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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE
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KARINE KAPPAUN

ESTUDOS ESTRUTURAIS E DE ATIVIDADES BIOLÓGICAS DO SOYURETOX, UM PEPTÍDEO DERIVADO DE UREASE. ZEBRAFISH (DANIO RERIO) COMO MODELO DE ESTUDO DO PEPTÍDEO

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Tese apresentada como requisito parcial para a obtenção do grau de Doutor pelo Programa de Pós-Graduação em Medicina e Ciências da Saúde da Escola de Medicina da Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS)

PORTO ALEGRE

Julho de 2018

Dedico aos meus pais

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RESUMO

Ureases (ureia amido-hidrolases; EC 3.5.1.5) são enzimas níquel dependentes, amplamente distribuídas em bactérias, fungos e plantas, que catalisam a hidrólise da ureia à amônia e dióxido de carbono. O jaburetox é um peptídeo derivado de uma isoforma de urease da planta *Canavalia ensiformis.* Estruturalmente, o jaburetox é um peptídeo intrinsecamente desordenado, demonstrado por ferramentas *in silico* de bioinformática, e experimentalmente, por dicroísmo circular (CD) e ressonância magnética nuclear (RMN). Diversas propriedades biológicas do jaburetox já foram descritas, tais como atividade inseticida, capacidade de interagir com bicamadas lipídicas, efeito fungitóxico, dentre outras. O soyuretox é um peptídeo colinear ao jaburetox, porém derivado da urease ubíqua de soja. Um dos objetivos dessa tese foi realizar estudos estruturais do peptídeo soyuretox por CD e RMN, que revelaram sua natureza intrinsecamente desordenada e aumento no conteúdo de estrutura secundária na presença de micelas de SDS. Propriedades biológicas do soyuretox foram avaliadas. O peptídeo tem atividade entomotóxica, induzindo agregação de hemócitos de *Rhodnius prolixus*, o vetor da doença de Chagas, tanto *in vivo* como *in vitro*. O efeito fungitóxico do soyuretox na levedura *Candida albicans* envolve a produção de ânions superóxido, sendo o peptídeo imunolocalizado ligado na superfície da levedura. Por fim, foram avaliados os efeitos comportamentais e morfológicos do soyuretox em larvas de *zebrafish (Danio rerio),* visando aprofundar o entendimento do mecanismo de ação tóxica e o perfil toxicológico dessa molécula, que apresenta potencial biotecnológico como biopesticida.

Palavras chaves: soyuretox, ureases, estrutura tridimensional, RMN, *zebrafish, Candida albicans, Rhodnius prolixus,* proteinas desordenadas.

ABSTRACT

Ureases (urea amidohydrolases, EC 3.5.1.5) are nickel-dependent enzymes, widely spread in bacteria, fungi and plants, which catalyze the hydrolysis of urea to ammonia and carbon dioxide. Jaburetox is a peptide derived from a urease isoform of the *Canavalia ensiformis* plant. Structurally, jaburetox is an intrinsically disordered peptide, demonstrated by bioinformatics tools, and experimentally, by circular dichroism (CD) and nuclear magnetic resonance (NMR). Several biological properties of jaburetox have already been described, such as insecticidal activity, ability to interact with lipid bilayers, fungitoxic effect, among others. Soyuretox is a peptide colinear to jaburetox derived from the soybean ubiquitous urease. One of the objectives of this thesis was to carry out structural studies of the soyuretox peptide by CD and NMR, which revealed its intrinsically disordered nature and that its secondary structure content is modified in the presence of SDS micelles. Biological properties of soyuretox were evaluated. The peptide has entomotoxic activity, inducing aggregation of hemocytes from *Rhodnius prolixus*, the vector of Chagas disease, both *in vivo* and *in vitro*. The fungitoxic effect of soyuretox on yeast *Candida albicans* involves the production of superoxide anions, and the peptide was immunolocalized on the surface of the yeast. Finally, the behavioral and morphological effects of soyuretox on zebrafish larvae (*Danio rerio*) were evaluated, aiming to deepen the understanding of the mechanism of toxic action and the toxicological profile of this molecule, which presents biotechnological potential as a biopesticide.

