (Matrix Science) against the NCBI database, and taxid was restricted to Viridiplantae (taxid:33090). Results were analyzed manually.

2.6. Electrophoresis

The peptides fractions of Jbtx and its mutant forms were visualized in SDS-Tricine gels [28]. The gels were stained with colloidal Coomassie G-250 (Sigma Chem. Co) according [29].

2.7. Western blot

Western blots were performed according to [30]. Briefly, peptides were electrophoresed, transferred to PVDF membranes (Millipore) and immersed in a blocking buffer consisting of 5%

nonfat dry milk in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl and 4.3 mM $Na_2HPO_4.7H_2O$, pH 7.3). After washing, the membrane was incubated with rabbit anti-jaburetox-2Ec polyclonal antibodies (1:7,500 dilution) for 2 h at room temperature, followed by a 2 h incubation with anti-rabbit IgG (1:20,000 dilution) alkaline phosphatase conjugate (Sigma Chem. Co.). Colorimetric detection was carried out using 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt and nitro-blue tetrazolium chloride.

2.8. Leakage experiment

Large unilamellar vesicles (LUVs) were produced and the leakage experiment was conducted as described previously [17]. LUVs were prepared using 10 mg of L-α-phosphatidic acid (egg chicken, Avant Polar Lipids), at a concentration of 20 mg/mL. The leakage promoted by Jbtx and its mutated forms at a final concentration of 5 μg/mL in 25 mM Tris, pH 7.0, was evaluated by the carboxyfluorescein release assay [17]. The concentration of LUVs in the experiment was estimated based on the absorbance of the fluorescent probe at 490 nm, and adjusted to a value of 0.1. In the leakage assays, fluorescence intensity of the reaction mixture (LUVs plus peptide or buffer) was recorded as a function of time. The samples were excited at 490 nm and the fluorescence was acquired at 518 nm. It was assumed that the absence of leakage (0%) corresponded to the fluorescence of the vesicles at time zero; 100% leakage was taken as the value of fluorescence intensity obtained after the addition of 1% (v/v) Triton X-100. All measurements were carried out in a Cary Eclipse fluorescence spectrophotometer (Varian).

2.9. Insecticidal activity

Fifth-instar *Rhodnius prolixus* were kindly provided by Dr. Hatisaburo Masuda and Dr. Pedro L. Oliveira (Institute of Medical Biochemistry, Universidade Federal do Rio de Janeiro, RJ, Brazil)

and by Dr. Denise Feder (Universidade Federal Fluminense, RJ, Brazil). The phytophagous milkweed bugs (*Oncopeltus fasciatus*) were reared in our laboratory as previously described [14].

2.9.1. Injection assays

Fifth instars of *O. fasciatus* or *R. prolixus* were injected into the hemocoel using a Hamilton Microliter 900 series syringe (Hamilton). Group of 10 insects (*O. fasciatus*) or 5 insects (*R. prolixus*) were injected with 20 mM sodium phosphate buffer (pH 7.5) containing peptides at a final dose of 0.015µg (*O. fasciatus*) or 0.05 µg (*R. prolixus*) per mg of insect body weight. Control insects received injections of buffer alone. Mortality rate within each group was recorded after 48 or 96 hours. Two independent bioassays were carried out for each peptide on each insect model. Results shown are means ± standard errors.

2.9.2. Feeding assays

Fifth instars *R. prolixus* were fed on *R. prolixus* saline solution (150 mM NaCl, 8.6 mM KCl, 2.0 mM $CaCl_2$, 8.5 mM $MgCl_2$, 4.0 mM $NaHCO_3$, 34.0 mM glucose, 5.0 mM HEPES, pH 7.0 (Lane et al, 1975)) containing 1 mM ATP and enough peptide (tested individually) to give final doses of 0.1 µg per mg of body weight. Groups of 5 insects for each peptide were fed for approximately 30 min, at 37 °C, by placing their mouth apparatus inside glass capillaries containing the test solutions. Control insects fed solely on *R. prolixus* saline solution containing 1 mM ATP, under the same conditions. Mortality rate within each group was recorded after 24 hours. One triplicated bioassay was carried out for each peptide. Results shown are means and standard errors.

2.10. Measurement of fluid secretion by *Rhodnius prolixus* Malpighian tubules

The assay was performed essentially as described in [18], using *Rhodnius prolixu*s serotonin-stimulated Malpighian tubules. Secretion rate was expressed as the percentage of fluid secretion

measured after the addition of Jbtx or mutated peptides as compared to serotonin (2.5×10^{-8} M) alone (control). For each peptide and dose, 5-6 replicates were done. Results shown are means ± standard error.

2.11. In vivo Cockroach Metathoracic Coxal-Adductor Nerve-Muscle Preparation

The *in vivo* cockroach metathoracic-coxal adductor muscle preparation was used [31] to characterize further the entomotoxic activity of Jbtx and its mutated versions. Male adult *Phoetalia pallida* (3-4 months after molting) were reared in our laboratory at controlled temperature (22-25°C) on a 12 h:12 h light:dark cycle. Animals were immobilized by chilling and mounted, ventral side up, in a Lucite holder covered with 1 cm soft rubber that restrained the body and provided a platform to which the metathoracic coxae could be firmly attached using entomologic needles. The left leg was then tied at the medial joint with a dentistry suture line connected to a 1 g force transducer (AVS Instruments, São Carlos, SP, Brazil). The transducer was mounted in a micromanipulator to allow adjustment of muscle length. The exoskeleton was removed from over the appropriated thoracic ganglion. Nerve 5, which includes the motor axon to the muscle, was exposed and a bipolar electrode was inserted to provide electrical stimulation. The nerve was covered with mineral oil to prevent dryness and stimulated at 0.5 Hz, 5 ms, with twice the threshold, during 120 min. Twitch tension were digitalized, recorded and retrieved using a computer based software AQCAD (AVS Instruments, São Carlos, SP, Brazil). Data were further analyzed using the software ANCAD (AVS Instruments, São Carlos, SP, Brazil). Jbtx and peptides were dissolved in insect physiological solution (214 mM NaCl, 3.1 mM KCl, 9 mM $CaCl_2$, 0.1 mM $MgSO_4$, 5 mM HEPES, pH 7.2 [32]). The test solutions were prepared daily and 20 µl were injected into the insect's third abdominal segment using a Hamilton syringe.

2.12. Molecular modeling and simulation

The three-dimensional model for Jbtx was built by comparative modeling with MODELLER9v10 [33] employing the structure of the *Canavalia ensiformis* major urease isoform (PDB ID: 3LA4, [19] as template. Ten models were built, stereochemically evaluated and theoretically validated for their three-dimensional profiles with PROCHECK [34] and Verify3D [35], respectively. The best scored model was then selected. The amino-terminal Met residue and the carboxy-terminal LEHHHHHH segment were added with SwissPDBviewer [36]. The Jbtx peptide was then subjected to molecular dynamics (MD) simulations with GROMACS 4.5 suite [37] using GROMOS96 53a6 force field [38] for 500 ns. The systems were solvated in triclinic boxes using periodic boundary conditions and SPC water models [39]. Counterions ($Na+$) were added to neutralize the systems. The Lincs method [40] was applied to constrain covalent bond lengths, allowing an integration step of 2 fs after an initial energy minimization using Steepest Descents algorithm. Electrostatic interactions were calculated with Particle Mesh Ewald method [41]. Temperature and pressure were kept constant by coupling proteins, ions, and solvent to external temperature and pressure baths with coupling constants of $\tau = 0.1$ and 0.5 ps [42], respectively. The dielectric constant was treated as $\varepsilon = 1$, and the reference temperature was adjusted to 300 K. The system was slowly heated from 50 to 300 K, in steps of 5 ps, each step increasing the reference temperature by 50 K, allowing a progressive thermalization of the molecular system. The simulation was performed to 500 ns, with no restraint, considering a reference value of 3.5 Å between heavy atoms for a hydrogen-bond, and a cutoff angle of 30° between hydrogen-donor-acceptor [37].

2.13. Statistical analysis

Data were evaluated by ANOVA followed by the Bonferroni's or Student *t* test using GraphPad Prism software (Version 5.0 for Windows). See legends to figures for more details. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. MD simulation of Jbtx

It has been previously suggested that a prominent β-hairpin in the predicted model of the urease-derived peptide Jbtx could be responsible at least in part for its membrane-disturbing activity and some of its biological properties[15, 17]. The presence of this β-hairpin was confirmed by x-ray crystallographic data of jack bean urease [19], and short simulations of the crystal-derived peptide were performed [43].

In order to establish if this β-hairpin would still be present in the peptide once it has been released from the urease molecule, a 3D-model of Jbtx was constructed using the crystal structure of jack bean urease as template and subjected to molecular dynamics for 500 ns (Figure 2, panels A and B). The MD simulation indicated that Jbtx becomes more globular when in aqueous solution (Figure 2, panel B), changing its conformation along the simulation with an increase of RMSD, as compared to the initial crystal-derived structure (Supplementary Figure 1). The secondary structure of Jbtx changed in solution, with loss of many helix turns and formation of a minute beta sheet. The β-hairpin at Jbtx's C-terminal half was conserved despite the increase in coil content (Figure 2, panels B and D).

Table 1 Complementary primers for the generation of Jaburetox mutants. The underline sequence shows the *Nde*I and *Xho*I restriction site, respectively.

	Size	Sequence
5' Del_β-hairpin	40 -Mer	AGTATGGTCCGACTATTGGTGAAAAGGATTTTGCCCTTTA
3' Del_ β-hairpin	40 -Mer	TAAAGGGCAAAATCCTTTTCACCAATAGTCGGACCATACT
5' N-terminal	25-Mer	CCAA<u>CATATG</u>GGTCCAGTTAAATGA
3' N-terminal	25-Mer	CCCC<u>CTCGAG</u>GGTGAAAGGACAATC
5' C-terminal	25-Mer	CCAA<u>CATATG</u>AAAGCCATTCCTCGT
3' C-terminal	25- Mer	CCCC<u>CTCGAG</u>TATAACTTTTCCACC

3.2. Expression of recombinant Jaburetox (Jbtx) and mutated forms

Aiming to identify motifs probably involved in the biological activities of Jbtx, mutated forms of the peptide lacking the internal β-hairpin (JbtxΔ-β), the N-terminal half (Jbtx C-ter) or the C-terminal half (Jbtx N-ter) domains were constructed. A schematic representation of these peptides is shown in Figure 1.

All the His-tagged peptides were purified and analyzed by SDS-PAGE (Figure 3A and B). The predicted molecular masses of the peptides based on their deduced amino acid sequences are 11193 Da for Jbtx, 9625.6 Da for JbtxΔ-β, 6325.8 Da for Jbtx N-ter and 6772.5 Da for Jbtx C-ter. As it can be observed from the SDS-PAGE results, all the recombinant peptides showed the expected mass, except for the Jbtx-N-ter peptide which behaved as a dimer with an estimated molecular mass of approximately 12 kDa (Figure 3B).

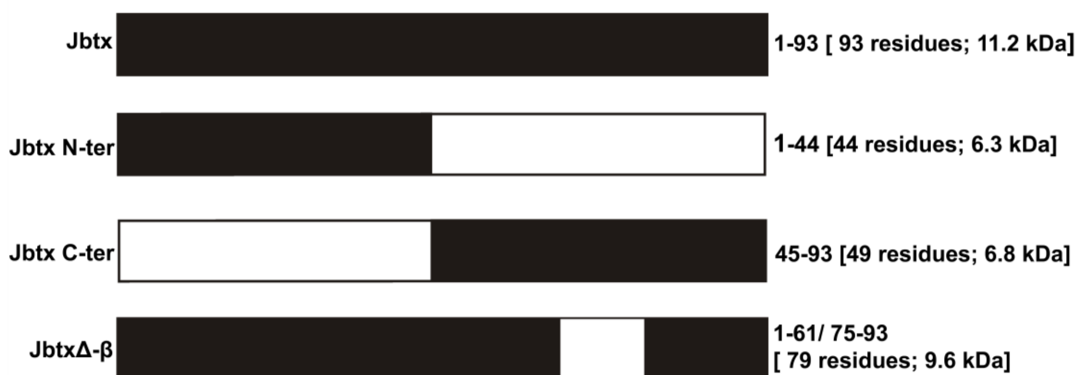

Figure 1. Schematic representation of the sequences of Jaburetox and mutants. The number of amino acids residues of each molecule (shown in black) is indicated on the right side.

Anti-Jbtx-2Ec polyclonal antibodies recognized equally Jbtx-2Ec, Jbtx and JbtxΔ-β (result not shown) and although with a weaker reactivity, also interacted with the two half-peptides (Figure 3C).

All bands seen in the lanes corresponding to each peptide were excised from the SDS-PAGE gels, digested with trypsin and submitted to MS/MS analysis. The identities of the peptides Jbtx, JbtxΔ-β, and Jbtx C-ter (and aggregated forms of the peptides) were confirmed by MS/MS analysis as shown in Figure 3D. On the other hand, the peptide Jbtx N-ter was not identified in the MS/MS assay. The band corresponding to the dimer of the peptide Jbtx N-ter in the SDS-PAGE (Figure 3B) reacted positively with the anti-Jbtx antibodies (Figure 3C), thus confirming its identity. The tendency to form aggregates previously described for jaburetox-2Ec [17, 44] persisted in Jbtx, as well as in all the mutated forms of this peptide, as confirmed by the MS/MS analysis. After a few days in aqueous solution, all the peptides formed insoluble precipitates. These aggregates did not revert to the monomeric state under a number of tested conditions [17].

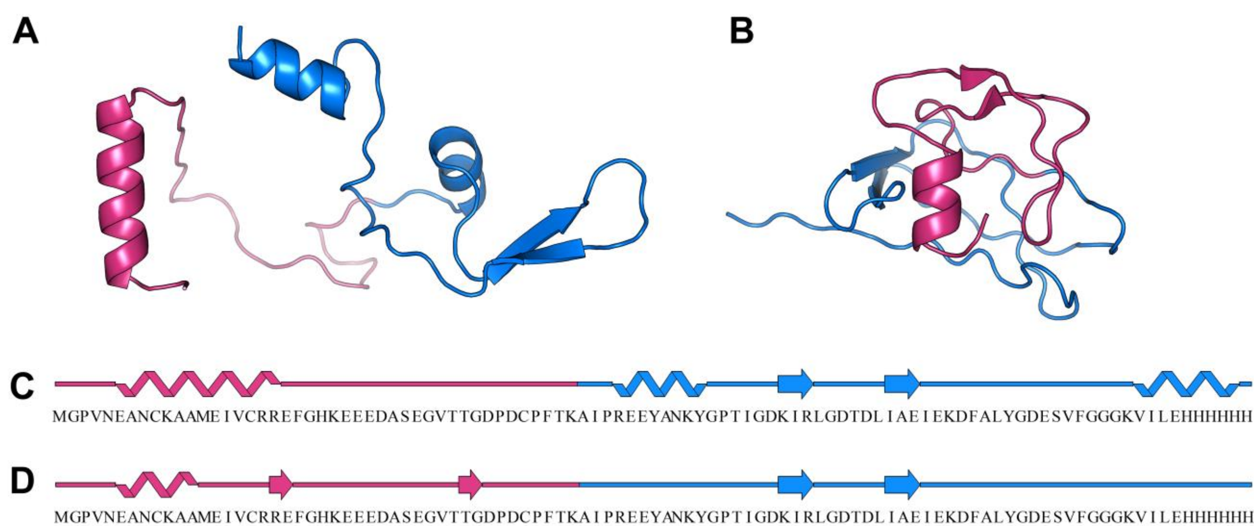

Figure 2. Structural changes in jaburetox and its mutated versions after MD simulation of 500 ns. Three dimensional representations of the full Jbtx peptide (A) initial and (B) final structures, with the N-terminal domain (residues 1-44) depicted in pink and the C-terminal domain in blue; (C) Jbtx N-ter (amino-terminal mutant): top, initial state; bottom, final state; (D) Jbtx C-ter (carboxy-terminal mutant): top, initial state; bottom, final state; Schematic representations of the secondary structure content of the (i) initial and (f) final structures are colored according to their three-dimensional counterparts. The corresponding amino acid sequences are also shown.

High ionic strength accelerates the aggregation of Jbtx (data not shown), suggesting hydrophobic interactions as a driving force for the oligomerization process. Because it was not possible to ascertain the oligomeric state of each peptide in solution, their monomeric states were considered when expressing molar concentrations in the subsequent assays.

Since all mutated peptides retained considerable antigenicity towards anti-Jbtx-2Ec polyclonal antibodies, they probably kept their tridimensional structures, resembling the corresponding portions in Jbtx. The CD spectrum of Jbtx (not shown) indicated the presence mainly of irregular structures, with a minor contribution of β-sheets and helices. This type of CD spectrum has been observed for Chab I, a charybdotoxin analog [45], and also for the acid unfolded state of equine β-lactoglobulin, which has residual helices and β-hairpins [46, 47].

Simulations were carried out with to establish the putative structures in solution of the mutated peptides representing the two half domains of Jbtx. The N-terminal mutant (residues 1 to 44) became completely unfolded after simulation (Figure 2, panel C), while the C-terminal mutant (residues 45 to 93) showed propensity towards stabilization of a newly-formed β-sheet (Figure 2, panel D).

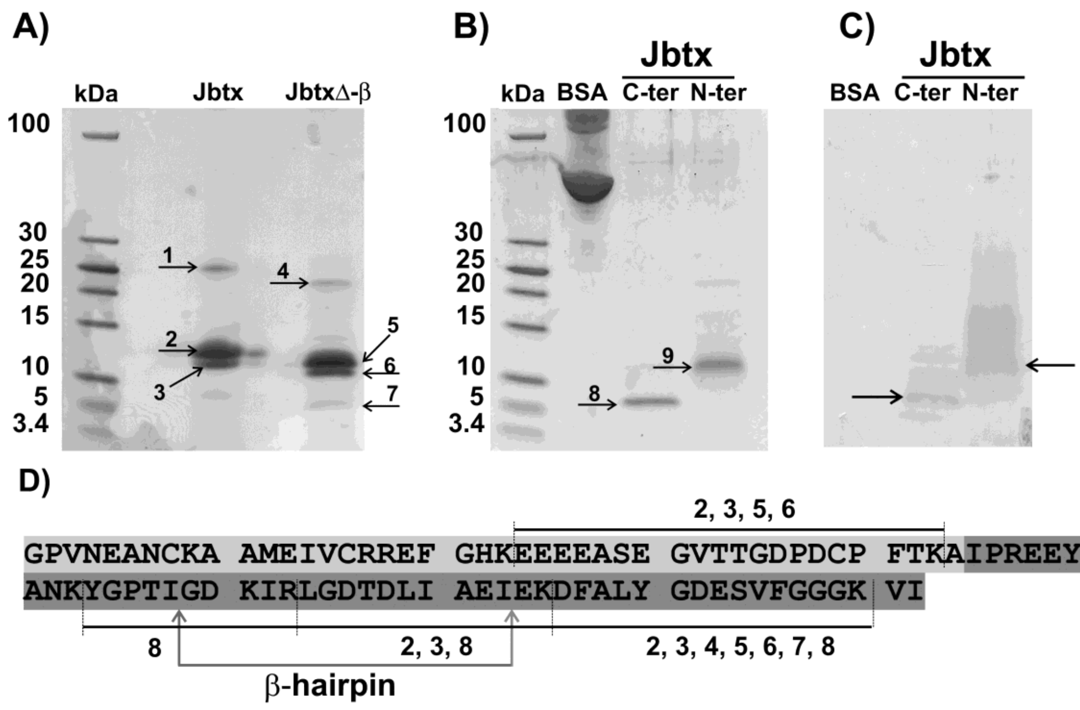

Figure 3 (A and B) SDS-Tricine PAGE of jaburetox and their derived peptides. Numbered arrows indicate the bands that were excised and analyzed by mass spectrometry. Lanes: Jbtx, jaburetox; JbtxΔ-β, jaburetox with deleted β-hairpin motif; BSA, bovine serum albumin; C-ter, carboxy-terminal region of jaburetox; N-ter, amino-terminal region of jaburetox. (C) Western blot analysis with polyclonal anti-jaburetox antibodies. Lanes: BSA, bovine serum albumin as negative control; C-ter, carboxy-terminal region of jaburetox; N-ter, amino-terminal region of jaburetox; (D) Amino acid sequence of jaburetox. The numbered lines above and below the sequence correspond to the arrows in panel A and B, showing parts of the jaburetox sequence identified by mass spectrometry. The sequence of Jbtx N-ter mutant is shown in light gray and that of the Jbtx C-ter in dark gray. The region corresponding to the β-hairpin is also indicated.