Keywords: soyuretox, ureases, three dimensional structure, NMR, zebrafish, *Candida albicans*, *Rhodnius prolixus*, disordered proteins.

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LISTA DE ABREVIATURAS

A absorbância

BSA albumina sérica bovina

CD Dicroísmo Circular (Circular Dichroism)

CFU unidades formadoras de colônia (ColonyFormingUnits)

CMC concentração micelar crítica

CNTX canatoxina

DMSO dimetilsulfóxido

dpf dias após a fertilização

eSBU urease embrião-específica de soja

IPTG isopropilβ-D-1-tiogalactopiranosídeo

Jbtx jaburetox

JBU isoforma da urease de *Canavalia ensiformis*

JBURE-IIB isoforma da urease de *Canavalia ensiformis*

kDa quilodalton (s) (1000 daltons)

LB meio de cultura Luria-Bertani

NBT cloreto de tetrazólio-nitroazul (Nitro blue tetrazoliumchloride).

OD densidade ótica

PDB Protein Data Bank

RMN Ressonância magnética nuclear

rpm rotações por minuto

SAXS espalhamento de raio X de baixo ângulo

SDS dodecil sulfato de sódio

Sytx soyuretox

TCEP tris(2-carboxietil)fosfina (Tris (2-carboxyethyl)phosphine)

uSBU urease ubíqua de soja

SUMÁRIO

APRESENTAÇÃO

A tese está organizada em três capítulos:

Capítulo 1. Introdução Geral – Ureases e peptídeos derivados de ureases

Capítulo 2. Artigo submetido à publicação

Soyuretox, a recombinant peptide derived from soybean (*Glycine max*) ubiquitous urease: biological and structural properties, and toxicity screening in zebrafish larvae.

Capítulo 3. Considerações finais, Conclusões e Perspectivas

Ao final da tese são apresentadas as referências de todos os capítulos.

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Capítulo I

Introdução Geral

1. INTRODUÇÃO GERAL

1.1 UREASES

As ureases (EC 3.5.1.5; ureia amido-hidrolase) são enzimas níquel dependentes que catalisam a hidrólise de ureia em amônia e carbamato, o qual se decompõe em uma segunda molécula de amônia e dióxido de carbono. Estas proteínas, que têm sido foco de estudo do nosso grupo por mais de três décadas, estão amplamente distribuídas em diversos organismos incluindo bactérias, plantas e fungos, mas não são sintetizadas por animais (LIGABUE-BRAUN et al., 2013; KRAJEWSKA, 2009). Independentemente de suas origens e de suas organizações quaternárias, ureases são proteínas homólogas que apresentam no mínimo 55 % de identidade na sequência de aminoácidos. As estruturas 3D de ureases de diversas fontes mostram ampla sobreposição sugerindo mecanismos catalíticos similares. Análises filogenéticas indicam que todas as ureases atuais evoluíram de uma proteína ancestral comum (LIGABUE-BRAUN et al., 2013).

O estudo das ureases fez marcos históricos importantes na Bioquímica: James B. Sumner em 1926 demonstrou, com a cristalização da urease JBU (Jack bean urease) isolada das sementes da planta feijão de porco (*Canavalia ensiformis*), a natureza proteica das enzimas (SUMNER, 1926), o que lhe rendeu o Prêmio Nobel de Química em 1946. Em 1975, a descoberta de átomos de niquel no sítio ativo da JBU, fundamentais para a atividade catalítica da enzima (DIXON et al., 1975), estabeleceu a relevância biológica desse metal. Anos mais tarde, em 2001, nosso grupo foi um dos pioneiros a descrever propriedades biológicas não-catalíticas das ureases (FOLLMER et al., 2001).

As ureases vegetais, como as de feijão-de-porco (*Canavalia ensiformis*), de soja (*Glycine max*), e fúngicas, como a levedura *Cryptococcus gattii*, são em geral trímeros (α_3) ou hexâmeros (α_6) compostos por um único tipo de subunidade com aproximadamente 90 kDa. Já as ureases bacterianas, em sua vasta maioria, possuem três tipos de cadeias polipeptídicas que se organizam como trímeros de trímeros ($[\alpha\beta\gamma]_3$), como descrito para as enzimas de *Sporosarcina* (*Bacillus*) *pasteurii e*

Klebsiella pneumoniae. Ureases compostas por dois tipos de subunidades são conhecidas, até o momento, somente para bactérias do gênero *Helicobacter*. A estrutura 3D da urease de *H. pylori* foi descrita em 2001 por Ha e colaboradores (HA et al. 2001), revelando um tetrâmero de trímeros de dímeros $([\alpha\beta]_3)_4$ (Mazzei, Musiani, and Ciurli, 2017). As subunidades estruturais das ureases são mostradas na Figura 1 (CARLINI & LIGABUE-BRAUN, 2016).

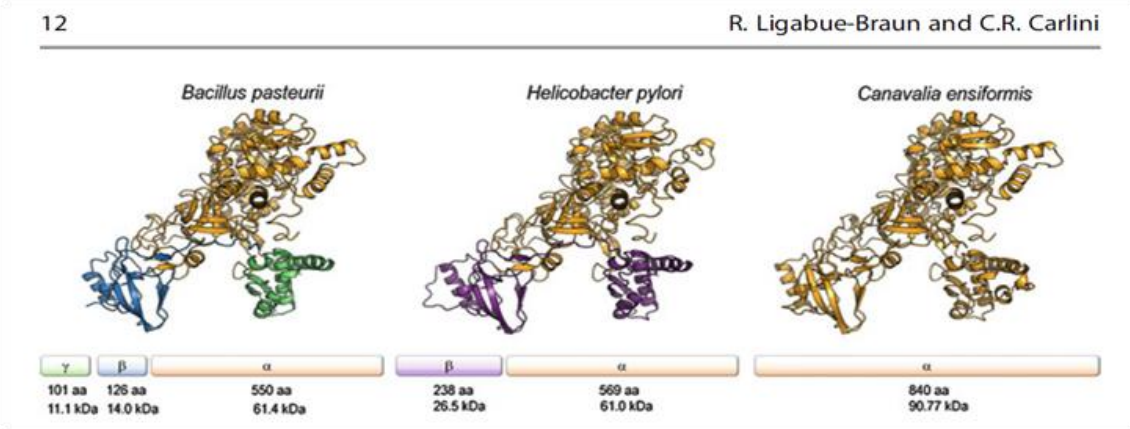


Figura 1. Estrutura das ureases**:** A estrutura 3D obtida por cristalografia das ureases bacterianas e vegetais são superponíveis (parte de cima da figura). As ureases bacterianas podem possuir três tipos de subunidades, como a de S. (*B.*) *pasteurii*, ou dois tipos, como a de *H. pylori.* Ureases fúngicas ou vegetais, como a leguminosa *Canavalia ensiformis*, são compostas por apenas um tipo de subunidade. Abaixo das estruturas estão representadas as suas respectivas subunidades, com indicação do número de aminoácidos e da massa molecular de cada cadeia polipeptídica (Figura retirada de Carlini & Ligabue-Braun, 2016).

As ureases fazem parte da classe de proteínas multifuncionais chamadas de *moonlighting*, e apresentam, além da atividade da hidrólise da ureia, outras propriedades biológicas não enzimáticas. Ureases tratadas com um inibidor covalente irreversível, o *p*-hidroximercuribenzoato, perdem a capacidade de hidrolisar ureia mas mantém outras atividades biológicas, sugerindo que existem domínios proteicos diferentes do sítio catalítico que são responsáveis por

desempenhar estas outras atividades (FOLLMER et al., 2001; FOLLMER, WASSERMANN & CARLINI, 2004).

1.2 A ATIVIDADE UREÁSICA EM PLANTAS

A atividade ureásica está presente em todas as espécies de plantas, e em todos os tecidos vegetais (POLACCO & WINKLER, 1984). O nitrogênio é o elemento essencial para o crescimento de plantas, porém está presente em baixas concentrações no solo. Quando está na forma de ureia, o nitrogênio não é assimilável pela planta. A conversão de ureia a amônia, uma forma assimilável de nitrogênio, é a principal função das ureases em plantas, sendo a ureia a fonte primária de nitrogênio para plantas (WITTE, 2011). Duas leguminosas em especial, a soja (*Glycine max*) e o feijão-de-porco (*Canavalia ensiformis*), são importantes fontes de ureases, as quais vêm sendo estudadas pelo grupo.