3.3. Vesicle leakage promoted by Jbtx peptides

We employed LUVs composed by L-α-phosphatidic acid [17] as a membrane model to evaluate which part of the Jbtx molecule interacts with phospholipid membranes and induces vesicle leakage. Figure 4 shows typical results. Vesicle leakage was more prominent when LUVs were treated with either Jbtx C-ter or Jbtx (5 μg/mL), although all peptides produced at least 80% of leakage at the end of the 10 min incubation period. Taken together, these findings showed that the β-hairpin is not essential for the membrane-disruptive activity of Jbtx. Moreover, the data indicated

that all Jbtx-related peptides are able to induce LUV leakage, while the C-terminal region of the peptide seems to contribute the greatest effect. In fact, hydropathicity plots indicated the presence of prominent hydrophobic regions in both, the N-terminal and the C-terminal domains of Jbtx (Supplementary Figure 2)

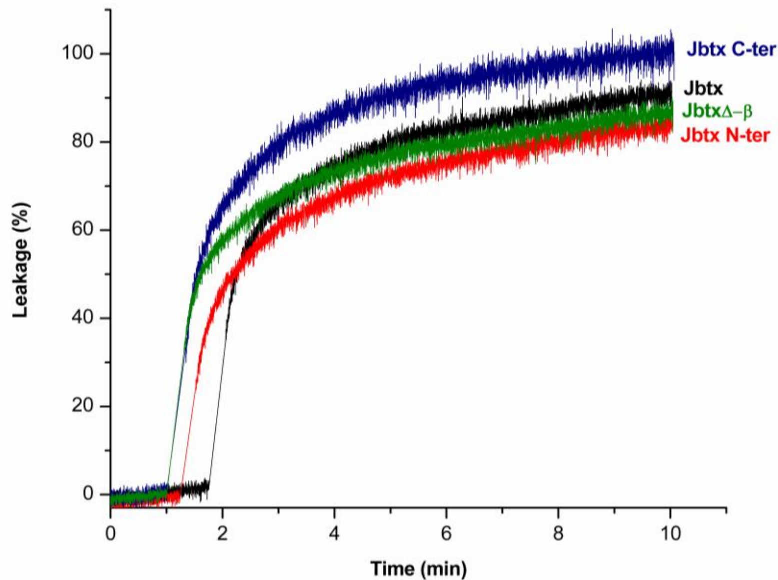

Figure 4. Effect of jaburetox and derived mutants on LUVs composed by L-α-phospatidic acid. The carboxyfluorescein release assay was performed for each peptide at a final concentration of 5 µg/mL (Jbtx, 0.44 µM; JbtxΔ-β, 0.51 µM; Jbtx N-ter, 0.79 µM; Jbtx C-ter, 0.73 µM) in 25 mM Tris, pH 7.0. The absence of leakage (0%) corresponds to the fluorescence of the vesicles at time zero; 100% leakage was taken as the value of fluorescence intensity obtained after addition of 1% (v/v) Triton X-100. The experiments were performed at 25°C. The figure shows superimposed tracings of a typical result for each peptide to facilitate comparison.

3.4. Insecticidal effect of Jbtx peptides

In order to compare the insecticidal activity of Jbtx to that previously described for Jbtx-2Ec [5, 16], we tested the entomotoxic effect of Jbtx upon injection into *R. prolixus* nymphs. Employing a dose of 0.05 µg/mg of insect weight, 100% mortality was observed 48 h after injection (result not shown), indicating that the absence of the V5 epitope in Jbtx did not affect its insecticidal property.

When the insecticidal activity of the β-hairpin-deleted form (JbtxΔ-β) was assayed in *R. prolixus* nymphs, it produced an entomotoxic (mortality) effect equivalent to that of the original Jbtx, either by injection (Figure 6A) or by feeding (Figure 6B).

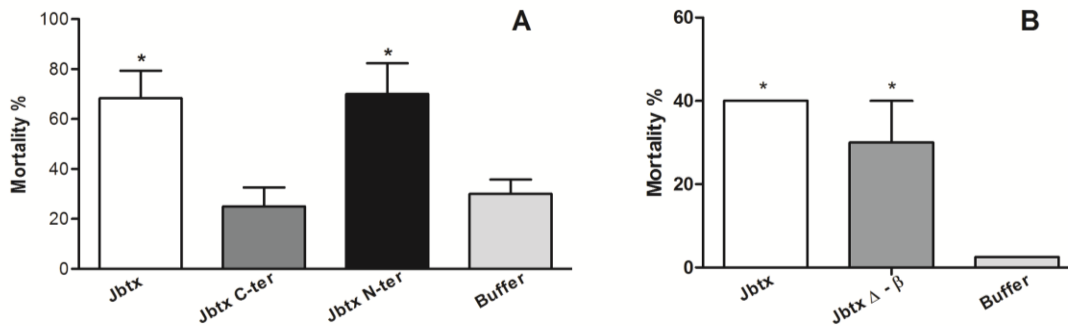

Figure 5. Insecticidal effect of Jbtx and derived peptides on fifth instar *Rhodnius prolixus.* **(A)** Groups of 5 insects were injected with each peptide separately at final doses of 0.05 µg per mg of body weight. Control insects were injected with *Rhodnius* saline. The mortality was recorded after 96 h. Two independent bioassays were carried out for each peptide. Results shown are means and standard error. **(B)** Groups of 5 insects were fed on *R. prolixus* saline plus 1 mM ATP and the peptides separately at final doses of 0.1 µg per mg of body weight. Control insects were fed solely on *R. prolixus* saline plus 1 mM ATP. Mortality rate within each group was recorded after 24 h. Two independent bioassays were carried out for each peptide. Results shown are means and standard error.

Four days after injection into fifth instars *R. prolixus,* we have observed that the Jbtx N-ter induced up to 60% mortality, while Jbtx C-ter caused less than 10% mortality (Figure 6A). On the other hand, 24 hours after feeding, both Jbtx N-ter and Jbtx C-ter had similar lethal effects on *R. prolixus* nymphs, ranging from 60 to 80% mortality (Figure 6B).

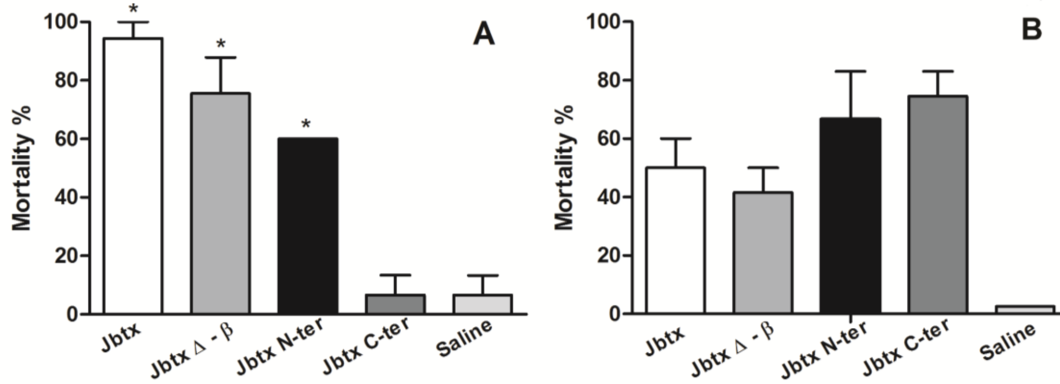

Figure 6. Insecticidal effect of Jbtx and derived peptides on fifth instars *Oncopeltus fasciatus*. **(A)** Groups of 10 nymphs were injected with 1.5 µL of Jbtx, Jbtx N-ter or Jbtx C-ter into the hemocoel (dose of 0.015 µg/mg of insect body weight) or 20 mM phosphate buffer, pH 7.5 (control group). **(B)** Groups of 5 nymphs were injected with 1.5 µL of Jbtx or Jbtx mutant peptides into the hemocoel (dose of 0.015 µg/mg of insect body weight) or 20 mM phosphate buffer, pH 7.5 (control group). The mortality rate was recorded after 96 h. Results are means ± standard error of triplicates of two independent experiments. (*) indicates statistically significant difference ($p \leq 0.05$) from the control group.

Fifth instars *O. fasciatus* were also injected with Jbtx and its mutant variants. Similarly to what was observed for *R. prolixus* upon injections, Jbtx N-ter (Figure 5A) and JbtxΔ-β (Figure 5B) displayed lethal effects comparable to that of Jbtx, while Jbtx C-ter was near to inactive (Figure 5A), suggesting that the N-terminal portion of the Jbtx carries its insecticidal domain.

3.5. Antidiuretic effect of Jbtx-related peptides on Malpighian tubules

We have previously described that, in the dose range of 10^{-16} to 10^{-15} M, Jbtx 2-Ec inhibited the serotonin-stimulated fluid secretion in *R. prolixus* Malpighian tubules [18]. Figure 7 shows that Jbtx and all its variants, at a concentration of 1 x 10^{-15} M, were able to inhibit fluid secretion in the tubules producing similar antidiuretic effect.

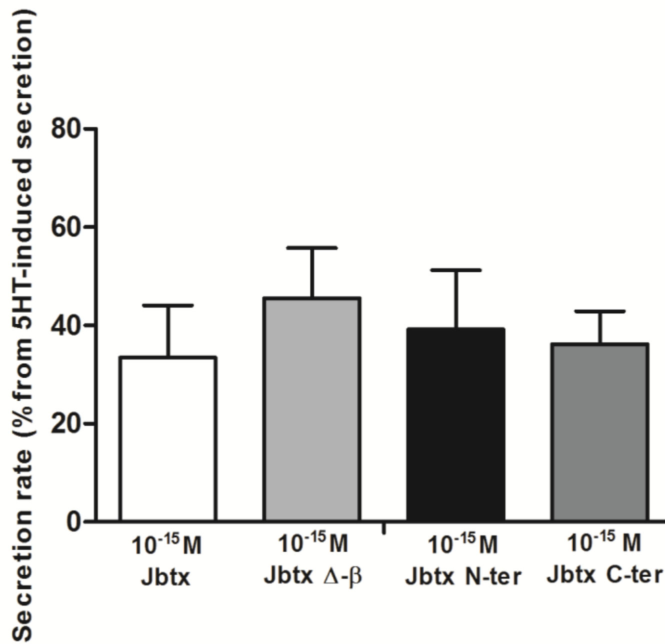

Figure 7. Effect of jaburetox and mutants on secretion of *Rhodnius prolixus* Malpighian tubules. The assay was performed as described by Stanisçuaski et al., 2009. Tubules were incubated with 2.5 x10^{-8} M serotonin (5-hydroxy-tryptamine, 5-HT) for 20 min to record the maximal secretion. After washing, the tubules were incubated with the peptides (1x10^{-15} M) in the presence of serotonin for another 20 min. The secretion rate was expressed as a percentage from the control (serotonin without peptides). Results shown are means ± standard deviation of 5-6 replicates for each peptide.

3.6. In vivo neuromuscular blockade of cockroach nerve-muscle preparations induced by Jbtx-related peptides

The injection of Jbtx or its mutant versions (32μg/g of animal weight) produced a time-dependent blockade of the cockroach nerve-muscle preparation (Figure 8). Jbtx was the most effective and induced a complete neuromuscular paralysis at 35 ± 10 min followed by Jbtx N-ter at 80 ± 2 min. In contrast, the neuromuscular blockades induced by Jbtx Δ−β or Jbtx C-ter were only partial at the end of the 120 min recording time. The administration of insect saline alone did not interfere with normal neuromuscular responses during 120 min recordings (Figure 8, panel A). Thus, similar to what was observed in the case of the insecticidal activity (upon injection), these data suggest that N-

terminal half of Jbtx carries its entomotoxic domain. In this type of assay, however, there is a contribution of the β-hairpin to the effect.

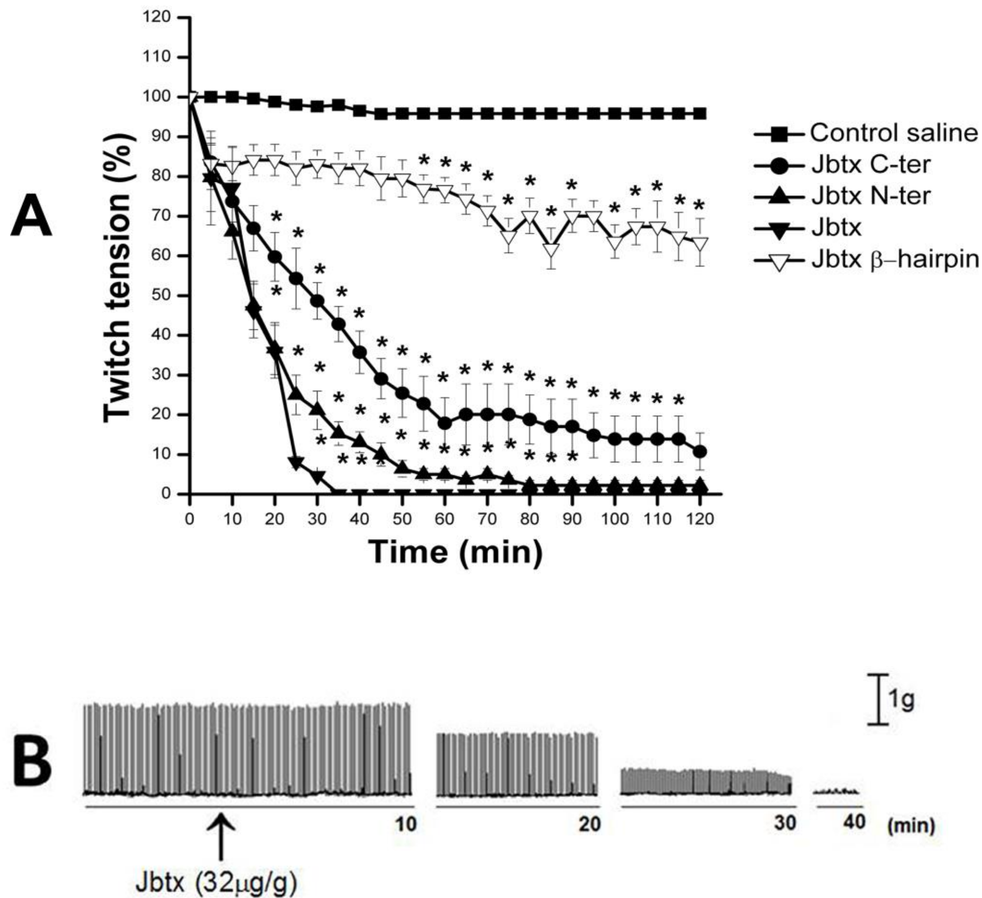

Figure 8. Neuromuscular paralysis induced by Jbtx and peptides on *in vivo* cockroach coxal-adductor methatoracic nerve-muscle preparation. **(A)** time-course of the blockade of the neuromuscular activity in the presence of 32 μg/g of each version of Jbtx peptides against control insects treated only with saline (means ± standard error, n=12). Note that Jbtx and Jbtx N-ter were able to induce complete paralysis. * indicates $p \leq 0.05$ in comparison to control saline, with ANOVA two way and Student *t* test). **(B)** representative myographic 120 min recording of the coxal-adductor methatoracic nerve-muscle preparation of a Jbtx-treated cockroach.

4. Discussion

In this study we evaluated the jack bean urease-derived peptide Jbtx and three domain-deleted variants in order to identify regions of the molecule that are critical for its entomotoxic activities. A previous version of Jbtx, harboring a large V5-antigen derived from the pET101/D-TOPO plasmid and called Jaburetox-2Ec (Jbtx-2Ec), was shown to be lethal to *R. prolixus* by oral route and hemocoel injection [48, 49] and to permeabilize vesicles composed of charged lipids [17]. Jbtx has the same 93 amino acid urease-derived sequence and the polyhistidine tail found in Jbtx-2Ec, but lacks the V5 epitope present in the later. Here we demonstrated that Jbtx displays insecticidal activity equivalent to that described for Jbtx-2Ec, evidencing that the epitope V5 is not implied in its entomotoxicity.

Comparing the structure obtained here for Jbtx with the model of Jaburetox-2Ec generated by comparative modeling [17] (prior to the first description of the crystal structure of a plant urease [19]), important conformational similarities can be seen in the region correspondent to the short helix as well as in the large content of random-coil conformation, even though Jaburetox-2Ec exhibited a more well-defined β-hairpin than Jbtx. The differences in the models generated for these peptides might be attributed in part to the distinct initial structures used in the MD simulation, as *H. pylori* urease and Jack bean urease served as template in the comparative modeling for Jaburetox-2Ec [17] and Jbtx (this work), respectively.

Balasubramanian and Ponnuraj [19] were the first to report in 2010 the crystal structure of a plant (*C. ensiformis*, jack bean) urease at 2.05 Å of resolution. These authors confirmed the presence of an internal β-hairpin motif in the jack bean urease, previously suggested by our group to be present in the structure of Jaburetox-2Ec [15, 17], and proposed to be involved in the insecticidal activity of both urease and its derived entomotoxic peptide. The same group described insecticidal and antifungal properties of the pigeon pea (*Cajanus cajan*) urease and reported the presence of a similar β-hairpin motif in the crystal structure of this urease [8]. Moreover, Balasubramanian and

coworkers, using molecular modeling studies and short (5 ns) molecular dynamics simulations of Jbtx, suggested that its β-hairpin could self-associated into a β-barrel able to anchor into a membrane-like environment, and hypothesized an insecticidal mode of action of Jbtx based on pore formation [43].

Also present in bacterial ureases, the microbial β-hairpin motif is formed with contributions from the α- and the β urease chains while its counterpart in the single chain of plant ureases is formed exclusively by amino acids located in a region corresponding to the bacterial α-chain [19]. Our group reported that, contrasting to plant ureases, *Bacillus pasteurii* urease has no insecticidal activity against *Dysdercus peruvianus* [50]. Since the β-hairpin motif is present in the *B. pasteurii* urease as well [19], a plausible explanation for this could be the fact that part of the sequence corresponding to the N-terminal half of Jbtx is missing in bacterial ureases.

Here we demonstrated that Jbtx and JbtxΔ-β, its β-hairpin deleted version, behaved almost indistinguishably regarding LUVs leakage, antidiuretic effect and insecticidal activity upon injection. These results strongly suggested that the β-hairpin motif is not involved in membrane-disturbing activity or in these biological properties of the peptide.

At that point we had no clues to any other possible motif in the Jbtx molecule that could be responsible for its biological properties, so we decided to produce two half-peptides, corresponding to the N-terminal and C-terminal half versions of Jbtx (Jbtx N-ter and Jbtx C-ter, respectively). The mutated peptides Jbtx N-ter and Jbtx C-ter were then tested for LUV leakage and for different types of entomotoxic activities. Two distinct groups of results were obtained depending on the assay: (i) Jbtx, JbtxΔ−β and Jbtx N-ter were equally active while and Jbtx C-ter was inactive or significantly less active; and (ii) all the peptides produced similar effects.

When tested for insecticidal activity upon injection into *O. fasciatus* (Figure 5) or *R. prolixus* (Figure 6A) nymphs, Jbtx, Jbtx Δ−β and Jbtx N-ter caused significant mortality after 96 h, while the survival rate of insects injected with Jbtx C-ter was equivalent to that of control group. It thus

became clear from these experiments that the N-terminal half of Jbtx (Jbtx N-ter) has the insecticidal domain of Jbtx. This conclusion agrees with the fact that the deletion of the β-hairpin, which is present in the Jbtx C-ter, did not interfere on the entomotoxicity.

On the other hand, all the peptides were able to induce blockade of the cockroach neuromuscular junction *in vivo* (Figure 8). The neuromuscular blockade induced by Jbtx resembles the effect of neurotoxins which act directly on receptor ion channels [51], among which are pore-forming neurotoxins [52]. In this work we did not attempt to elucidate the pharmacological interactions of Jbtx and related peptides at specific sites of insect neuromuscular junctions. The Jbtx N-ter peptide had an effect comparable to that of the intact peptide producing almost complete neuromuscular blockade after 40 min of recordings while JbtxΔ−β and Jbtx C-ter were clearly less active. The loss of activity of JbtxΔ−βin this biassay may reflect some critical alteration of the peptide 3D-structure affecting also its N-terminal domain, which alone is capable of producing full effect in the absence of the β-hairpin.

Upon feeding to *R. prolixus* (Figure 6B) all the peptides were lethal, even Jbtx C-ter, contrasting with its lack of activity when injected into the hemolymph. This fact points to the presence of two active domains in the Jbtx molecule, with the amphipatic β-hairpin in the C-terminal domain probably interacting with insect's gut membranes as predicted, when given by oral route. This conclusion could also be drawn from the fact that all the peptides had equivalent antidiuretic effects (Figure 7) and that both terminal domains of Jbtx were able to induce leakage of vesicles (Figure 4). Preliminary results using Planar Lipid Bilayers, another artificial membrane composed only of lipids, also showed that all mutant forms of Jbtx display membrane disturbing properties and form ion channels (Piovesan A., unpublished data). On the other hand, Jbtx C-ter showed significantly lower activity than Jbtx or Jbtx N-ter, when its first contact within the insect was with the hemolymph probably due to a "saturating" effect of the lipid-rich medium on its membrane-disturbing property.

All the peptides, including Jbtx C-ter, produced antidiuresis or were lethal given by oral route, circumstances where their first interaction happened with single cell layered tissues such as the Malpighian tubules [53] or the gut [54]. One hypothesis to explain the lack of specifity of these assays to discriminate the different Jbtx variants could be that biological multilayered tissues systems, such as the neuromuscular junction [55] and the whole insect (by injection, skipping the first contact with the gut), probably add additional levels of tissue- or cell specificity to the entomotoxic effects of Jbtx-related peptides. Altogether, our data indicate that the main entomotoxic domain of the urease-derived peptide Jbtx is located in its N-terminal half. However, depending on the bioassay, the C-terminal domain and/or its β-hairpin motif could also contribute part of the biological activity of Jbtx.

From the molecular dynamics simulation, it seems that the monomeric Jbtx peptide is mostly formed by coils (Figure 2). Our simulation results confirm and expand previous theoretical observations [17], such as the compaction of the peptide in solution. Simulations of the half-peptides indicated that after 500 ns Jbtx N-ter adopts a random coil conformation while Jbtx C-ter acquires a newly-formed β-sheet (Figure 2). These data may explain why Jbtx is highly prone to aggregation[17], and the instability of Jbtx N-ter in aqueous solution (unpublished results), possibly a consequence of the unfolding of the highly hydrophobic N-terminal of Jbtx that would require protein-protein (or protein-membrane) contact to stabilize.

Presently, to the best of our knowledge, it is not possible to compare the MD simulated structure of Jbtx to that of any other known insecticidal or membrane-disrupting peptide. The high level of coils, especially in the N-terminus, may be related to the peptide toxicity, since some toxins employ these unfolded states as recognition motifs. One example of such toxins is colicin, from *E. coli* [56]. These unfolded recognition domains may be advantageous for the toxins that carry them, since they allow these proteins to overcome steric restrictions while providing large average interaction surfaces per residue [56-58]. There are many reports in the literature of folding and

oligomerization of proteins and peptides that acquire their biologically active state upon interaction with lipids or membranes. Examples are cecropin A, a 37-residue insect antimicrobial peptide [59, 60], the Cyt1Aa toxin produced by *Bacillus thuringiensis* [61], anticancer β-hairpin peptides [62], antimicrobial, cell-penetrating peptides and fusion peptides such as the HIV fusion peptide FP23 [63], to cite a few.

The tendency to oligomerize and to interact with lipids exhibited by Jbtx brings the question whether the active form of the peptide (or its N-ter and C-ter versions) is an oligomer rather than a monomer. The oligomerization/aggregation phenomenon was also observed for Jbtx-2Ec, causing an enormous impact of the membrane-disruptive ability of the peptide [17].

Jbtx has promising biotechnological potential as a biopesticide. We are currently testing transgenic Jbtx expressing sugar cane (*Saccharum officinarum*) plants, and so far we have observed an increase of resistance to several species of lepidopterans in greenhouse conditions (Becker-Ritt et al., unpublished data). These data indicate the effectiveness of the peptide as an environment friendly insecticide with practical application, reducing crop losses while avoiding the use of chemical toxic agents.

We conclude that the urease-derived peptide Jbtx probably represents a new example of membrane-active peptide with insecticidal and fungitoxic activities. Its insecticidal activity was tracked down mostly to its N-terminal region and does not require the prominent β-hairpin present in the C-terminal region, although this part of the molecule probably contributes to its overall entomotoxic properties. Understanding the complex behavior of these peptides in solution as well as in the presence of lipids and biological membranes is a critical step towards unraveling their mechanisms of action and exploiting their potential as insecticidal agents.

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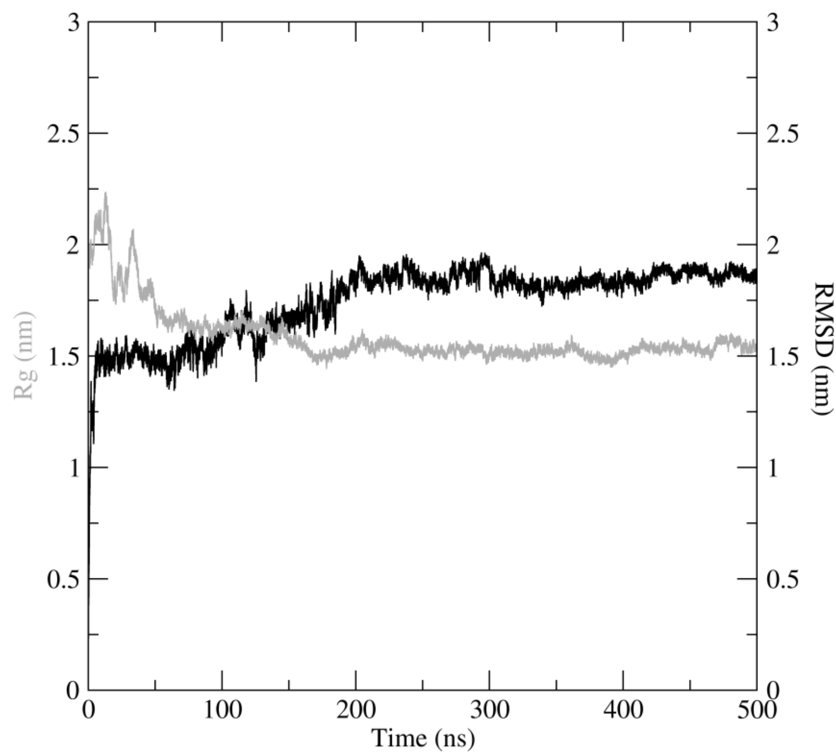
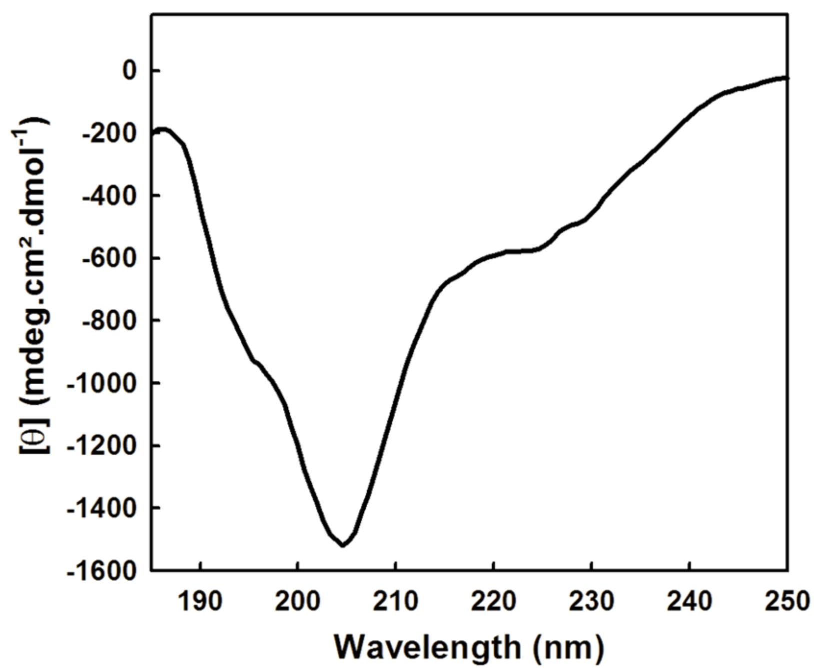
Supplementary Figure



Supplementary Figure 1. Time course of conformational changes of Jbtx in aqueous solution. All-atom root mean square deviation (RMSD, grey line), and radius of gyration (black line) of the Jbtx molecular dynamic simulation during 500 ns.



Supplementary Figure 2. Hydropathicity plot for jaburetox. The profile was calculated considering the Kyte-Doolittle scale (see Methods) and a window size of 7 residues. The position of the β-hairpin motif in the C-terminal domain is indicated.

CAPITULO II

Eletrofisiologia: estudo sobre os canais iônicos formados por Jaburetox e seus mutantes derivados

Justificativa:

Considerando a obtenção dos mutantes do jaburetox descrita no Capítulo I dessa tese, e os dados que mostram a capacidade desses em alterar a permeabilidade de lipossomos, o Capitulo II relata estudos eletrofisiológicos no modelo de *Planar Lipid Bilayer* (PLB) foram testados os diferentes peptídeos visando identificar motivos estruturais envolvidos na interação destes com as membranas lipídicas e formação de canais iônicos.

Objetivos específicos

- Analisar a capacidade do peptídeo jaburetox (jbtx) e seus mutantes derivados jaburetox Δβ-hairpin (jbtxΔ-β), jaburetox N-terminal (jbtx N-ter) e jaburetox C-terminal (jbtx C-ter) de formar canais iônicos em bicamadas lipídicas no ensaio com *Planar Lipid Bilayers* (PLB);
- Caracterizar os canais iônicos formados pelos diferentes peptídeos, quantificando a corrente iônica e suas condutâncias. Além disso, determinar a seletividade iônica dos canais, verificando se o canal tem capacidade de discriminar ions K^+ ou Cl^-.
- Comparar a atividade dos diferentes mutantes deste peptídeo, visando identificar os motivos estruturais envolvidos neste processo.

Materiais e Métodos

Reagentes

Os fosfolipídeos PE (L-α-phosphatidylethanolamine Heart, Bovine) e PC (L-α-phosphatidylcholine Heart, Bovine) foram adquiridos de Avanti Polar Lipids. O Colesterol (C3045) foi adquirido de Sigma Aldrich.

Jaburetox e mutantes derivados

Os peptídeos recombinantes Jbtx, JbtxΔ-β, Jbtx N-ter e Jbtx C-ter foram purificados de acordo com a descrição do capítulo I. Após a purificação, os peptídeos foram quantificados pelo método de Bradford para realização dos ensaios.

Ensaio de bicamadas lipídicas planares (PLB)

Este método foi inicialmente descrito por Mueller *et al*. (1962), consiste em estudar canais iônicos formados em uma bicama lipídica artificial. Esta bicamada é inserida ou "pintada" em um orifício que separa dois compartimentos aquosos. Resumidamente, um bloco feito de teflon, também chamado de câmara, é cortado em dois compartimentos circulares. O primeiro compartimento chamado *trans* fica interligado ao segundo compartimento *cis*, que irá comportar uma cubeta que possui um orifício de 250 μm. A bicamada lipídica será inserida nesta abertura entre dois compartimentos aquosos *cis* e *trans*. O compartimento *cis* é conectado a um gerador de voltagem através de um eletrodo de Ag/AgCl, para controlar o potencial transbicamada. O compartimento *trans* é conectado a entrada do amplificador que mede a corrente, através do segundo eletrodo de Ag/AgCl A proteína a

ser testada é adicionada no lado *cis.* Assim que ocorre a incorporação da proteína na membrana, sob voltagem controlada, a corrente que flui pelo canal pode ser monitorada.

O ensaio eletrofisiológico com membranas lipídicas planares foi realizado segundo protocolo previamente descrito (Peyronnet, *et al*, 2001; Peyronnet *et al*., 2000). As bicamadas lipídicas foram formadas a partir de uma mistura lipídica composta de fosfatidiletanolamina (PE), fosfatidilcolina (PC) e colesterol (Ch) na proporção de 7:2:1 respectivamente, conforme Schwartz *et al.*(1993). Os lipídios foram preparados em *n*-decano (solvente apolar) na concentração final de 25 mg/mL. Na figura 6 está a foto do sistema de PLB. Os compartimentos aquosos *cis* e *trans* foram preenchidos com tampão contendo 500 mM de KCl, 1 mM de $CaCl_2$ e 10 mM de HEPES, pH 7,5 (condição simétrica). Antes de montar o sistema, o orifício de 250 µm da cubeta, feita de resina poliacetal, foi pré-tratado com a mistura lipídica, cobrindo as paredes internas do orifício também com uma camada da mistura lipídica, para posteriormente a membrana poder se fixar. Após o pré-tratamento, a cubeta foi submetida a um leve jato de nitrogênio para secar a camada lipídica formada em volta do orifício.

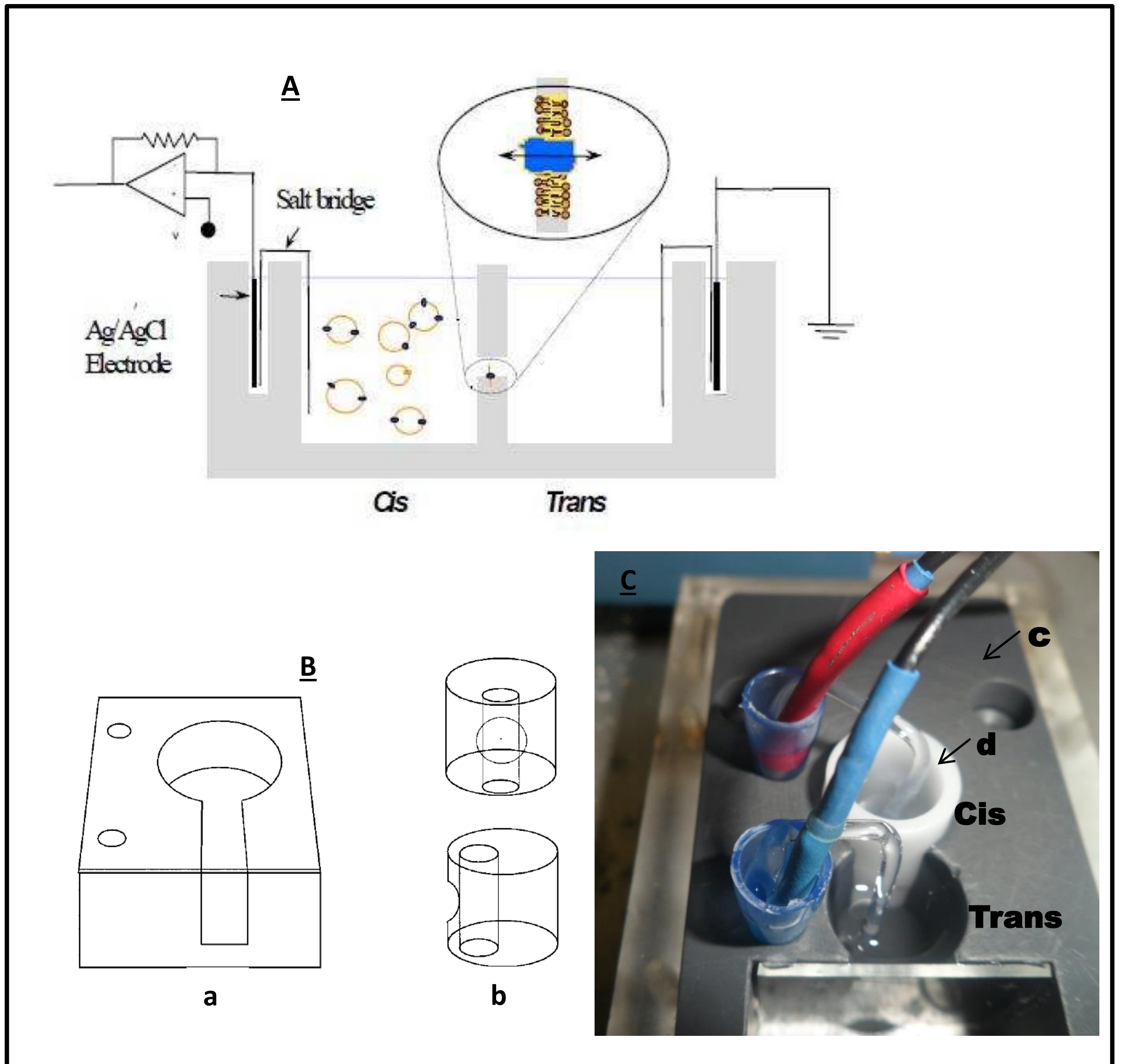


Figura. 5. A. Esquema do sistema da técnica de PLB, em que a bicamada lipídica é formada na abertura de 250 μm entre os compartimentos *cis* e *trans*, como exemplificado na figura. Extraído de Ghazi, 2003. **B**. Esquema representativo da câmara utilizada para os experimentos de PLB. (a)**.** Mostra a câmara com compartimento *cis e trans*. (b) Representa o compartimento cis que comporta a "cup" que contem o orifício de 250 μm em que a bicamada lipídica será pintada. Figura retirada de William et al. 1994.**C.** Imagens do sistema da técnica de PLB. (c). A câmara com os compartimentos *cis* e *trans* feita de Teflon. (d)**.** cubeta de poliacetal (cor branca), com orifício de 250 μm (local que a camada lipídica é "pincelada").

Estando as cubetas já imersas no tampão do experimento, a bicamada lipídica foi “pincelada” através do orifício usando um fino bastão de vidro de ponta arredondada, mergulhado previamente na solução lipídica. A pequena gota lipídica formada na extremidade deste bastão foi então espalhada no orifício (Figura 6).

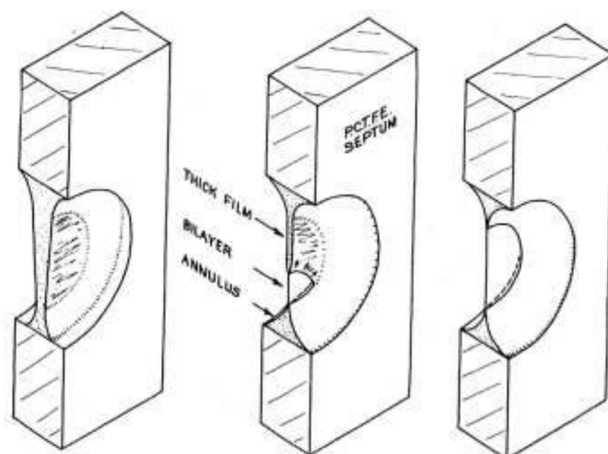


Fig.6. Formação da bicamada lipídica na abertura de 250 µm. No primeiro momento tem-se uma multicamada (multilayer) que em poucos segundos e estendida em uma bicamada (bilayer). A bicamada e mantida pelo “annulus”, uma estrutura com massa lipídica milhares de vezes maior que a bicamada constituída de micelas lipídicas. Fonte: Miller, 1986.

A membrana funciona como um capacitor, pois é um ótimo isolante elétrico e acumula cargas. Nos experimentos foram utilizados valores de capacitância típicos entre 150 e 200 pF (picoFarad), sendo que a espessura da membrana é inversamente proporcional ao valor de capacitância, ou seja, quanto maior o valor de capacitância, mais fina é a espessura da membrana. A incorporação dos peptídeos às membranas foi realizada adicionando alíquotas da proteína (15 µg) ao volume de 1 ml da câmara *cis*, após a formação da bicamada. No momento da inserção da proteína começava o registro de corrente, indicando a formação de um canal iônico. A atividade do canal foi monitorada por mudanças em forma de degraus no registro de corrente iônica com a aplicação de

voltagem através da PLB. Todos os experimentos foram executados a temperatura ambiente (20-25°C).

Para verificar a capacidade dos peptídeos em discriminar espécies iônicas, experimentos de seletividade iônica foram realizados com os peptídeos já inseridos na membrana e apresentando atividade de canal. Então, foi aplicado voltagem zero no sistema e criado um gradiente eletroquímico entre os dois compartimentos através da adição de uma solução concentrada de KCl de forma a estabelecer o gradiente 1.5/0.5 M de KCl (*cis:trans*), monitorando-se uma mudança na linha de base de corrente iônica. Esta mudança indicará a espécie iônica que flui pelo canal (condição assimétrica).

Gravações dos dados de PLB e análises

As conexões elétricas entre a câmara e instrumentos de gravação foram feitas por eletrodos da Prata/cloreto de prata e ponte salina/ágar (4% em 1 mol $^{-1}$ KCl). A corrente que flui pelos canais foi gravada utilizando-se o amplificador Axopatch-1D patch-clamp (Axon instruments, Foster City, California, USA), exibido em um osciloscópio (Kikusui 5040, Tokyo, Japan) e armazenado em um gravador digital CRC VR-100A (Instrutech Corp., Great Neck, New York, USA). As análises foram realizadas com o programa pClamp versão 6.02. A condutância dos canais foi estimada a partir das inclinações das regressões lineares de curvas corrente/voltagem (I/V). O potencial de reversão V_R, foi obtido pelo ponto que intercepta o eixo x na regressão linear na curva corrente/voltagem e foi comparado com o valor teórico calculado para K^+, de acordo com a equação de Nernst (Hille, 2001).

Resultados

Em condições simétricas, a atividade de canal para Jaburetox e os diferentes mutantes foi observada quando concentrações de 15 µg/ml de peptídeo foram acionados ao compartimento *cis*. O tempo de inserção das proteínas na membrana artificial variou de minutos a horas, mas em geral ocorria inserção após 20-30 minutos de experimento. Traçados de corrente representativos de alguns experimentos podem ser vistos na figura7.

Os peptídeos jbtx e jbtxΔ-β, que possuem tamanhos muito próximos, foram testados na concentração de 15 µg/ml, equivalente a 1,3 nM. Os resultados mostrados na figura 8 indicam que ambos, jbtx e jbtxΔ-β, se inserem na PLB e formam canais iônicos, com cinéticas bastante semelhantes. Os mutantes jbtx N-ter e jbtx C-ter possuem metade do tamanho dos outros peptídeos, ou seja, na mesma concentração de 15 µg/ml representam a dose molar de 2,6 nM. Conforme ilustrado na figura 8, esses mutantes também são capazes de se inserir na PLB e formar canais iônicos, mas são menos ativos em induzir a abertura de canais.