1.3 UREASES DE PLANTAS

1.3.1 AS UREASES DE SOJA (*Glycine max*)

A soja produz três isoformas de urease: a urease embrião específica (eSBU), a urease ubíqua (uSBU) e uma terceira isoforma, a SBU-III. A eSBU é codificada pelo gene *Eu1* (MEYER-BOTHLING & POLACCO, 1987), que é expresso apenas no embrião em desenvolvimento, sendo a proteína acumulada nas sementes maduras (POLACCO & WINKLER, 1984). A uSBU, codificada pelo gene *Eu4*, está presente em todos os tecidos da planta em baixas concentrações (TORISKY et al., 1994). A eSBU é cerca de 1000 vezes mais abundante na semente do que a uSBU (TORISKY & POLACCO, 1990). A SBU-III foi recentemente caracterizada como uma nova isoforma de urease de soja, codificada pelo gene *Eu5.* Seus transcritos foram detectados nas sementes, embriões em desenvolvimento e em raízes da planta. Por serem proteínas muito conservadas, as mutações encontradas na SBU-III em aminoácidos

conservados no sítio ativo “consenso” de ureases sugerem que não há atividade ureolítica nesta proteína, e que, provavelmente, a SBU-III não está envolvida em mecanismos de disponibilização de nitrogênio na planta (WIEBKE-STROHM et al., 2016).

Mutantes de soja com lesões no gene da uSBU apresentam anormalidades características do acúmulo de ureia, como necroses nas extremidades das folhas e raízes, acúmulo de ureia nas folhas e sementes, e retardo na germinação, o que sugere que a uSBU está relacionada à reciclagem de derivados de ureia. Em contraste, em mutantes de soja com ausência da eSBU, não houve mudança no fenótipo e nem aumento dos níveis de ureia, sugerindo que essa proteína possa estar envolvida em outro tipo de função, provavelmente relacionada à defesa da planta (HOLLAND & POLACCO, 1992). A eSBU purificada direto das sementes de soja mostrou ter diversas propriedades biológicas não enzimáticas, como indução de agregação de plaquetas de coelho, atividade inseticida contra o percevejo manchador do algodão, *Dysdercus peruvianus,* e atividade antifúngica contra fungos fitopatogênicos, como *Curvularia lunata* (BECKER-RITT et al., 2007), o que corrobora a hipótese de ser a eSBU uma proteína de defesa, como proposto no estudo citado anteriormente.

Nosso grupo clonou o gene da uSBU em *E.coli*, e obteve a expressão da proteína recombinante fusionada com GST (glutationa S-transferase), possibilitando o estudo de suas propriedades biológicas. A uSBU~GST apresentou atividade contra fungos filamentosos e leveduras patogênicas como a *Candida albicans* e *C. tropicalis*. A uSBU-GST também mostrou ser entomotóxica para o barbeiro *Rhodnius prolixus,* e induziu exocitose, evidenciada no ensaio de agregação de plaquetas de coelho, mostrando ter atividades biológicas similares a eSBU (MARTINELLI et al., 2017).

1.3.2 AS UREASES DA *CANAVALIA ENSIFORMIS*

A planta feijão de porco (*C. ensiformis*) é uma leguminosa que produz três isoformas de urease: a urease clássica, JBU ou JBURE-I (SUMNER, 1926), a canatoxina, CNTX (CARLINI & GUIMARAES, 1981) e a JBURE-II (PIRES-ALVES et

al., 2003; MULINARI et al., 2011). A JBU é a isoforma mais abundante encontrada na semente madura.

A CNTX foi isolada como uma proteína neurotóxica em 1981 e, anos depois, foi identificada como sendo uma isoforma de urease (CARLINI & GUIMARAES, 1981; FOLLMER et al., 2001). A CNTX possui 30-40 % da atividade ureolítica da JBU, mostrando similaridade de sequência com a JBU mas diferindo desta no estado de oligomerização, conteúdo de metais e ainda no comportamento em cromatografia de afinidade por metais (FOLLMER et al., 2001).