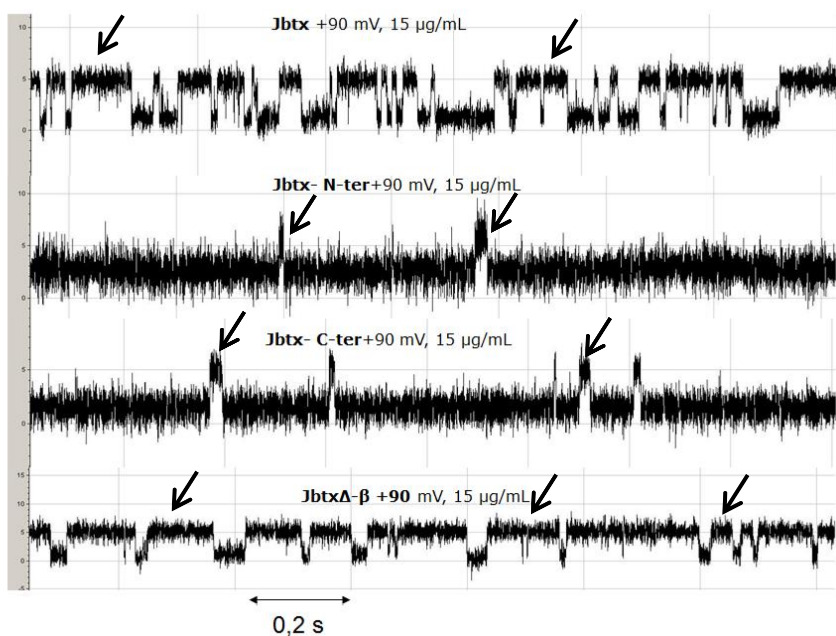


Figura 7. Segmentos representativos de traçados de corrente de canais formados por jbtx, jbtx N-ter, jbtx C-ter e jbtxΔ-β. Os traços foram gravados em condição simétrica com 500 mM KCl, sob voltagem de + 90 mV. As setas exemplificam alguns momentos em que os canais estão abertos.

Quando quantidades menores dos peptídeos jbtx N-ter e jbtx C-ter foram adicionadas (5 e 10 μg) ao ensaio, não foi observado atividade de canal e/ou o tempo de inserção nas membranas era muito demorado. Para todos os peptídeos foi observada abertura de canais em diferentes voltagens, variando de -20 a -100 mv e de +20 a +100 mv.

Os gráficos que correlacionam voltagem aplicada X corrente observada, mostram diferentes níveis de condutância resultantes dos canais formados pelos diferentes peptídeos (Figuras 8, 9, 10 e 11). Cada reta caracteriza canal formado, indicando que os peptídeos formam apenas um tipo de canal quando inseridos nas membranas. As condutâncias encontradas variaram de 32,10 ± 0,36 pS e 43,30 ± 2,73 pS (tabela I).

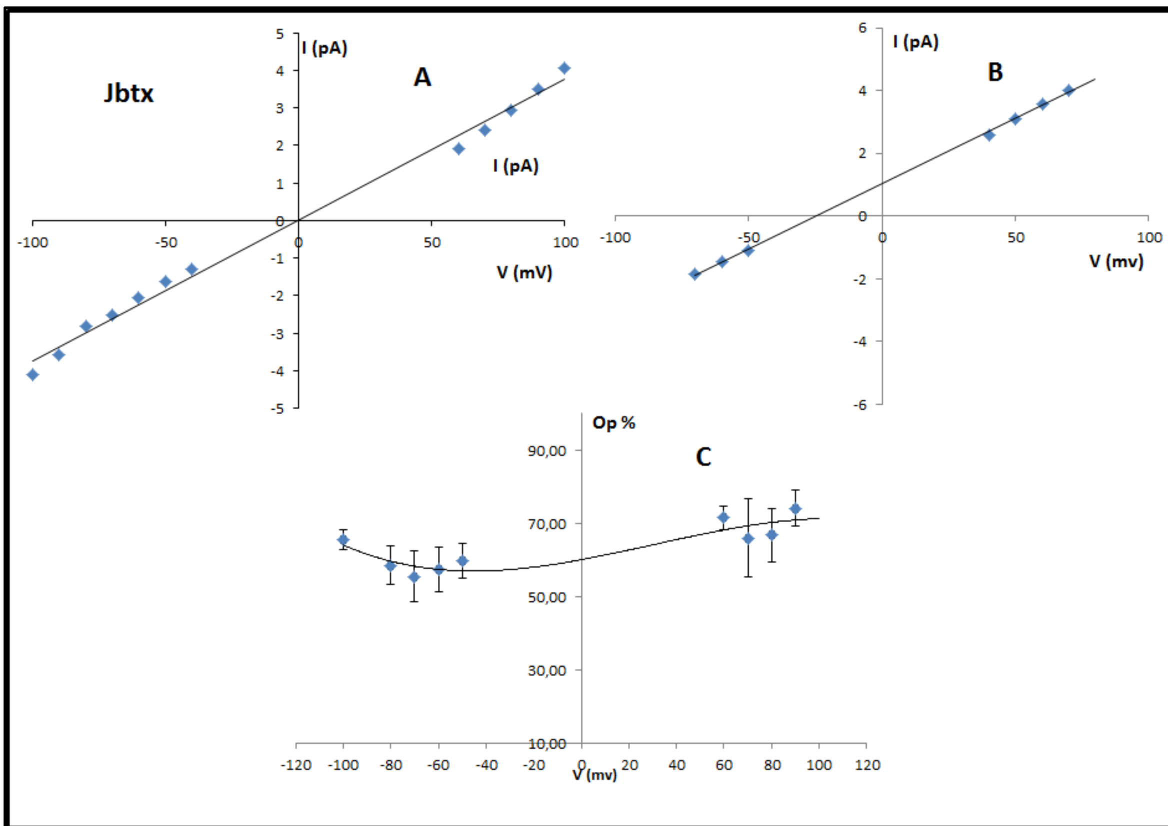


Figura 8. Propriedades biofísicas do canal formado pelo jbtx. **A.** gráfico mostrando a relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição simétrica contendo 500 mM de KCl, pH 7,5. O canal apresentou condutância de 36,43 ± 0,71 pS. **B.** relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição assimétrica 0,5:1,25 M de KCl (cis:trans). O canal apresentou condutância de 52,93 ± 7,48 pS. **C.** Probabilidade de abertura do canal em diferentes voltagens.

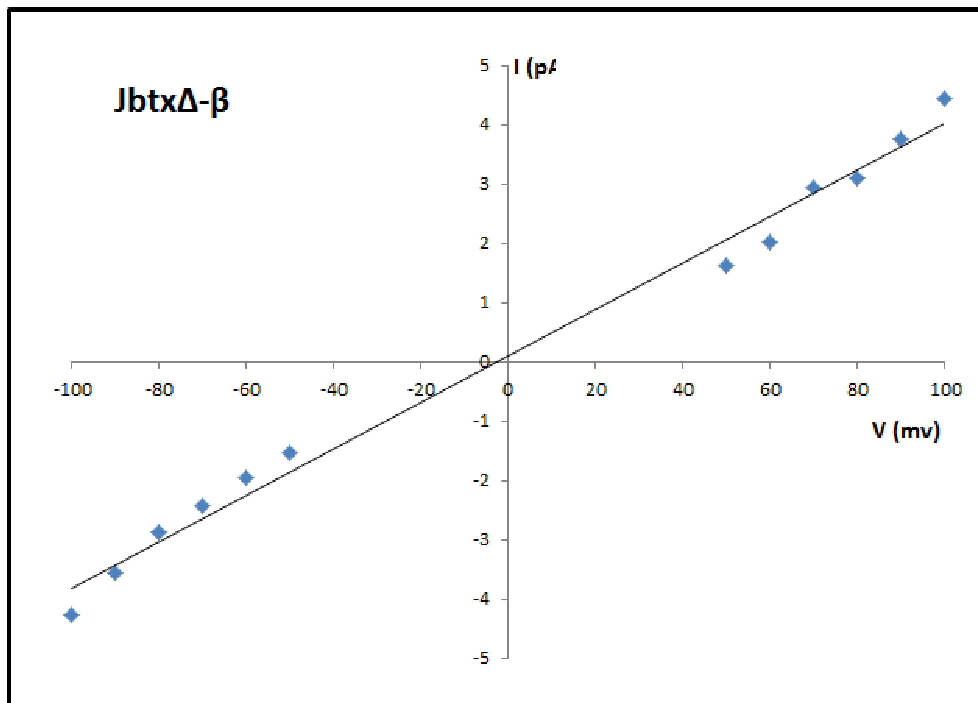


Figura 9. Propriedades biofísicas do canal formado pelo JbtxΔ-β. A relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição simétrica contendo 500 mM de KCl, pH 7,5. **A.** o canal formado por JbtxΔ-β apresentou condutância de 37,7 ± 1,5 pS.

Nos experimentos de seletividade iônica (condição assimétrica), constatou-se que o jbtx e os mutantes têm seletividade maior para cátions (K+). Esta característica do canal é determinada através do gradiente eletroquímico gerado no sistema, enquanto a voltagem é zero. Por definição, quando a mudança na linha de base da corrente é positiva, indica que cátions estão fluindo através do canal, neste caso, K^+. É importante ressaltar que íons cloreto também conseguem fluir através do canal, mas em quantidade menor, em relação a potássio. Este resultado pode ser visto na figura (8B, 10B e 11B), que mostra a equação da reta deslocada em relação á condição simétrica.

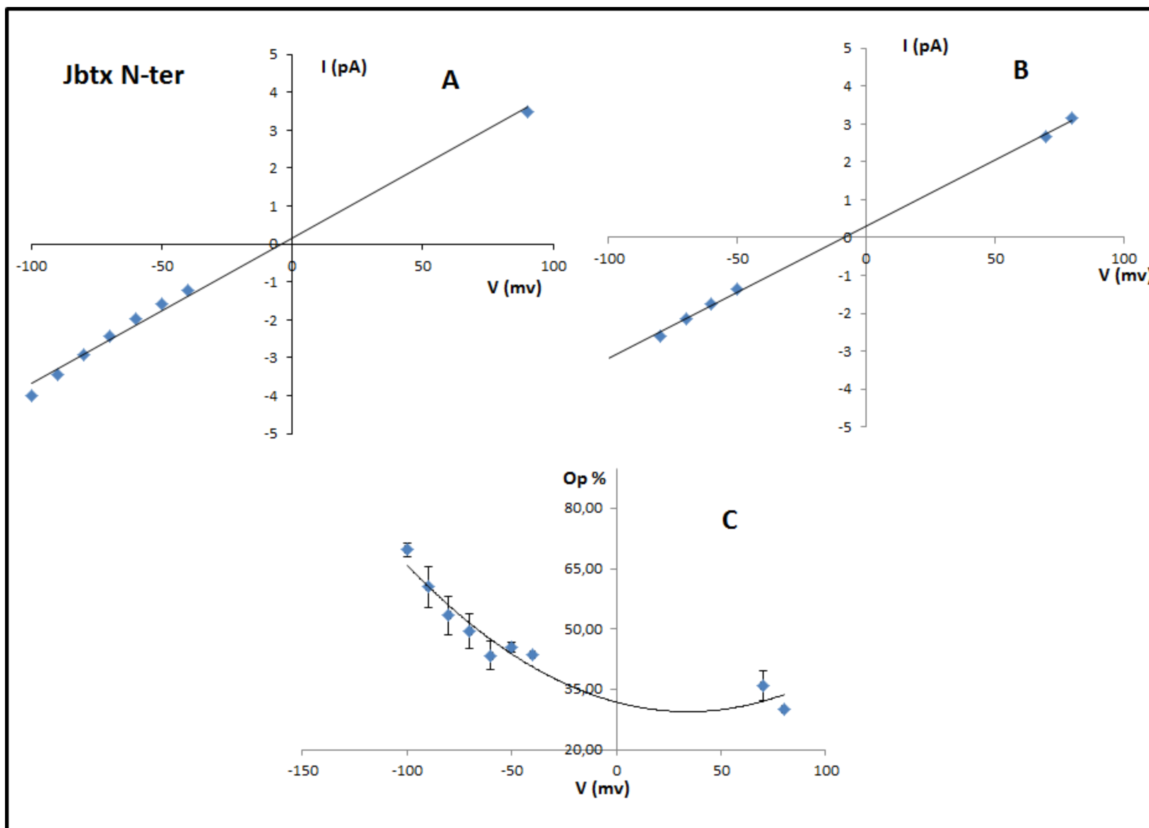


Figura 10. Propriedades biofísicas do canal formado pelo jbtx N-ter. **A.** A relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição simétrica contendo 500 mM de KCl, pH 7,5. O canal apresentou condutância de 32,10 ± 0,36 pS. **B.** relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição assimétrica 0,5:1,25 M de KCl (cis:trans). O canal apresentou condutância de 46,20 ± 3,60 pS. **C.** Probabilidade de abertura do canal em diferentes voltagens.

O potencial de reversão para os diferentes peptídeos ficaram deslocados de -20,08 ± 7,63 mV até -27,82 ± 4,78 mV para voltagens negativas, comparado com condição simétrica de KCl (tabela I).

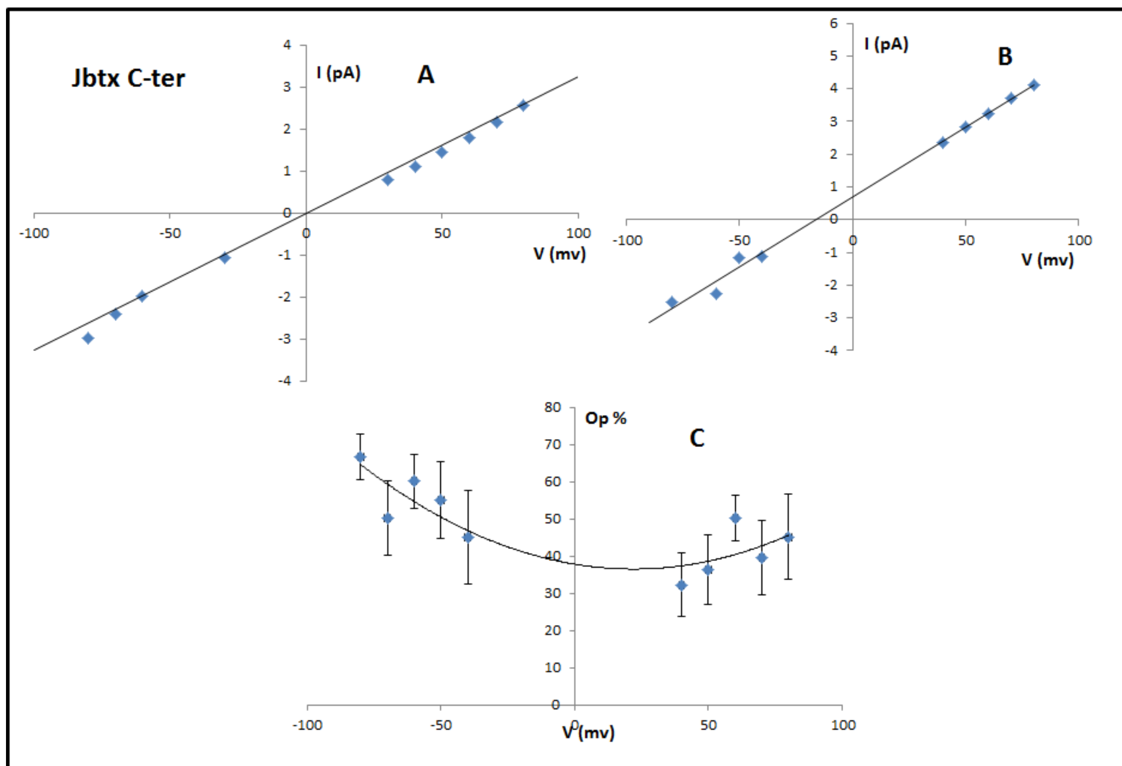


Figura 11. Propriedades biofísicas do canal formado pelo jbtx C-ter. **A.** relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição simétrica contendo 500 mM de KCl, pH 7,5. O canal apresentou condutância de 32,10 ± 0,36 pS. **B.** relação corrente (pA) X voltagem (mV) do canal sob diferentes voltagens em condição assimétrica 0,5:1,25 M de KCl (cis:trans). O canal apresentou condutância de 46,20 ± 3,60 pS. **C.** Probabilidade de abertura do canal em diferentes voltagens.

Para o mutante jbtxΔ-β não foi realizado o ensaio de seletividade, mas como os demais peptídeos foram seletivos para potássio, acreditamos que este possua a mesma seletividade para cátions.

Tabela 1. Propriedades biofísicas dos canais iônicos formados por jbtx e os mutantes derivados.

Propriedade dos canais / Peptídeos	Condutâncias (pS) (Condição simétrica)	Condutâncias (pS) (condição assimétrica)	Potencial de reversão (mv)
Jbtx	36,43 ± 0,71 (n=3)	52,93 ± 7,48 (n=3)	-27,82 ± 4,78 (n=3)
JbtxΔ-β	37,7 ± 1,5 (n=2)	_	_
Jbtx C-ter	43,30 ± 2,73 (n=3)	38,5 ± 3,5 (n=3)	-23,17 ± 7,90 (n=3)
Jbtx N-ter	32,10 ± 0,36 (n=3)	46,20 ± 3,60 (n=3)	-20,08 ± 7,63 (n=3)

A probabilidade de abertura dos canais (Po) formados pelos diferentes peptídeos pode ser visto na figura (8C, 10C, 11C e tabela II). De modo geral os peptídeos não apresentaram canais voltagem dependentes, pois a probabilidade de abertura na mesma voltagem positiva e negativa não variou muito,como pode ser verificado na tabela II.

Tabela II. Probabilidade de abertura dos canais iônicos formados pelos diferentes peptídeos.

Peptideo	Probabilidade de abertura %			
	+ 80 mV	- 80 mV	+ 70 mV	-70 mV
Jbtx	67±7,23	59±5,16	66±10	55±6,89
Jbtx N-ter	-	53±4,8	36±6,7	49±4,32
Jbtx C-ter	45±11,4	67±6,14	40±10,05	50±10,01

Discussão

A habilidade da urease de *C. ensiformis* (JBU) de interagir com membranas lipídicas planares foi inicialmente demonstrada por Piovesan (2009). Na mesma ocasião, ensaios preliminares com o jaburetox-2Ec indicaram que o peptídeo teria atividade formadora de canais iônicos no mesmo modelo. A partir desses dados, nesta tese testamos os peptídeos jbtx, jbtxΔ-β, jbtx N-ter e jbtx C-ter no modelo de bicamadas lipídicas planares visando identificar motivos estruturais envolvidos nesta atividade. Há na literatura a descrição de muitos peptídeos antimicrobianos que possuem um β-hairpin em sua estrutura e conseguem permeabilizar membranas artificiais, como as protegrinas de leucócitos porcinos (Fahrner, 1996). O peptídeo antimicrobiano Arecina, isolado do poliqueta marinho *Arenicola marina* possui um amplo espectro de atividade antimicrobiana e habilidade de formar poros em bicamadas lipídicas, através da interação do motivo β-hairpin com estas membranas (Zakhar *et al.*, 2011). Como já citado anteriormente, foi identificado por modelagem molecular o motivo β- hairpin na região carboxi-terminal do jbtx. A fim de averiguar se este domínio estaria envolvido nesta atividade, o mutante jbtxΔ-β (em que o β- hairpin foi deletado) foi testado em bicamada lipídica, verificando-se que este apresentou atividade de canal, produzindo a mesma condutância de canal mostrada pelo jbtx, sugerindo que este domínio não é importante para atividade de permeabilização de membranas lipídicas.

Obtivemos a expressão de peptídeos recombinantes correspondendo apenas às metades carboxi terminal e amino terminal do jbtx e testamos estes dois peptídeos nas bicamadas lipídicas. Para nossa surpresa, os dois peptídeos permeabilizaram as membranas formando canais iônicos para cations, como também demonstrado para o jbtx. É importante observar que foi necessário usar o dobro da concentração molar do peptídeo

jbtx N-ter e jbtx C-ter em relação ao jbtx completo, sugerindo que estes peptídeos são menos ativos que a molécula inteira do peptídeo. No conjunto, os dados sugerem a existência de pelo menos dois domínios distintos que interagem com membranas no peptídeo jbtx, que foram separados nos mutantes correspondendo às metades N- e C-terminal.

A condutância dos canais formados pelo jbtx e seus mutantes derivados variou de 32 a 43 pS. Esses canais podem ser considerados pequenos, se comparados com outras proteínas formadoras de canais catiônicos descritas como, por exemplo, a coronatina-1 isolada do fungo entomopatogênico *Conodiobolus coronatus*. Esta proteína apresenta atividade inseticida e em bicamadas lipídicas planares forma canais iônicos de condutâncias de 322 pS (Wieloch *et al*., 2011). Valores semelhantes de condutância descritos para o jbtx e mutantes foram demonstrados para a toxina Cry1Aa de Bt, que apresentou diferentes níveis de condutância variando de 11 a 52 pS. A maior condutância observada foi um valor aproximado de 2 a 5 múltiplos do valor da condutância do menor canal, sugerindo oligomerização das pequenas unidades de canal (Peyronnet *et al*., 2001).

Um dos fatores que deve ser considerado para compreender as atividades descritas para o jbtx e mutantes é o estado de oligomerização destes peptídeos. Já foi descrito para o jaburetox-2Ec que este peptídeo tende a formar agregados (Barros, *et al*., 2009; Moro, 2010), e o seu estado de oligomerização afeta diretamente no sua interação com lipossomos (Barros, *et al*., 2009) e, muito provavelmente, outras de suas atividades biológicas conhecidas. Estudos de espalhamento de luz mostraram que tanto o jaburetox-2Ec (Moro, 2010) como o jbtx (dados não publicados) formam grandes oligômeros (trímeros e/ou decâmeros) solúveis em meio aquoso, aparentemente não existindo o monômero livre em solução. O mesmo comportamento de oligomerização foi verificado

também para todos os mutantes do jbtx. Como ainda não conhecemos os fatores que controlam a oligomerização do peptídeo e como isso afeta suas atividades biológicas, nessa tese optamos por expressar as concentrações molares de cada peptídeo considerando as massas dos monômeros.

Durante os experimentos com os peptídeos em bicamadas lipídicas observou-se que, após um longo tempo da formação do canal, outros canais com amplitudes maiores (dados não ilustrados) ocasionalmente eram formados. Esses canais que se formavam após algum tempo de experimento, sugeriam várias moléculas do peptídeo interagindo com a membrana ou até mesmo agregados deste. A análise deste fenômeno revelou-se muito difícil nesse momento, e por isso foi descrito aqui apenas o canal principal, que ocorria sempre em todos experimentos realizados com os diferentes peptídeos. Um achado semelhante foi descrito para delta endotoxinas Cry1A(c) e Cry3A de *Bt*, tendo sido sugerido que estas toxinas em diferentes ordens de agregação interagiriam com a membrana formando subestados de um mesmo canal (Slatin *et al*., 1990).

No capítulo I desta tese foi mostrada a modelagem e simulação do comportamento do peptídeo jbtx em solução, tendo este apresentado uma alfa-hélice na região amino terminal e duas alfas-hélices na região carboxi terminal, além do β-hairpin. Em Nardi *et al.* (2001), os autores estudaram o domínio ativo da toxina colicina que tem atividade bactericida e forma canais em membranas lipídicas artificiais. A colicina possui 10 alfa-hélices em sua molécula, e estas foram deletadas aos pares para identificar as estruturas importantes. Foi verificado que as alfas-hélices do número 5 até o número 10, na região carboxi terminal, da toxina eram importantes para atividade *in vitro* e *in vivo* desta proteína. Na literatura há a descrição de peptídeos antimicrobianos, chamados de cecropinas, que possuem uma estrutura randômica em espiral, quando em solução, e que

assumem uma estrutura em alfa hélice anfipática quando interage com superfície de membrana (Epand *et al.*, 1999).

O mecanismo de ação do jbtx e mutantes derivados que resulta na atividade de formação de canais iônicos ainda é desconhecido. No entanto, com base nos estudos com as PLBs, nossa hipótese para futura investigação é que as alfa-hélices presentes nas duas metades da molécula do peptídeo possam estar envolvidas nesta atividade, ou que regiões desenoveladas do peptídeo, também presentes nas duas metades da molécula, possam interagir com as membranas formando estruturas importantes para a permeabilização. Ainda que os resultados apresentados no capítulo I dessa tese indiquem que a metade N-terminal do jbtx contem o domínio responsável pela atividade inseticida e antifúngica da molécula, os resultados com os mutantes frente à permeabilização de lipossomos evidenciaram que a metade C-terminal do peptídeo também é ativa nesse modelo. Estudos adicionais são necessários para obtenção de novos dados, que nos auxiliem na elucidação da estrutura e funcionamento dos canais formados pelo jbtx, bem como para entender a relação desta atividade formadora de canais com a toxicidade do peptídeo para insetos e fungos.

Conclusões Gerais e Perspectivas

No presente tivemos como objetivo identificar motivos estruturais importantes para as atividades tóxicas descritas para o peptídeo jaburetox (jbtx) derivado da Urease de *C.ensiformis*. A partir deste estudo podemos concluir que :

- O motivo estrutural β-*hairpin*, sugerido como fator importante para atividade inseticida e interações com membranas lipídicas, não parece estar envolvido nestas atividades (capítulo I);
- Todos os diferentes peptídeos utilizados neste estudo (jbtx, jbtxΔβ, jbtx N-ter e jbtx C-ter) mostraram capacidade de interagir com lipossomos, bem como com membranas lipídicas artificiais planares, sugerindo que ambos os domínios amino terminal e carboxi terminal do jbtx possam estar envolvidos nestas atividades (capítulo I e II);
- Os resultados obtidos nos ensaios de atividade inseticida sugerem que o domínio envolvido nestas toxicidades seja a região N-terminal do peptídeo (capítulo I);
- Ainda não foi esclarecido como e se o estado de oligomerização destes peptídeos auxiliam ou interferem nas atividades tóxicas descritas.

Os dados obtidos trouxeram contribuições importantes para o entendimento do mecanismo de ação do peptídeo jbtx e sugerem que este represente um novo tipo de peptídeo ativo em membrana e com propriedades inseticida e fungitóxica. Entretanto estudos adicionais são necessários para elucidação do seu mecanismo de ação. Neste sentido, os nossos passos seguintes para o trabalho serão: (1) identificar o estado de oligomerização dos peptídeos jbtx, jbtx N-ter e jbtx C-ter em solução por gel-filtração e por espalhamento de luz; (2) identificar as estruturas secundárias formadas pelos peptídeos em solução aquosa por dicroísmo circular (CD) e ressonância nuclear

magnética (NMR); (3) Identificar as estruturas secundárias formadas pelos peptídeos na presença de lipídeos (CD e NMR); (4) estudar as interações destes peptídeos com lipossomos por ressonância nuclear magnética (CD e NMR).

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Stanisçuaski F., Carlini C.R. Plant ureases and related paptides: Understanding their entomotoxic properties.Toxins 4 (2012) 55-67.

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Wassermann G.E., Oliveira-Severo D., Uberti A.F., Carlini C.R. *Helicobacter pylori* uréase activates blood platelets through a lipoxygenase-mediated pathway. J. Cell Mol. Med. 14 (2010) 2025-2034.

Wieloch W., Boguś M.I., Ligęza M., Koszela-Piotrowska I., Szewczyk A. Coronatin-1 isolated from entomopathogenic fungus *Conidiobolus coronatus* kills *Galleria mellonella* hemocytes *in vitro* and forms potassium channels in planar lipid membrane 58 (2011) 369-79.

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Zerner, B. Recent advances in the chemistry of an old enzyme, Urease. Rus. J. Bioorg. Chem. 19 (1991) 116-131

Anexos

Anne Helene Souza Martinelli

Curriculum Vitae

Dezembro/2012

Dados pessoais

Nome Anne Helene Souza Martinelli

Endereço profissional Universidade Federal do Rio Grande do Sul
Avenida Bento Gonçalves 9500, Prédio 43431, Laboratório 110.
Agronomia - Porto Alegre
91501-970, RS - Brasil
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Endereço eletrônico ahsmartinelli@yahoo.com.br
martinelli@cbiot.ufrgs.br

Formação acadêmica/titulação

2008 - 2012 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
Título: Peptídeos tóxicos das ureases vegetais: caracterização de suas propriedades por mutagênese sítio-dirigida, Ano de obtenção: 2012.
Orientador: Célia Regina Carlini
Co-orientador: Giancarlo Pasquali

2005 - 2007 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
Título: Expressão da urease ubíqua de soja em Escherichia coli, Ano de obtenção: 2007.
Orientador: Célia Regina Carlini

1999 - 2004 Graduação em Ciências Biológicas - Licenciatura Plena
Universidade do Vale do Rio dos Sinos, UNISINOS, São Leopoldo, Brasil.
Título: "Toxicidade de Urease de Soja [Glycine max (L.) Merril] a Fitopatógenos".
Orientador: Lídia Mariana Fiuza e Célia Regina Carlini

2000 - 2002 Ensino Profissional de nível Técnico em Curso Técnico em Biotecnologia.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.

Formação complementar

2011 - 2011 Doutorado sanduíche.
Université de Montreal, UdeM, Montreal, Canadá.

Atuação profissional

1. Universidade Federal do Rio Grande do Sul – UFRGS.

Vínculo institucional

2004 - Atual Vínculo: Servidor público, Enquadramento funcional: Técnica de Laboratório-Biologia, Carga horária: 40h, Regime: Integral.

Atuo como técnica em laboratório de aulas práticas, auxiliando nas aulas dos cursos de graduação, e como pesquisadora colaboradora no Laboratório de Proteínas Tóxicas-UFRGS.

Possuo experiência na área de Microbiologia, Biologia Molecular, Bioquímica de proteínas e Biossegurança atuando nos temas de clonagem, purificação de proteínas, proteínas recombinantes, mecanismos de ação de peptídeos fungitóxicos e inseticidas.

Projetos

Projetos de pesquisa

2011 - Atual Ureases e peptídeos derivados: interação como membranas biológicas e canais iônicos.
Descrição: Ureases (EC 3.5.1.5) são enzimas níquel dependentes, que catalisam a hidrólise da ureia à amônia e CO2, sintetizadas por plantas, bactérias e fungos. A proteína neurotóxica Canatoxina (CNTX), um dímero de cadeias de 95 kDa, ligadas não covalentemente, é uma isoforma de urease da leguminosa Canavalia ensiformis, causando convulsão e morte de ratos e camundongos com DL50 2 mg/kg. Demonstramos que ureases induzem exocitose, ativação de plaquetas, e ligam-se a gangliosídeos. Esses efeitos não dependem da atividade ureolítica, implicando que há outros "domínios" proteicos biologicamente ativos nessas moléculas. Ureases microbianas também possuem essas propriedades, que podem contribuir para a patogênese das úlceras gástricas causadas por Helicobacter pylori, na urolitíase e meningite do neonato por Proteus mirabilis, ou ainda na meningite por leveduras do complexo Cryptococcus. Estudos mostraram que a urease de H.pylori agrega plaquetas, induz edema de pata em camundongos, e intensa ativação de neutrófilos e monócitos, efeitos mediados por eicosanoides derivados da rota das lipoxigenases. O estudo do mecanismo de ação da urease e peptídeo derivado mostra que, em concentrações nanomolares, esses polipeptídeos interagem com membranas, formando e/ou interferindo com canais iônicos, principalmente canais de cálcio e de sódio. Essa propriedade de ureases está relacionada ao efeito neurotóxico da CNTX e pode ter implicada nos quadros neurológicos e convulsivos decorrentes da infecção por microrganismos produtores de urease.
Situação: Em andamento; Natureza: Pesquisa.
Integrantes: Anne Helene Souza Martinelli (Colaboradora); Celia Regina Carlini - Coordenador.

2008 – Atual Ureases: Aspectos Estruturais e Propriedades Biológicas

Descrição: Ureases hidrolisam ureia a amônia e CO2. Isoformas de urease têm alta homologia, mas certas atividades biológicas são próprias de uma isoforma em particular. Por análises cromatográficas, eletroforéticas, espectroscópicas, cristalografia, modelagem molecular, estudamos relações estrutura X atividade biológica de ureases de plantas (Canavalia ensiformis; Glycine max; Gossypium hirsutum); bactérias (Helicobacter pylori, Bradyrhizobium sp., Bacillus pasteurii) e do fungo Cryptococcus neoformans.
Situação: Em andamento Natureza: Projetos de pesquisa
Integrantes: Anne Helene Souza Martinelli (Colaboradora); Célia Regina Carlini – Coordenador.

2008 – Atual Proteínas Tóxicas e Ureases em Sementes de Soja (Glycine max)

Descrição: Os estudos visam isolamento, caracterização físico-química de ureases e proteínas relacionadas (ex: soyatoxina, soybean toxin), clonagem de genes de ureases e de proteínas acessórias que incorporam Ni nas ureases. Estudos de ativação de apoureases heterólogas e da eficiência de nodulação de soja por Bradyrhizobium japonicum mutantes para genes de ureases.
Situação: Em andamento Natureza: Projetos de pesquisa
Integrantes: Anne Helene Souza Martinelli (Colaboradora); Célia Regina Carlini (Responsável).

Projeto de extensão

2008 – Atual Ureases de C.ensiformis: Clonagem e Expressão Gênica

Descrição: Objetivamos a caracterização dos genes da família das urease de Canavalia ensiformis, bem como a compreensão dos fatores que regulam a expressão desses genes. Pelo menos três membros da família de genes de urease foram identificados. Outros estudos visam expressão heteróloga de cDNAs completos ou truncados de ureases em plantas, bactérias e leveduras, e estudos com as proteínas acessórias que incorporam níquel nas ureases.
Situação: Em andamento Natureza: Projeto de extensão
Integrantes: Anne Helene Souza Martinelli (Colaboradora); Célia Regina Carlini (Responsável).

Idiomas

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

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

Produção

Produção Bibliográfica

Artigos completos publicados em periódicos

1. Postal, Melissa, **Martinelli, Anne H.S.**, Becker-Ritt, Arlete B., Ligabue-Braun, Rodrigo, Demartini, Diogo R., Ribeiro, Suzanna F.F., Pasquali, Giancarlo, Gomes, Valdirene M., Carlini, Celia R.
Antifungal properties of Canavalia ensiformis urease and derived peptides. Peptides (New York, N.Y. 1980).v.38, p.22 - 32, 2012.

2. Wiebke-Strohm, Beatriz, Pasquali, Giancarlo, Margis-Pinheiro, Márcia, Bencke, Marta, Bücker-Neto, Lauro, Becker-Ritt, Arlete B., **Martinelli, Anne H. S.**, Rechenmacher, Ciliana, Polacco, Joseph C., Stolf, Renata, Marcelino, Francismar C., Abdelnoor, Ricardo V., Homrich, Milena S., Del Ponte, Emerson M., Carlini, Celia R., Carvalho, Mayra C. C. G., Bodanese-Zanettini, Maria Helena
Ubiquitous urease affects soybean susceptibility to fungi. Plant Molecular Biology.v.79, p.75 - 87, 2012.

3. Menegassi, Angela, Wassermann, German E., Olivera-Severo, Deiber, Becker-Ritt, Arlete B., **Martinelli, Anne Helene S.**, Feder, Vanessa, Carlini, Celia R.
Urease from Cotton (Gossypium hirsutum) Seeds: Isolation, Physicochemical Characterization, and Antifungal Properties of the Protein. Journal of Agricultural and Food Chemistry.v.56, p.4399 - 4405, 2008.

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

5. Mitidieri, S., **Martinelli, A. H. S.**, Vainstein, M.
Enzymatic detergent formulation containing amylase from Aspergillus niger: A comparative study with commercial detergent formulations. Bioresource Technology. v.97, p.1217 - 1224, 2005.

6**. Martinelli, A. H. S.**, Mitidieri, S.
Detergentes Biológicos Biodegradáveis. Avaliação das Formulações do Mercado. Biotecnologia Ciência & Desenvolvimento (Impresso). , v.26, p.56 - 60, 2002.

Trabalhos publicados em anais de eventos (resumo)

1. Lopes, F. C., **Martinelli, A. H. S.**, Postal, M., Kappaun, K., Tichota, D. M., Pasquali, G., Brandelli, A., Carlini, C. R.
Atividade fungitóxica de urease ubíqua de soja (Glycine max) recombinante. In: XIV reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular, 2012, Porto Alegre, Rio Grande do Sul.
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2. **Martinelli, A. H. S.**, Kappaun, K., Postal, M., Piovesan, A. R., Pasquali, G., Carlini, C. R.
Jaburetox-2EC, peptídeo recombinante derivado da urease: estudo de mutagênese sítio-dirigida. In: XIV Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular, 2012, Porto Alegre, Rio Grande do Sul.
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3. Kappaun, K., **Martinelli, A. H. S.**, Pasquali, G., Carlini, C. R.
Estudo da estabilidade dos peptídeos derivados da urease de Canavalia ensiformis: jaburetox-2Ec e jaburetox -v5. In: XXIII Salão de Iniciação Científica UFRGS, 2011, Porto Alegre, Rio Grande do Sul.
XXIII Salão de Iniciação Científica UFRGS. 2011.

4. **Martinelli, A. H. S.**, Kappaun, K., Pasquali, G., Carlini, C. R.
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XIII Reunião Anual do programa de Pós-Graduação em Biologia Celular e Molecular. 2011.

5. Kappaun, K., **Martinelli, A. H. S.**, Pasquali, G., Carlini, C. R.
Estudos Biológicos de Peptídeos derivados da urease de Canavalia ensiformis. In: XXII Salão de Iniciação Científica, 2010, Porto Alegre, Rio Grande do Sul.
XXII Salão de Iniciação Científica. 2010.

6. **Martinelli, A. H. S.**, Kappaun, K., Becker-Ritt, A. B., Pasquali, G., Carlini, C. R.
Urease-Derived insecticidal peptide: Site-directed mutagenesis studies. In: XII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular, 2010, Porto Alegre, Rio Grande do Sul.
XII Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. 2010.

7. Becker-Ritt, A.B., **Martinelli, A. H S**., Kappaun, K., Carlini, C. R.
Fungitoxic and/or fungistatic properties of ureases In: In: XVI World Congress of the International Society on Toxinology X Congresso da Sociedade Brasileira de Toxinologia, 2009, Recife.

XVI World Congress of the International Society on Toxinology X Congresso da Sociedade Brasileira de Toxinologia. 2009.

8. **Martinelli, A. H. S.**, Kappaun, K., Mulinari, F., Staniscuaski, F., Becker-Ritt, A.B., Bertholdo-Vargas, L.R., Pasquali, G., Carlini, C. R.
Jaburetox-2EC, a Recombinant Urease-Derived Insecticidal Peptide: Site-Directed Mutagenesis Studies In: XXXVIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. 2009. Águas de Lindóia.
XXXVIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. 2009.

9. **Martinelli, A. H. S.**, Mulinari, F., Staniscuaski, F., Kappaun, K., Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
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10. Kappaun, K., **Martinelli, A. H. S.**, Becker-Ritt, A.B., Pasquali, G, Carlini, C. R.
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11. Kappaun, K., **Martinelli, A. H. S.**, Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
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12. Kappaun, K., **Martinelli, A. H. S.**, Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
Expressão de urease ubiqua de soja em Escherichia coli In: XX Salão de Iniciação Científica UFRGS, 2008, Porto Alegre.
XX Salão de Iniciação Científica UFRGS. 2008.

13. Kappaun, K., **Martinelli, A. H. S.**, Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
Expressão de urease ubíqua de soja em Escherichia coli.
Reunião anual do PPGBCM. 2008.

14. **Martinelli, A. H. S.**, Kappaun, K., Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
Expression of soybean ubiquitous urease in Escherichia coli In: XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2008, Águas de Lindóia.
XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular. 2008.

15. **Martinelli, A. H. S.**, Becker-Ritt, A.B., Kappaun, K., Polacco, J.C., Pasquali, G., Carlini, C. R.
Peptídeo tóxico de ureases vegetais: caracterização de suas propriedades por mutagênese sítio dirigida In: Reunião Anual do Programa de Pós-Graduacao em Biologia Celular e Molecular, 2008, Porto Alegre.
Reunião Anual do Programa de Pós-Graduacao em Biologia Celular e Molecular. 2008.

16. Becker-Ritt, A.B., **Martinelli, A. H. S.**, Mitidieri, S., Soares, V. F., Wassermann, G., Pasquali, G., Carlini, C. R.
Antifungal activity of ureases from Glycine max and Canavalia ensiformis seeds and a recombinant Helicobacter pylori urease In: Plant Biology and Botany 2007 Joint Congress, 2007, Chicago.
Plant Biology and Botany 2007 Joint Congress. 2007.

17. **Martinelli, A. H. S.**, Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
Expressão de urease ubíqua de soja em Escherichia coli e avaliação de suas atividades biológicas In: VIII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular, 2006, Porto Alegre.
VIII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular. 2006.

18. **Martinelli, A. H. S.**, Ferreira,T.B., Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
Expressão da urease de soja em Escherichia coli e avaliação da sua toxicidade a fungos fitopatogênicos In: VII Reunião Anual do programa de Pós-Graduação em Biologia Celular e Molecular do centro de Biotecnologia, 2005, Porto Alegre.
VII Reunião Anual do programa de Pós-Graduação em Biologia Celular e Molecular do centro de Biotecnologia. 2005.

19. Becker-Ritt, A.B., **Martinelli, A. H. S.**, Pasquali, G., Carlini, C. R.
Expression of soybean [Glycine max (L) Merrill] ubiquitous urease in tobacco (Nicotiana tobacum) and antifungal activity of soybean ureases In: XXXIV Reunião Anual SBBq, 2005, Águas de Lindóia.
XXXIV Reunião Anual SBBq. 2005.