A CNTX, quando administrada intraperitonealmente em ratos e camundongos, induz bradicardia, hipertensão e hipotermia, precedendo convulsões e a morte dos animais (DL_{50} = 0,5-2,0 mg/kg) (CARLINI et al., 1984). O mesmo não ocorre ao se administrar a CNTX por via oral, provavelmente devido à instabilidade da proteína no pH ácido do estômago (CARLINI & GUIMARAES, 1991). Embora exista alta similaridade, a JBU não apresenta ação convulsionante como a CNTX. Ao se injetar a proteína intraperitonealmente em uma dose de 20 mg/kg em camundongos, nenhuma letalidade foi observada (FOLLMER et al., 2001).

Tanto a CNTX como a JBU apresentam outras atividades biológicas, não relacionadas à ureolise, como a propriedade secretagoga, observável *in vivo* e *in vitro* em vários tipos celulares de mamíferos, e facilmente medida em plaquetas sanguíneas através da resposta de agregação plaquetária (BARJA-FIDALGO, GUIMARAES & CARLINI, 1991; CARLINI, GUIMARAES & RIBEIRO, 1985; FOLLMER et al., 2001). Posteriormente, em estudos comparando duas ureases vegetais, a JBU e eSBU, e a urease de *S*. (*B*.) *pasteurii*, verificou-se que todas estas proteínas possuem atividade secretagoga, induzindo agregação plaquetária como observado para a CNTX, ainda que com diferenças em relação às doses efetivas 50 % (CARLINI, GUIMARAES & RIBEIRO, 1985; FOLLMER et al., 2004). As ureases vegetais apresentam também atividade inseticida contra insetos de diferentes ordens, como por exemplo: o caruncho do feijão-de-corda *Callosobruchus maculatus* (Bruchidae), o barbeiro *Rhodnius prolixus* (Hemiptera) (CARLINI et al., 1997; FERREIRA-DASILVA et al., 2000), os percevejos (Hemiptera) pragas na agricultura *Nezara viridula*e *Dysdercus peruvianus* (CARLINI & GROSSI-DE-SA, 2002; Staniscuaski et al., 2005), e o “milkweed bug” *Oncopeltus fasciatus* (Hemiptera) (DEFFERRARI et al., 2011). A atividade antifúngica das ureases foi inicialmente descrita para a JBU, eSBU e a

urease de *H. pylori* em fungos filamentosos (BECKER-RITT et al., 2007), e depois demonstrada em leveduras (POSTAL et al., 2012).

A terceira isoforma de urease da *C. ensiformis*, designada JBURE-II, foi clonado por nosso grupo (PIRES-ALVES et al., 2003; MULINARI et al., 2011). Um estudo proteômico permitiu a identificação da proteína em sementes de *C. ensiformis* e em plântulas em diferentes estádios de desenvolvimento, comprovando que o gene *jbure-II* é funcional na planta (DEMARTINI, CARLINI & THELEN, 2011). A urease recombinante JBURE-II foi produzida em *E. coli*, na ausência das proteínas acessórias que promovem a incorporação de Ni^{2+} no sítio ativo, obtendo-se a apourease enzimaticamente não ativa (MULINARI et al., 2011). Comprovando mais uma vez a existência de propriedades biológicas independentes da atividade enzimática, a apourease JBURE-II apresentou atividade antifúngica e efeitos entomotóxicos equivalente aos da JBU (MULINARI et al., 2011).

1.4 PEPTIDEOS DERIVADOS DE UREASES

1.4.1 JABURETOX

Estudando o mecanismo da atividade inseticida da CNTX, observou-se que a suscetibilidade dos insetos dependia da sua hidrólise após ingestão pelo inseto e formação de peptídeos entomotóxicos, liberados por enzimas digestivas tipo-catepsina dos insetos (CARLINI & GUIMARAES, 1991; FERREIRA-DASILVA et al., 2000; PIOVESAN et al., 2008; REAL-GUERRA, CARLINI & STANISCUASKI, 2013). Por meio da hidrólise *in vitro* da CNTX pelas enzimas digestivas do inseto *C. maculatus,* foi isolado um peptídeo com propriedades inseticidas (FERREIRA-DASILVA et al., 2000), denominado Pepcanatox, que despertou o interesse no estudo de peptídeos derivados de urease.