20. **Martinelli, A. H. S.,** Becker-Ritt, A.B., Pasquali, G., Carlini, C. R.
Obtenção E Purificação de Urease de Soja [Glycine max (L) Merril] e Avaliação de sua Toxicidade a Microrganismos Fitopatogênicos. In: Mostra de Iniciação Científica da Unisinos, 2004, São Leopoldo.
Mostra de Iniciação Científica da Unisinos. 2004.

21. MARTINELLI, A. H. S., BECKER-RITT, A.B., PASQUALI, G., CARLINI, C. R.
Soybean (Glycine max (L.) Merril) ureases: expression in tobacco (Nicotiana tobacum) In: XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular- SBBq, 2004, Caxambu.
XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular- SBBq. , 2004.

22. **MARTINELLI, A. H. S.**
Produção de Ácido Cítrico por Fungos Filamentosos In: IX Reunião Acadêmica da Biologia da Unisinos- RABU, 2003, São Leopoldo.
IX Reunião Acadêmica da Biologia da Unisinos- RABU. 2003.

23. **Martinelli, A. H. S.**, Mitidieri, S.
Produção de Amilase para Formulação de Detergentes Biodegradáveis In: ENZITEC 2002, 2002, BRASÍLIA.
5° Seminario Brasileiro de Tecnologia Enzimática. BRASÍLIA: 2002. v.5.

24. **Martinelli, A. H. S.**, Mitidieri, S., Vainstein, M.H.
Produção de Amilase para Formulação de Detergentes Biodegradáveis In: Salão de Iniciação Científica, 2002, Porto Alegre.
XIII Salão de Iniciação Científica. , 2002.

25. **Martinelli, A. H. S.**, Mitidieri, S., Vainstein, M.H.
Produção e Caracterização de uma Amilase para Formulação de Detergentes Biodegradáveis In: Salão de Iniciação Científica da UFRGS, 2002, Porto Alegre.
XIV Salão de Iniciação Científica da UFRGS. 2002.

Orientações e supervisões

Trabalhos de conclusão de curso de graduação

1. Karine Kappaun. **Efeito tóxico do peptídeo Jaburetox -V5 na célula bacteriana liofilizada a insetos modelos e análise da influência do epitopo V5 na formação de agregados.** 2011. Curso (Farmácia) - Universidade Federal do Rio Grande do Sul.

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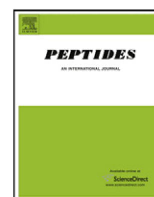

Antifungal properties of *Canavalia ensiformis* urease and derived peptides

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ABSTRACT

Ureases (EC 3.5.1.5) are metalloenzymes that hydrolyze urea into ammonia and CO_2. These proteins have insecticidal and fungicidal effects not related to their enzymatic activity. The insecticidal activity of urease is mostly dependent on the release of internal peptides after hydrolysis by insect digestive cathepsins. Jaburetox is a recombinant version of one of these peptides, expressed in *Escherichia coli*. The antifungal activity of ureases in filamentous fungi occurs at submicromolar doses, with damage to the cell membranes. Here we evaluated the toxic effect of *Canavalia ensiformis* urease (JBU) on different yeast species and carried out studies aiming to identify antifungal domain(s) of JBU. Data showed that toxicity of JBU varied according to the genus and species of yeasts, causing inhibition of proliferation, induction of morphological alterations with formation of pseudohyphae, changes in the transport of H^+ and carbohydrate metabolism, and permeabilization of membranes, which eventually lead to cell death. Hydrolysis of JBU with papain resulted in fungitoxic peptides (~10 kDa), which analyzed by mass spectrometry, revealed the presence of a fragment containing the N-terminal sequence of the entomotoxic peptide Jaburetox. Tests with Jaburetox on yeasts and filamentous fungi indicated a fungitoxic activity similar to ureases. Plant ureases, such as JBU, and its derived peptides, may represent a new alternative to control medically important mycoses as well as phytopathogenic fungi, especially considering their potent activity in the range of 10^{-6}–10^{-7} M.

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1. Introduction

Ureases (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 [13,20]. Urease of jackbean (*Canavalia ensiformis*) seeds was the first enzyme ever to be crystallized [41], consisting of a hexamer of a single chain of 840 amino acid residues, with a molecular mass of 97 kDa [16,20,38]. It has been postulated that in plants these proteins contribute to the bioavailability of nitrogen and participate in defense mechanisms [12,16].

C. ensiformis produces several urease isoforms: the more abundant jackbean urease (JBURE-I), and two less abundant proteins, canatoxin (CNTX) [17] and JBURE-IIB [26]. CNTX-like proteins and urease accumulate in the mature seed, consistent with the proposed defense role associated with both insecticidal [40] and fungicidal properties [7,26].

Insecticidal activity of Jackbean urease depends mostly on the release of an entomotoxic peptide formed by proteolytic enzymes upon ingestion by the insect [15]. This peptide, Pepcanatox, was characterized and based on its sequence, a recombinant peptide named Jaburetox-2EC was produced using the corresponding sequence of the urease isoform JBURE-II as template [27]. This peptide has 93 amino acids and its toxicity to several insects, including some species that were not affected by the native urease, has been demonstrated [40].

CNTX was the first urease shown to inhibit the radial growth of several filamentous fungi [29]. In 2007, Becker-Ritt et al. [7] reported the fungicidal activity of the embryo specific urease from *Glycine max* (soybean), the major urease from *C. ensiformis* and of a bacterial urease from *Helicobacter pylori*, regardless of their ureolytic activity, toward different phytopathogenic fungi. Urease from other sources also display fungicidal activity, such as the cotton (*Gossypium hirsutum*) seed urease [23] and the

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recombinant JBURE-IIb apourease from *C. ensiformis* [26]. Recently, it was demonstrated that soybean plants lacking urease due to gene silencing were more susceptible to fungal infection, reinforcing the hypothesis of participation of these proteins in plant defense [44]. The fungitoxic activity of ureases occurs at submicromolar doses, making these proteins 2–3 orders of magnitude more potent than any other known antifungal proteins of plant origin, producing injuries to the cell wall and/or cell membrane and plasmolysis [6,7].

Infectious diseases, mainly candidiasis and aspergillosis, caused by yeasts and filamentous fungi are a serious problem worldwide, especially in tropical and subtropical countries where the number of immunosuppressed patients (who often develop these diseases), has increased over the last decade. The drugs available for treating these mycoses have low efficiency, low solubility and high toxicity, causing severe collateral effects. Besides these problems, the emergence of strains resistant to current therapeutic agents makes essential and urgent the identification of new antifungal compounds [35]. Despite numerous reports on the occurrence and activity of proteins and antimicrobial peptides originated from plant, some have already been successfully tested as transgenes to confer resistance to plants against fungi and/or insects [6], only a few have been evaluated for therapeutic potential in human mycoses [3]. The search for new antifungal compounds from plants became extremely urgent considering the spread of invasive mycoses, particularly in immunocompromised patients, caused by pathogenic fungi or in plants by soil fungi (e.g., *Alternaria*, *Curvularia* and *Rhizopus*), before considered as fungi of low virulence, and which are currently being considered as emerging pathogens [14].

Plants are an excellent source of compounds having antifungal activity, since they are continuously exposed to a broad range of phytopathogenic fungi in the environment. Plant antifungal peptides include defensins, lipid transport proteins, chitinases, lectins, thionins, cyclopeptide alkaloids and other less common types [6,14,28].

In this work we describe the toxic activity of JBU and of Jaburetox in pathogenic yeast. Studies on the mechanisms of their antifungal action have shown interference on energy metabolism and proton transport, morphological changes and permeabilization of the fungal membrane. Fungitoxic urease-derived peptides were obtained by enzymatic hydrolysis and provided clues to the location of antifungal domain(s) of the protein.

2. Materials and methods

2.1. *C. ensiformis urease (JBU)*

Urease type C-III from Jack bean (Sigma Aldrich) was used in all experiments. The protein (hexameric form, Mr 540 kDa) was solubilized in 50 mM Tris buffer, pH 7.0, and quantified by absorbance at 280 nm (0.604 A280 was considered equivalent to a 1.0 mg/mL protein solution). Enzyme-inactivated JBU was obtained by treating the protein with the active site inhibitor p-hydroxy-mercurybenzoate (Sigma Aldrich) as described in [17]. Excess of the inhibitor was removed by extensive dialysis against Tris buffer.

2.2. *PCR amplification and cloning of Jaburetox*

Jaburetox-2Ec, the recombinant peptide obtained by Mulinari et al., 2007 [27], contained 93 urease-derived amino acids, plus a V5 antigen and a C-terminal His-tag. The vector pET 101-D-TOPO containing Jaburetox-2Ec coding sequence was used as template in a polymerase chain reaction. In order to obtain a recombinant peptide containing the His-tag and lacking the V5 antigen, a set of primers were designed, the cDNA was amplified by PCR, cloned into pET 23-a vector and expressed in BL21-CodonPlus (DE3)-RIL (Stratagene). This new peptide was called Jaburetox.

The forward primer sequence was Jaburetox 5′ CCAACATATGGGTCCAGTTAA TGAAGCCAAT 3′ (the underline shows the NdeI site) and the reverse primer sequence was Jaburetox 5′ CCCCCTCGAGTATAACTTTTCCACCTCCAAAAACA 3′ (the underline shows the XhoI site). The PCR reaction was carried out in the following conditions: denaturation at 95 °C for 3 min, annealing at 55 °C for 30 s and elongation at 72 °C for 2 min. A total of 35 cycles were used and the final product was then digested with NdeI (Fermentas, Eugene, OR, USA) and XhoI (Fermentas, Eugene, OR, USA), dephosphorylated with thermosensitive alkaline phosphatase (Promega, Madison, WI, USA). The plasmid pET 23a::Jaburetox was sequenced using a ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster city, CA).

2.3. *Expression and purification of recombinant Jaburetox*

For isolation and purification of Jaburetox, 200 mL of Luria broth medium containing 100 μg/mL ampicillin and 40 μg/mL chloramphenicol were inoculated with 2 mL of the overnight culture. The cells were grown 2 h at 37 °C under shaking (OD600 = 0.7) and then 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. After 3 h, the cells were harvested by centrifugation and suspended in 10 mL of lysis buffer (50 mM tris buffer, pH 7.5, 500 mM NaCl, 5 mM imidazole), sonicated, centrifuged (14,000 × g, 30 min) and 10 μL of supernatant or 5 μL of the pellet sample were analyzed by SDS-PAGE. The supernatant was loaded onto a 2 mL Ni affinity column (Ni-NTA, QIAGEN, Hilden, Germany), which was previously equilibrated with the equilibration buffer (50 mM Tris buffer, pH 7.5, 500 mM NaCl, 5 mM imidazole). After 30 min, the column was washed with 20 mL of the same buffer, containing 50 mM imidazole. The recombinant peptide was eluted with the equilibration buffer containing 200 mM imidazole and quantified by the Bradford method [9]. The samples were dialyzed against the 50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol. A molecular mass of 10,128.2 Da (ExPASY ProtParam tool) was considered for Jaburetox.

2.4. *Fungi and yeast*

The yeasts *Candida parapsilosis* (CE002), *Candida tropicalis* (CE017), *Candida albicans* (CE022), *Kluyveromyces marxiannus* (CE025), *Pichia membranifaciens* (CE015), and *Saccharomyces cerevisiae* (1038) and filamentous fungi *Colletotrichum lindemuthianum*, *Colletotrichum musae*, *Colletotrichum gloeoporioides*, *Fusarium laterithium*, *Fusarium solani*, *Fusarium oxysporum*, *Phomopsis* sp., *Mucor* sp., *Trichoderma viridae*, *Pythium oligandrum*, *Lasiodiplodia theomobrae*, *Cercospora chevalier* and *Rhizoctonia solani* were kindly provided by Dr. Valdirene Gomes from the Laboratory of Physiology and Biochemistry of Microorganisms, Center of Bioscience and Biotechnology, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil or by Dr. José Tadeu Abreu de Oliveira from the Department of Biochemistry, Universidade Federal of Ceará, Fortaleza, Ceará, Brazil. The yeasts were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar). The fungi were maintained on potato agar (PDA) at 4 °C.

2.5. *Hydrolysis of JBU*

JBU was hydrolyzed using different commercial enzyme: trypsin (EC 3.4.21.4 – Sigma–Aldrich, St. Louis, MO, USA), chymotrypsin (EC 3.4.21.1 – Sigma–Aldrich, St. Louis, MO, USA) papain (Merck, Darmstadt, Alemanha), pepsin (EC 3.4.23.1 – Sigma, St. Louis, MO, USA). Different conditions of hydrolysis were tested, varying pH, incubation time and enzyme:substrate ratio. The reaction mixture after

hydrolysis with papain was submitted to ultrafiltration (4000 × g, 10 min) using 10,000 mw cut-off Amicon cartridges (Millipore, Billerica, MA, USA) to separate a pass-through filtered fraction containing peptides with Mr below 10,000 d and a retained fraction, with molecules bigger than 10,000 d.

2.6. Electrophoresis

The hydrolyzed fractions of JBU were visualized in SDS-Tricine gels [36]. The gels were stained with Colloidal Coomassie.

2.7. Analysis by liquid chromatography coupled to mass spectrometry

The filtered fractions (<10 kDa) after hydrolysis of JBU were desalted on reverse-phase column (C-18) in a HPLC system (Shimadzu). The column was equilibrated with 0.1% TFA (trifluoroacetic acid) and the retained fraction were eluted with a gradient (0–100%) of 99.9% acetonitrile in 0.1% TFA. The eluted peptides were pooled and lyophilized.

The lyophilized material was suspended in 0.1% formic acid (20 μL) and 5 μL were subjected to reversed phase chromatography (NanoAcquity UltraPerformance LC®-UPLC®, Waters, Milford, United States chromatograph) using a Nanoease C18, 75 μm ID at 35 °C. The column was equilibrated with 0.1% TFA and the peptides were eluted in 20 min gradient, ramping from 0 to 60% acetonitrile in 0.1% TFA at 0.6 nL/min constant flow. Eluted peptides were subjected to electro spray ionization and analyzed by mass spectrometry using a Q-TOF Micro™ spectrometer (Micromass, Waters, Milford, United States). The voltage applied to the cone for the ionization was 35 V. The three most intense ions in the range of *m*/*z* 200–2000 and +2 or +3 charges were selected for fragmentation.

The acquired MS/MS spectra were processed using Proteinlynx v.2.0 software (Waters, Milford, US) and the generated .mgf files were used to perform database searches using the MASCOT software (version 2.4.00) (Matrix Science, London, UK) against the NCBI database, restricting the organism to taxonomy "green plants_taxid 33,090." No digestion enzyme was selected. Search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 1.2 Da, and MS/MS tolerance of 1.2 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. Data was manually checked for validation.

2.8. Homology of urease with plant antifungal proteins

The N-terminal sequences of French bean thaumatin-like protein, French bean antifungal peroxidase, pinto bean chitinase (phasein A), and pea defensins (PSDs) were taken from [28]. The alignment of these sequences with the major urease of *C. ensiformis* (NCBI gi 167228) was performed with the ClustalW program [21], using the BLOSUM matrix [19]. The regions of urease which are similar to these antifungal proteins were colored manually with the UCSF Chimera molecular viewer [30].

2.9. Antifungal assays

2.9.1. Yeasts

The growth assays were performed according to [34]. Yeast cells of *C. tropicalis*, *C. albicans*, *C. parapsilosis*, *S. cerevisiae*, *K. marxianus* and *Pichia membranisfaciens* were set to multiply in Petri dishes containing Sabouraud agar for 24 or 48 h at 30 °C. For the assay, cells were removed with the aid of a sowing handle, and added to 10 mL of Sabouraud culture medium. The test samples were added to cells (1×10^4 per mL) and growth was evaluated by turbidity readings at a wavelength of 620 nm for a period of 24–48 h. The tests were performed in 96 well plates, U-bottom and read in a plate reader (Reader 400 EZ – Biochrom).

To evaluate the reversibility of the antifungal effect and discriminate fungistatic versus fungicidal activity, yeasts (10^4) were incubated with 0.36 μM JBU or buffer for 24 h at 28 °C. Then 10-fold serial dilutions of the incubated yeasts were made in fresh Sabouraud medium and plated in Sabouraud agar. The number of CFU in the 10^6-fold dilution after 24 h at 28 °C was determined under a microscope.

2.9.2. Filamentous fungi

The fungi were grown for 14 d on PDA at 28 °C. To obtain the spores, 5 mL of sterile saline were added to each Petri dish and the colonies gently washed with the tip of a pipette. To evaluate the hyphal growth, the experiment was made according to [7]. The spore suspension (1×10^6 spores per mL) was inoculated into 96 well plates containing potato dextrose broth (PDB), incubated at 28 °C for 16 h, and then the test samples (up to 80 μL) were added. The final volume in each well was 200 μL. The dialysis buffer (Tris 10 mM pH 6.5) was used as negative control and 0.1% hydrogen peroxide (H_2O_2), as a positive control. The plates were incubated at 28 °C and monitored turbidimetrically at 620 nm at 12 h intervals for 96 h. Alternatively, spores were incubated with the samples for 96 h at 28 °C and then germination was monitored by turbidity.

The tests were performed in triplicate and data presented as means and standard deviations.

2.9.3. Inhibition of glucose-stimulated acidification

Glucose-stimulated acidification of the medium results from extrusion of H^+ by the cells, through a H^+-ATPase pump in the plasma membrane [18]. We evaluated the effects of JBU and peptide(s) on this metabolic activity of *S. cerevisiae* and *C. albicans*, as described in [34]. Yeast cells were grown in Sabouraud agar medium for 48 h at 30 °C and then each plate was washed with 4 mL of Sabouraud medium to collect the cells. 8 μL of this suspension was added to 200 mL of liquid medium and incubated in a shaker (200 rpm) bath for 16 h at 30 °C. The culture was then centrifuged at 3000 x g for 5 min at 4 °C, the supernatant was discarded and 20 mL of Milli-Q water was added to the pelleted cells, which were suspended and centrifuged again. This process was repeated three times. Finally the yeast cells were resuspended in 3 mL of Milli-Q water.

Aliquots of 10^7 cells were pre-incubated for 30 min in 800 μL of protein or peptide samples in 10 mM Tris–HCl, pH 6.0 or the dilution buffer alone. After pre-incubation, 200 μL of 500 mM glucose was added to the cells and the medium pH was measured every minute for 30 min. The amount of H^+ released by the cell was calculated as the difference between the initial pH and the final pH (ΔpH), considering the equation $pH = -\log[H^+]$. The values are averages of triplicates for each experiment.

2.9.4. Evaluation of cell permeability

The permeabilization of the plasma membrane was assessed by measuring absorption of SYTOX *Green* (Invitrogen, Grand Island, NY, USA) as described by [22]. This dye forms a fluorescent complex with nucleic acids, entering cells when the integrity of their plasma membrane is compromised. Fungal cells were incubated with different concentrations of the test samples for 24 h and then exposed to 0.2 μM SYTOX *Green* for 30 min at room temperature. The cells were observed under a microscope (Axioskop 40 – Zeiss) equipped with a filter for fluorescein detection (excitation wavelength 450–490 nm and emission 500 nm).

2.9.5. Viability and metabolic activity

Fluorescent probes (LIVE/DEAD® Yeast Viability Kit – Invitrogen, Grand Island, NY, USA) were used to evaluate the viability and metabolic activity of yeasts in the presence of test samples. The yeast cells were grown overnight (16 h) in Sabouraud medium at 28 °C in the presence of either JBU, Jaburetox, dialysis buffer, or H_2O_2, and then centrifuged (3000 × *g*, 10 min) to remove the medium. Yeasts were suspended in buffer GH (2% D-(+) glucose, 10 mM Na-HEPES, pH 7.2) and then 1 μM of FUN-1 and 12.5 μM of calcofluor were added. After more 2 h of incubation at 28 °C, the cells were viewed under a fluorescence microscope (Axioskop 40 – Zeiss) equipped with filters for different wavelengths to allow visualization of fluorescein (green), rhodamine (red) and DAPI (blue).

Alternatively, cells were grown overnight in Sabouraud medium at 28 °C in the absence of test samples, followed by centrifugation to remove the medium. Yeasts were suspended in buffer GH (2% D-(+) glucose, 10 mM Na^+-HEPES, pH 7.2). 100 μL of cell suspension were mixed with the samples JBU, Jaburetox, dialysis buffer, or H_2O_2, and maintained for 2 h at 28 °C. Then, 1 μM of FUN-1 and 12.5 μM of calcofluor were added and after 2 h at 28 °C, the cells were viewed under the microscope Axioskop 40 – Zeiss with different filters, as described above.

2.9.6. Statistical analysis

All experiments were run in triplicates. Data were evaluated using "one-way" ANOVA followed by the *t* test of Bonferroni or Dunnett. A $p < 0.05$ was considered statistically significant. All analyzes were performed using GraphPad Prism software (version 3.0 for Windows).

3. Results

3.1. Antifungal activity of JBU against yeast

The activity of JBU was evaluated on six different species of yeasts: *S. cerevisiae*, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *P. membranisfaciens* and *K. marxiannus* (Fig. 1).

JBU inhibited the growth of *C. tropicalis* (Fig. 1A) and of *P. membranisfaciens* (Fig. 1C) at the lower dose tested – 0.18 μM. For the other yeasts, such as *K. marxiannus* (Fig. 1B), the cell culture became more turbid than the control culture in the presence of JBU up to 0.72 μM, suggesting increased growth and lack of effect antifungal effect. In contrast, the determination of colony forming units of the treated yeasts indicated a fungicidal effect upon all species after 24 h of exposure to 0.36 μM JBU (Fig. 2).

Enzyme-inactivated JBU (after treatment with the irreversible active site inhibitor p-hydroxy-mercurybenzoate) retained its fungitoxic effect on *P. membranisfaciens* (Fig. 1C), demonstrating that the antifungal effect of JBU on yeasts is independent of its enzymatic activity. Similarly, we have previously reported that the antifungal effect of JBU on filamentous fungi is not dependent on its enzymatic activity [7].

The ability of the JBU to permeabilize yeast membranes was studied with SYTOX *Green*, a fluorescent label with affinity for nucleic acids. After incubation of *C. tropicalis*, *P. membranisfaciens*, *K. marxiannus* and *C. parapsilosis* cells with JBU, the dye was added to the culture and maintained for 10 min under shaking at room temperature. All JBU-treated yeasts showed higher fluorescence when compared to controls, indicating permeabilization of cells, particularly associated to the formation of pseudohyphae in *C. tropicalis* (Fig. 3, panels B and C), *P. membranisfaciens* and *K. marxiannus*. Cell viability of JBU-treated *S. cerevisiae* was assessed using the LIVE/DEAD kit (Invitrogen) (Fig. 4). The fluorescent label FUN-1 indicates viable and metabolically active cells by formation of red fluorescent cylindrical intravacuolar structures (CIVs). Cells were

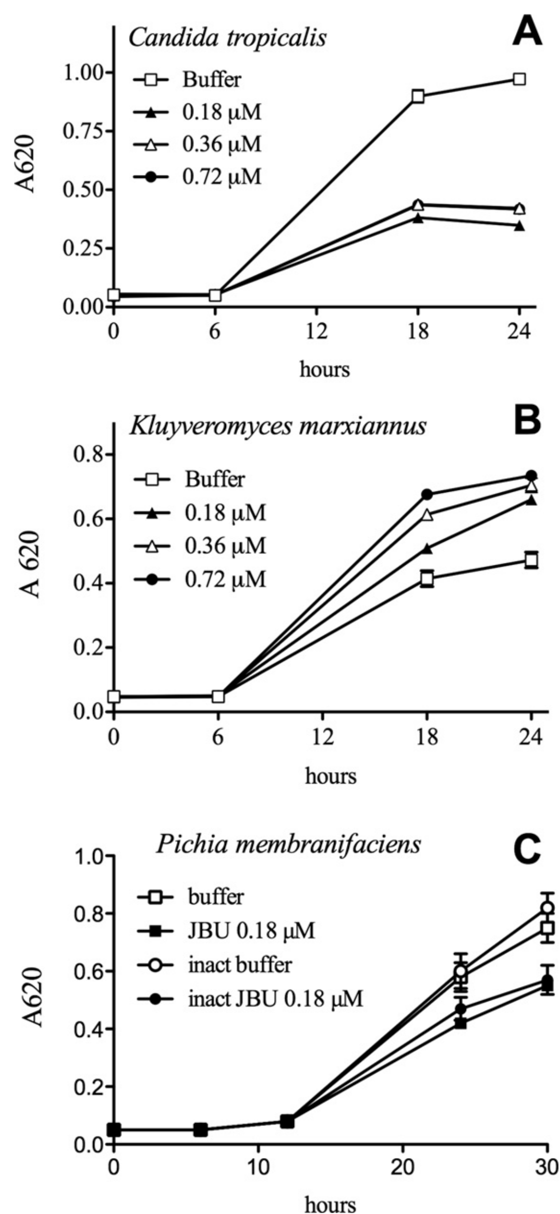

Fig. 1. Turbidimetric assay of *C. tropicalis* (panel A) and *K. marxiannus* (panel B) cells grown in the presence of different concentrations of JBU. In panel C, *P. membranifaciens* cells were exposed to native JBU or enzyme-inactivated JBU (inact JBU), after treatment with p-hydroxy-mercurybenzoate. Cells (10^4) in Sabouraud medium were incubated with the samples at 30 °C for 30 h and the absorbance of the culture was measured every 6 h. Each point represents the mean ± sd of triplicated points.

incubated with JBU and/or buffer for 2 h at 28 °C and then incubated with the fluorescent probes for 1 h. Control viable cells formed CIVs (Fig. 4, panels F and H), indicative of active metabolism. On the other hand, most cells treated with JBU showed a diffuse red/green fluorescence indicating lack of metabolic activity (Fig. 4, panel B and C), although cell walls are preserved (Fig. 4, panel D).

H^+-ATPase plasma membrane plays an essential role in the physiology of fungal cell. Interference in its function by classical antagonists leads to cell death [18,42]. Here, the effect (direct or indirect) of JBU on the activity of H^+-ATPase was evaluated by monitoring the glucose-stimulated medium acidification by *S. cerevisiae* and *C. albicans*. Cells were pre-incubated in buffer with samples for 30 min, then glucose was added and the pH was measured at 1 min intervals during 30 min. As shown in Fig. 5, JBU (0.09 μM) inhibited the acidification produced by *S. cerevisiae* and *C. albicans* cells by

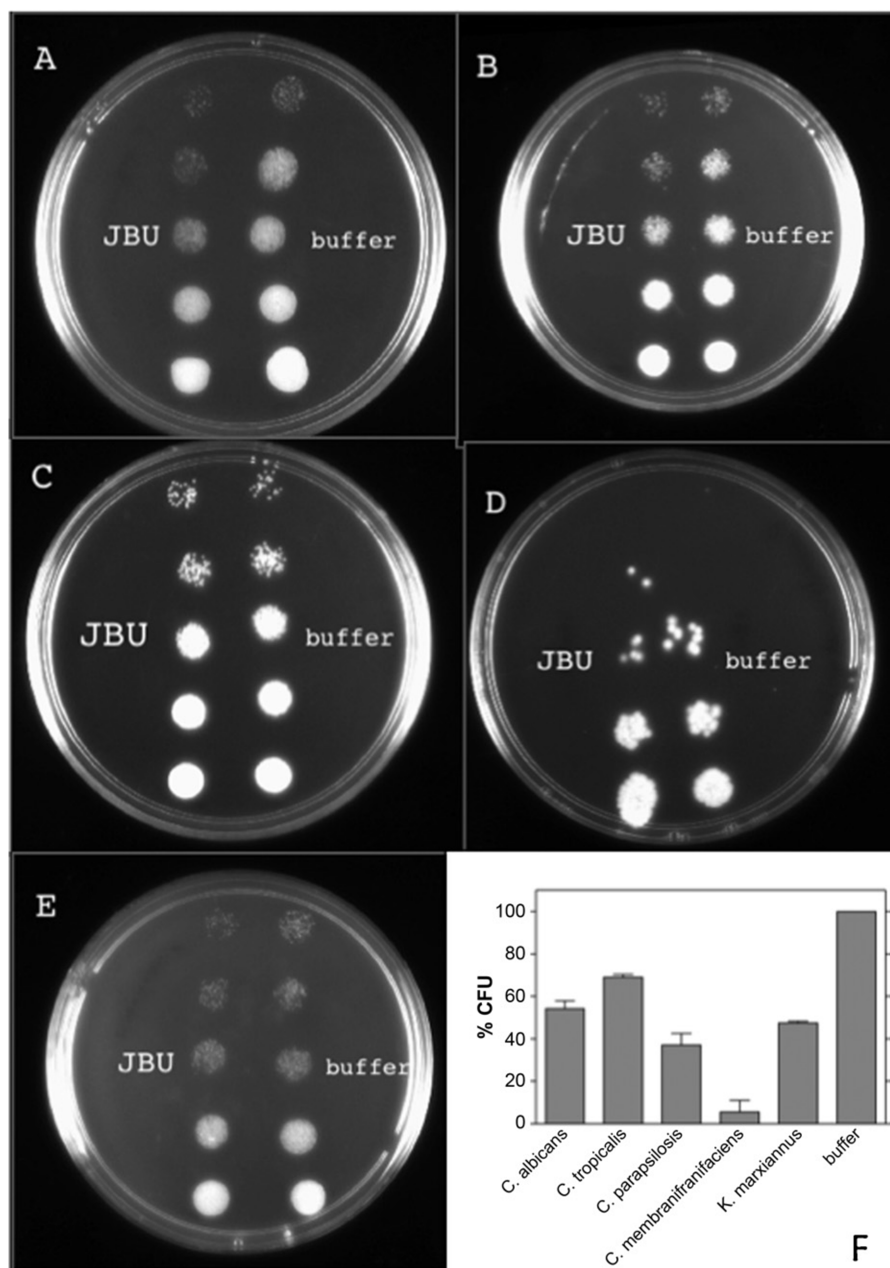

Fig. 2. Colony forming unit assay of yeasts after 24 h exposition to 0.36 μM JBU or buffer. Treated cells were 10-fold serial diluted in fresh Sabouraud medium and 10 μL of each dilution was plated and CFUs were counted after 24 h at 28 °C. Panels: (A) *C. albicans*; (B) *C. parapsilosis*; (C) *C. tropicalis*; (D) *P. membranisfaciens*; (E) *K. marxiannus*. Typical results are shown. In panel D, colony forming units of the yeasts were counted in the 10^6-fold dilution. The number of CFUs of control cells (buffer) was taken as 100%. Mean of two experiments.

92% and 95%, respectively.

3.2. Homology of urease with antifungal plant proteins – identification of putative antifungal domains

Alignments of the sequences of ureases revealed the presence of homologous regions with plant antifungal proteins, such as pea defensins, phasein A (a chitinase of *Phaseolus vulgaris cv. chick*), thaumatin and antifungal peroxidases [28] (Supplemental Figs. 1 and 2).

Although the degree of homology of ureases with these antifungal proteins is not high, it is noteworthy the fact that most of the homologous regions are close to each other, located in the alpha domain of JBU. This observation motivated the search of a putative antifungal domain in JBU.

3.3. Antifungal peptides derived from JBU

In a similar approach previously used to identify the insecticidal domain of *C. ensiformis* ureases [11,15,40], we tested different proteolytic enzymes (chymotrypsin, pepsin, trypsin and papain) for their ability to hydrolyze JBU producing antifungal peptide(s). Among the enzymes tested, papain hydrolyzed JBU generating fungitoxic fragment(s) after 2 h at 37 °C, pH 6.5, at an 1:10 enzyme/substrate ratio. Besides yeasts, JBU-derived peptides obtained by papain hydrolysis were also active against *Mucor* sp. and *F. oxysporum*, being more potent than the native protein (Fig. 6, panels A–D). Tryptic peptides derived from JBU were also fungitoxic, however trypsin alone or products of its auto hydroly-

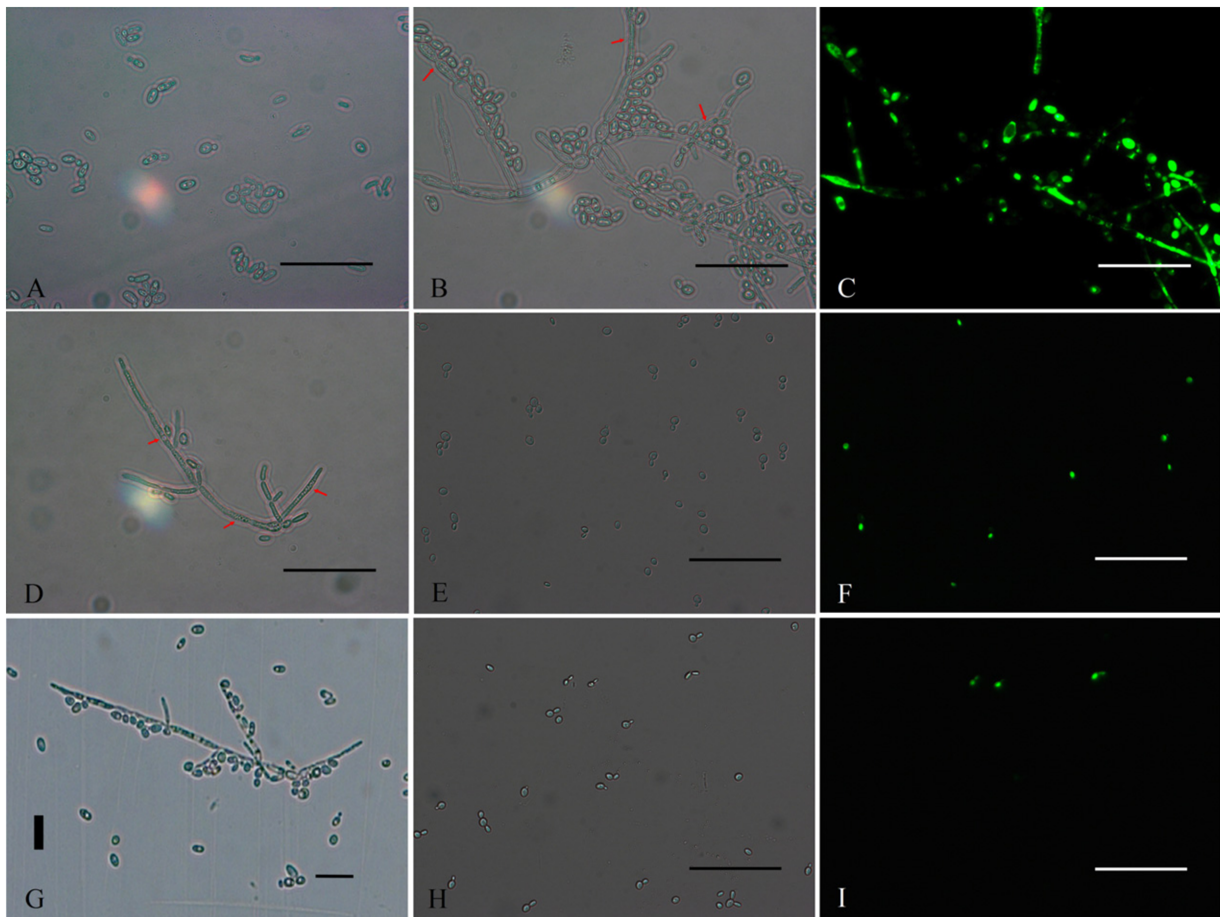

Fig. 3. (Panel A–D) JBU disrupts cell permeability and alters morphology in yeasts. *C. tropicalis* cells were grown at 28 °C for 24 h in the presence of 10 mM Tris pH 7.0 (A), 0.36 μM of JBU (B and C) and 0.1% H_2O_2 (D). SYTOX *Green* (C). Vacuoles (red arrows) can be seen within pseudohyphae cells in (B) and (D). (Panels E–I) Membrane permeabilization and change in morphology induced by Jaburetox in yeasts. *S. cerevisiae* cells were treated with 0.72 μM of Jaburetox (E and F) or buffer (H and I) for 24 h and the incubated with 0.2 μM of SYTOX *Green* (F and I). (E and H): bright field, (F and I): fluorescence. (Panel G) *C. tropicalis* cells were grown in the presence of 0.36 μM of Jaburetox at 28 °C for 24 h. Bars correspond to 50 μm in the panels A–D; 100 μm in the panels E, F, H and I; 20 μm in the panel G. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sis also presented inhibitory activity to some fungi, such as *Mucor* sp. without inhibiting others, like *F. oxysporum*.

3.4. Analysis of JBU fractions hydrolyzed with papain

JBU samples hydrolyzed by papain were analyzed by SDS-PAGE in Tricine buffer, showing the disappearance of the JBU (∼100 kDa) band and the presence of smaller bands, particularly in the 10 kDa region (Fig. 6, panel E).

Starting from 1 mg of JBU, the papain-hydrolyzed fraction containing peptides smaller than 10 kDa was desalted, lyophilized and analyzed to liquid chromatography coupled to mass spectrometry. Five peptides, corresponding to 7.1% sequence coverage of JBU (Table 1), were identified. The sequences of these peptides within JBU are highlighted in Supplemental Fig. 3. Interestingly, none of the peptides found matched any of the JBU regions that are homologous to the plant antifungal proteins shown in Supplemental Fig. 2, or showed homology to any other known antifungal proteins. No results were found searching these peptides against the Antimicrobial Peptide Database (APD2) [43]. Among the peptides identified, one (sequence in italics in Table 1) contained a partial sequence of the entomotoxic peptide Pepcanatox [29], which displays 10 kDa, similar to the most abundant peptides resulting from JBU hydrolysis by papain (Fig. 6, panel E).

Table 1
Identification by mass spectrometry of peptides (fraction <10 kDa) obtained by hydrolyzing JBU with papain.

Condition	Coverage sequence	Peptide
JBU + PAPAIN <10 kDa	7.14%	A-AEDVLNDIGAI-S
		G-KGSSSKPDELHEIIK-A
		G-KGSSSKPDELHEIIKAG-A
		N-IHTDTLNEAGFVE-H
		S-IEGNKVIRGGNAIADGPVN-E[a]

[a] The five last amino acids corresponds to the N-terminal of Jaburetox-2Ec [27].

Based on these data, a possible antifungal activity of a recombinant peptide equivalent to Pepcanatox [10] was evaluated. The peptide used in this study, named Jaburetox, contains the same 93 amino acids sequence derived from JBU (shown in Supplemental Fig. 3, panel B) present in Jaburetox-2Ec [27], and a poly-histidine tail, but lacking the V5 epitope of viral origin (A. H. S. Martinelli, PhD thesis).

3.5. Fungitoxic tests with Jaburetox

3.5.1. Filamentous fungi

The fungitoxic activity of Jaburetox was evaluated on germination and growth of *Penicillium herguei*, *Mucor* sp. and *R solani*, as shown in Fig. 6, panels F–H. *Mucor* sp. showed the highest susceptibility, its growth at 48 h being inhibited at the lowest tested

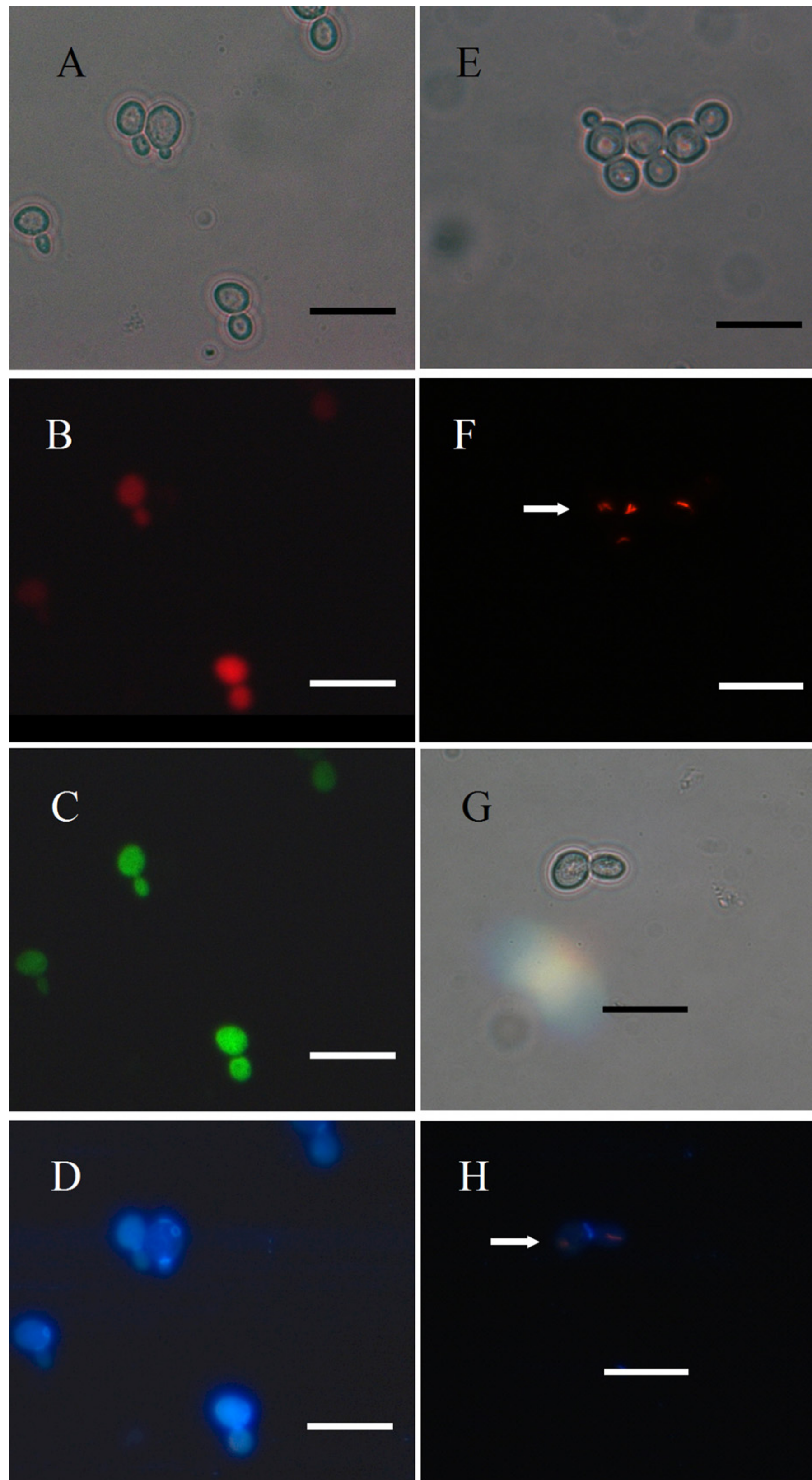

Fig. 4. Metabolic activity assay using the fluorescent probes, FUN-1 and CalcoFluorWhite. *S. cerevisiae* cells were incubated for 2 h at 28 °C in the presence of 10 mM Tris pH 7.0 (E–H), or 0.36 μM of JBU (A–D). After this period 1 μM of FUN-1 and 12.5 μM de CalcoFluorWhite were added and maintained for 1 h at 28 °C. Cells were then visualized under a fluorescence microscope. Panel A is the bright field of panels B–D; E is the bright field of F, and G is the bright field of H. Arrows in F and H point to CIVs (cylindric intracellular vesicles) which are formed by metabolic active cells, while the diffuse red (panel B) or green (panel C) fluorescence indicate lack of metabolic activity. Blue fluorescence shows that cell walls are preserved in JBU-treated cells. Bars correspond to 20 μm.

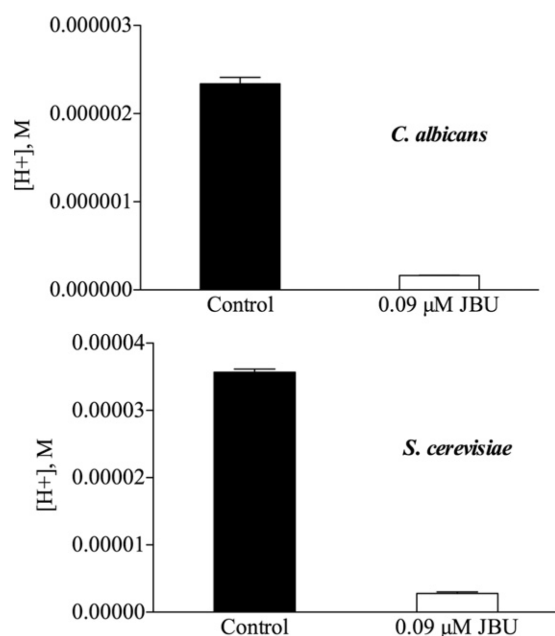

Fig. 5. Effect of JBU on the glucose-stimulated acidification of the medium by *S. cerevisiae* and *C. albicans*. Glucose (100 mM final concentration) was added to the medium 30 min after cells were exposed to 0.09 μM of JBU. The release of H^+ was measured as variation in the pH during 30 min. The bars represent the average ± sd of triplicated points.

dose (10 μM). For *P. herguei*, doses of 20 and 40 μM were inhibitory after 72–96 h, affecting also the production of pigments (data not shown) after hyphae development. In contrast, growth of *R. solani* was not affected at the highest dose of Jaburetox, 40 μM (Fig. 6, panel H).

3.5.2. Yeasts

Jaburetox at 9 μM inhibited the growth of *S. cerevisiae*, *C. parapsilosis*, *P. membranisfaciens* (Fig. 7). The other tested yeasts, *C. tropicalis*, *K. marxiannus* and *C. albicans*, were inhibited with 18 μM Jaburetox (not shown). The antifungal effect of Jaburetox did not persist after washing of the treated cells. Additional studies are needed to clarify whether the effect is fungistatic, if the peptide is being hydrolyzed/inactivated, or if the repeated administration of the peptide could lead to the death of the yeasts.

Permeabilization of the plasma membrane by Jaburetox was evaluated in *S. cerevisiae* showing that the treated cells are more permeable to SYTOX *Green* than controls (Fig. 3, panels E–F and H–I). As observed for the JBU, the peptide also induces morphological changes in yeasts (Fig. 3, panel G). The induction of pseudohyphae in *C tropicalis* and the membrane permeabilization effect in *S. cerevisiae* occurred at much lower doses (0.36–0.72 μM) than those required to arrest fungi propagation.

4. Discussion

In this work we have shown that, besides filamentous fungi, JBU is also toxic against yeasts. The fungitoxic effects consisted in inhibition of proliferation, induction of morphological alterations with formation of pseudohyphae, changes in transport of H^+ and in energy metabolism, permeabilization of membranes, eventually leading to cell death.

The antifungal effect of the JBU in yeasts or filamentous fungi [7] is independent of its catalytic activity, since the enzymatically inactivated protein, after treatment with the covalent inhibitor p-hydroxy-mercurybenzoate, maintained its fungitoxic properties. The generation of antifungal peptides upon proteolysis of urease further reinforce this fact. On the other hand, the presence of intact urease in the supernatant of cultures after 24 h was observed for most yeasts except for *K. marxiannus*, which extensively degraded JBU (data not shown). Thus at this point, it is not clear to us whether hydrolysis of JBU by the yeasts is required for expression of its fungicidal effect.

Similar to our observation in filamentous fungi [7], the fungicidal activity of JBU in yeasts is also specie-specific, affecting differently in terms of effective dose and type of toxic effects the six yeast species under study. The antifungal activity of ureases from other sources, such as the soybean urease and *H. pylori* urease, on yeasts should be assessed to give a more comprehensive idea of the antifungal property of ureases in general.

Turbidimetric evaluation of growth curves was not a reliable method to detect the antifungal effect of JBU as in some cases treated cultures became more turbid than controls not exposed to the toxic protein. The fungicidal activity of JBU was demonstrated for all the yeast species by counting colony forming units after incubation with the toxic protein. The lack of correlation between the increase in turbidity of cell cultures and the antifungal effect of JBU is probably consequent to morphological alterations of the treated yeasts, such as increased cell volume, aggregation, formation of hyphae and pseudohyphae, as shown in Fig. 3, panels B and C. Ribeiro et al. [33] reported increased turbidity of yeast cultures in the presence of antifungal proteins homologous to 2S albumins isolated from seeds of *Passiflora edulis f. flavicarpa* and *Capsicum annuun*, and associated this effect to cell agglomeration and formation of pseudohyphae, as visualized by microscopy.

At least part of the antifungal effect is due to permeabilization of membrane cells by JBU and derived peptides. Several plant proteins and peptides have the ability to permeabilize membranes, such as 2S albumins and LTPs [2,32], and defensins, which interfere on ion channels [1]. It has been reported that NaD1, a defensin from *Nicotiana alata*, permeates the membrane of hyphae and generates ROS [1]. Similarly, JBU also causes changes of cellular permeability in filamentous fungi accompanied by morphological changes, visualized in *P. herguei* by scanning electron microscopy, leading to plasmolysis and cell death [7].

Other studies have shown that both JBU and Jaburetox are capable of inserting themselves into lipid membranes making liposomes leaky and forming ion channels, which can lead to dissipation of ionic gradients essential for maintaining cell homeostasis [5,31]. Additionally, small angle X-ray scattering (SAXS) studies have demonstrated the insertion of JBU into the lipid bilayer of liposome membranes, affecting several physical parameters of the membranes [24].

Exposition to JBU induced the formation of pseudohyphae in *C. tropicalis* (Fig. 3, panel B), *P membranisfaciens* and *K. marxiannus* (not shown). In addition, JBU induced alterations in the cytoplasm of pseudohyphae, with the appearance of vacuoles similar to that seen in cells treated with H_2O_2 (Fig. 3, panels B–D – red arrows). Morphogenesis in fungi is determined by the expression of different genes induced by environmental factors. This regulation involves a cyclin specific isoform [8]. In the case of alkaline pH, the route of Rim101 (a transcription regulator) is activated through an "upstream" cascade, which starts at membrane receptors (Rim21 and DG16) [37]. Conditions that affect the cell cycle, such as treatment with hydroxy-urea, which by depleting ribonucleotides inhibits DNA replication, or nocodazol, that disrupts microtubules and blocks mitosis, make the elongated cells unable to divide and may cause the formation of pseudohyphae or hyphae-like cells, which eventually die [8]. Induction of pseudohyphae is described for other antifungal molecules such as PvD1 [22], 2S albumin [33], and peptides of *C. annuunn* [34]. These authors suggest that changes in pH caused by interference of these proteins in the H^+ flow could be responsible for the morphological variations seen in yeasts. The

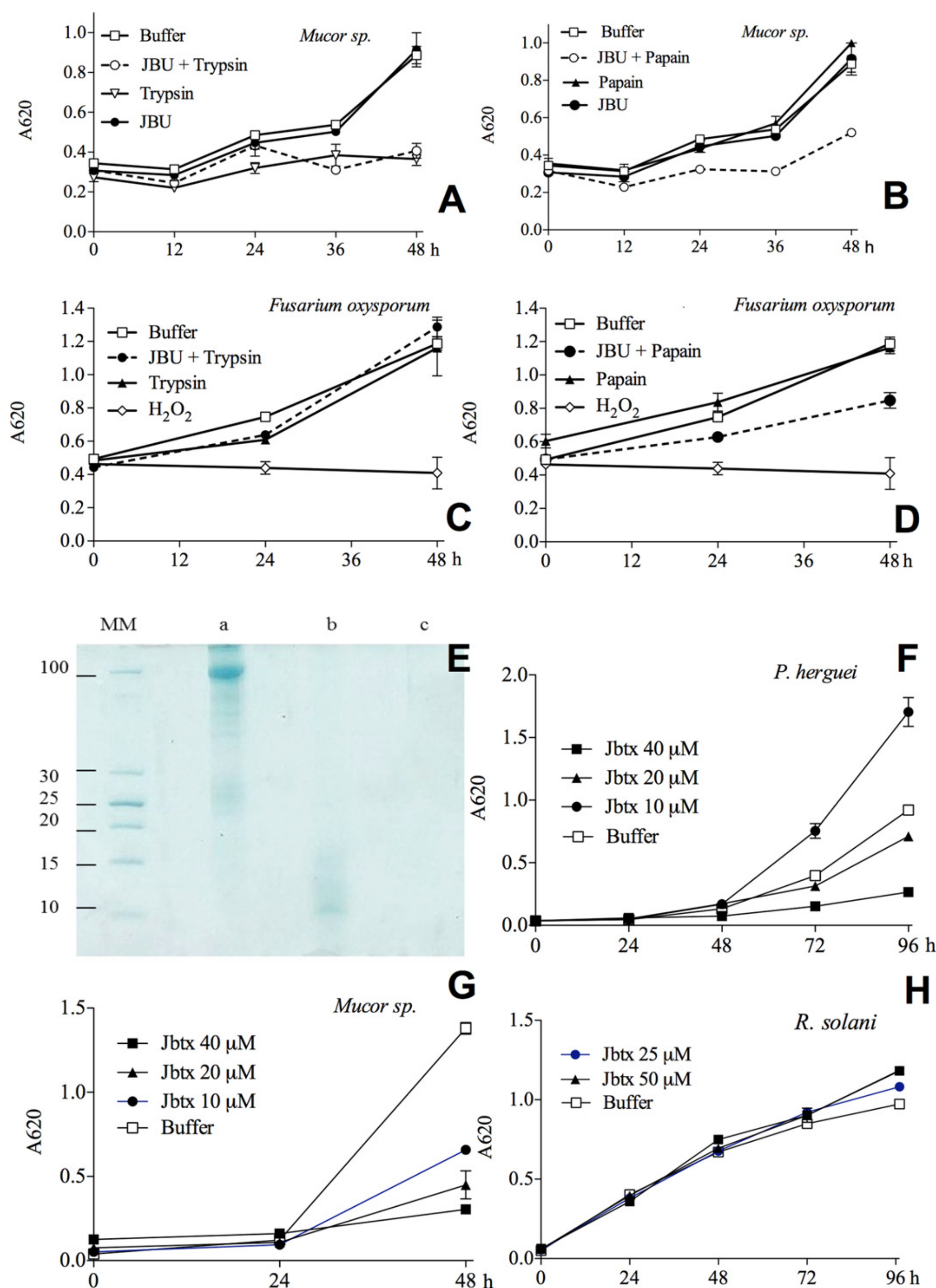

Fig. 6. Effect of urease-derived peptides on the growth of filamentous fungi. (Panels A–D) Growth curves of *Mucor* sp. (A and B) and *F. oxysporum* (C and D) in the presence of native JBU, hydrolyzed JBU, buffer, trypsin, papain and H_2O_2 (positive control). Spores were inoculated into PDB medium in a 96 well plate, incubated at 28 °C for 16 h, and then test samples were added. The plate was incubated at 28 °C and absorbance was read every 24 h. Each point represents mean ± sd of triplicated points. (Panel E) SDS-Tricine PAGE of papain-hydrolyzed JBU. Lanes: (a) = JBU, (b) = JBU hydrolyzed of papain for 2 h, 37 °C, pH 6.5, enzyme: substrate ratio 1:10, (c) = papain. MW = molecular mass markers. The gel was stained with colloidal Coomassie. (Panels F–H) Growth curves of *P. herguei*, *Mucor* sp. and *R. solani* in the presence of different concentrations of the recombinant peptide Jaburetox (Jbtx). The 96-well plate was incubated at 28 °C and absorbance at 620 nm was read every 24 h. Each point represents the mean ± sd of triplicated points.

apparent increased size of yeast cells treated with JBU may reflect the formation of pseudohyphae and considering the increased permeability of these cells (Fig. 3, panels B and C), it may indicate a “terminal phenotype”.

Here we showed that JBU at 0.09 μM affected the carbohydrate metabolism and inhibited by 92% and 95% the glucose-stimulated medium acidification in *S. cerevisiae* and *C. albicans*, respectively. Inhibition of acidification may be consequent to the membrane permeabilization, leading to dissipation of the H^+ gradient, as demonstrated for the 2S albumin protein of *P. edulis f. flavicarpa* on cells of *S. cerevisiae* and *C. albicans* [33]. Mello et al. [40], showed that PvD1, a defensin from common bean *Phaseolus vulgaris*, inhibited acidification in *S. cerevisiae* and *Fusarium* species, and ascribed this effect to disturbances caused by the protein on the plasma

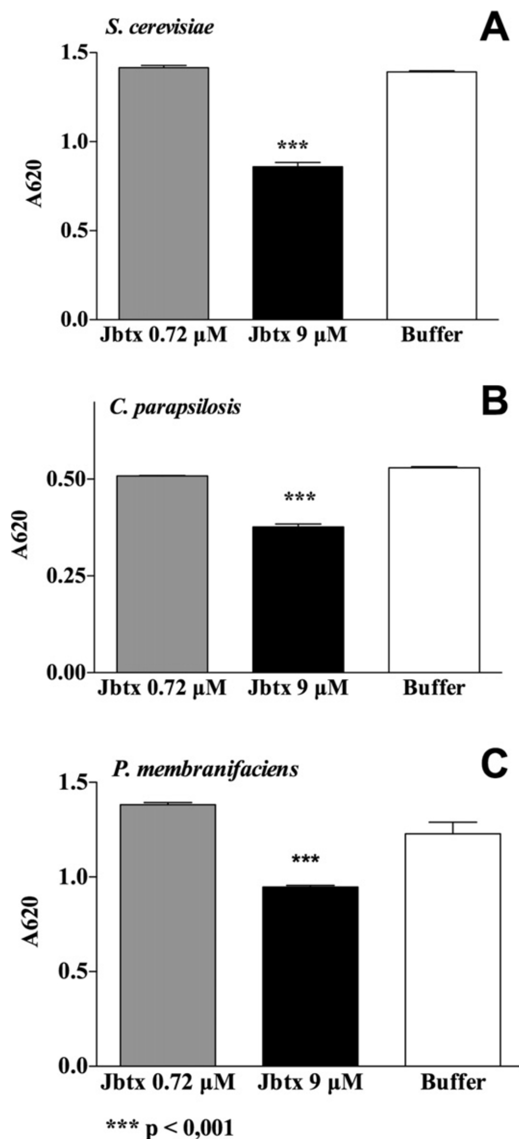

Fig. 7. Growth of *S. cerevisiae*, *C. parapsilosis*, *P. membranisfaciens* in the presence of the urease-derived peptide Jaburetox or buffer. The 96 well plate containing the cells was incubated at 28 °C and the absorbance at 620 nm was read after 18 h. Each point represents mean ± sd of points in triplicates.

membrane of fungal cells. The plasma membrane H^+-ATPase has a central role in the physiology of fungi cells and interference on its function by a number of antagonists can lead to cell death [42].

Interference caused by *C. ensiformis* urease isoforms on the activity of ATPases has been previously described. CNTX was shown to uncouple Ca^{2+} transport by the Ca^{2+} Mg^{2+} ATPase in sarcoplasmic reticulum vesicles [4]. Inhibition of a V-type H^+ ATPase in the Malpighian tubules of *Rhodnius prolixus* by the JBU-derived peptide Jaburetox-2Ec was reported [39].

JBU-treated *S. cerevisiae* cells failed to form cylindrical intravacuolar structures (CIVs) in the presence of the FUN-1 fluorescent probe (Fig. 4, panels B and C). The formation of CIVs involves the transport of FUN-1 ([2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-iodide-phenylquinolinium]) molecules to the vacuole, an ATP dependent process which is inhibited by sodium azide or when the H^+ gradient across the mitochondrial membrane is disrupted [25]. Metabolically active cells, growing in aerobic or anaerobic conditions, form CIVs, visualized as red-orange fluorescent cylinders inside the cells. Cells treated with JBU showed a diffuse fluorescence in cytosol. According to the manufacturer, this staining pattern indicates cells with intact membranes, but metabolically compromised. There was no change in the staining of Calcofluor White M2R (which labels the cell wall) in cells treated with JBU as compared to controls, indicating the integrity of cell walls after a 2 h treatment (Fig. 4, panel D). These data show that 2 h exposure of *S. cerevisiae* to JBU interferes on the energy metabolism of the cells, with no visible changes in membrane permeability. As the exposure of *C. tropicalis* (Fig. 3, panel C), *P. membranisfaciens*, *C. parapsilosis* and *K. marxiannus* cells to JBU for 24 h caused membrane permeabilization, monitoring of JBU-treated *S. cerevisiae* for a longer time is required to evaluate if progression of antifungal effect would eventually lead to cell death.

Hydrolysis of JBU with papain produced fungitoxic peptides smaller than 10 kDa. Five of these peptides were identified by mass spectrometry and none of them match putative antifungal domains of JBU homologous to other plant antifungal proteins. At this point, two possibilities should be considered: these peptides are not associated with antifungal(s) domain(s) of JBU, or the JBU antifungal(s) domain(s) are unlike any other fungitoxic proteins already known.

One of these peptides contained part of the N-terminal sequence of the insecticidal peptide Jaburetox-2Ec. Becker-Ritt et al. [7], reported that Jaburetox-2Ec did not affect the micellar growth of phytopathogenic fungi, including that *P. herguei*. In that study, the peptide was added to the medium at a lower dose (0.57 µM), after 16 h of culture, at a later stage of germination of the spores. Here, Jaburetox was added simultaneously with the spores, leading to inhibition of germination and growth, and delaying development of hyphae. This result indicates that besides its insecticidal activity, this internal peptide of *C. ensiformis* urease is also antifungal, affecting the early stages of development of the mycelium, a step also susceptible to ureases [7]. The variations in methodology used in the two studies may have influenced the different results obtained. The time course and characteristics of the fungitoxic effects indicated similar antifungal mechanisms for JBU and Jaburetox, probably based on the ability of these polypeptides to insert in membranes, altering the cell permeability.

The antifungal activity of Jaburetox on yeasts required 2–3 times larger doses as compared to the holoprotein JBU, indicating the possibility that other protein domains are involved in this activity. Becker-Ritt et al. reported the antifungal activity of the two-chained urease from *H. pylori*. Bacterial ureases lack part of the amino acid sequence (the N-terminal half) of Jaburetox, which in single-chained plant ureases corresponds to a linker region between bacterial subunits. This fact strongly suggests that other antifungal domain(s) besides the region corresponding to the entomotoxic domain are present in ureases.

The discovery of new antifungal agents becomes increasingly important due to the increasing number of cases of invasive mycoses. JBU, a protein with multiple functions and peptides derivatives may represent a new alternative to control clinically important and phytopathogenic fungi, especially considering their potent activity in the 10^{-6}–10^{-7} M range. Structure versus activity studies deepening the identification of protein domains and the construction of biologically active recombinant peptides containing these domains are some of steps toward unraveling the real fungicidal/fungistatic potential of ureases and derived peptides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2012.08.010.

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