Baseado na sequência N-terminal do Pepcanatox, e utilizando como molde para PCR o cDNA da urease JBURE-II (PIRES-ALVES et al., 2003), um peptídeo recombinante análogo foi produzido em *E. coli*, e denominado jaburetox-2Ec (MULINARI et al., 2007). O jaburetox-2Ec mostrou ter potente atividade inseticida contra o lepidóptero *Spodoptera frugiperda* e contra o hemiptero *Dysdercus peruvianus*, apresentando neste último uma toxicidade maior do que a observada para a CNTX na mesma dose. Ao contrário da CNTX, que causa convulsão e morte em poucas horas quando injetado intraperitonealmente em ratos e camundongos, o

jaburetox-2Ec não produziu efeito tóxico nesses animais, mesmo em doses 5 vezes maiores (MULINARI et al., 2007). Posteriormente, a molécula do jaburetox-2Ec foi otimizada para conter somente a sequência peptídica derivada da urease JBURE-II, eliminando-se sequências exógenas introduzidas pelo sistema de expressão (epitopoV5), resultando no peptídeo chamado apenas de jaburetox (POSTAL et al., 2012), expresso em vetor pET-23a. Ambos os peptídeos, jaburetox-2Ec e jaburetox, mostraram propriedades entomotóxicas equivalentes (POSTAL et al., 2012).

Como parte da sua atividade entomotóxica e inseticida, o jaburetox apresenta efeito antidiurético em *R. prolixus* (STANISCUASKI et al., 2009), e causa inibição da secreção de urina em túbulos de Malpighi isolados desse inseto (STANISCUASKI et al., 2009; MARTINELLI et al., 2014). O jaburetox também apresenta neurotoxicidade para o barbeiro *Triatoma infestans* (Hemíptera), tendo sido imunolocalizado no cérebro do inseto, onde altera atividades enzimáticas relevantes (GALVANI et al., 2015); o mesmo tipo de efeito também foi descrito para *R. prolixus* (FRUTTERO et al., 2017). Além disso, o jaburetox mostrou ter efeito neurotóxico em preparações neuromusculares de baratas, como a *Phoetalia pálida* (Orthoptera) (MARTINELLI et al., 2014).

Em estudo do nosso grupo, foi demostrado que parte da ação entomotóxica do jaburetox em *R. prolixus* ocorre por interferência no sistema imune, o que torna o inseto mais sensível à infecção por bactérias entomopatogênicas, provavelmente por “consumo” dos fatores envolvidos na defesa do inseto contra patógenos. Nesse estudo foram feitos ensaios com hemócitos (que são células envolvidas na resposta imune do inseto), demonstrando-se que o jaburetox ativa e promove agregação dessas células, tanto *in vitro* (incubação de hemócitos isolados com jaburetox), como *in vivo* (injeção de jaburetox no inseto e posterior coleta dos hemócitos) (FRUTTERO et al., 2016).

Considerando que o jaburetox, em função de sua atividade inseticida e antifúngica, possa ter aplicações biotecnológicas como biopesticida, foram produzidas plantas de soja, milho e cana de açúcar que expressam o jaburetox sob controle do promotor 35S. Testes com folhas destacadas das plantas mostraram que o jaburetox pode conferir resistência contra o ataque de pragas como *Helicoverpa armigera* (soja e milho) (DIDONÉ, 2017) e *Spodoptera frugiperda* (polífaga) (MULINARI, 2004).

Outros estudos mostraram que o jaburetox afeta a permeabilidade de lipossomos (BARROS et al., 2009; MARTINELLI et al., 2014) e induz a formação de canais iônicos cátion-seletivos em membranas bilipídicas planares (PIOVESAN et al., 2014). A permeabilização de membranas de células em leveduras induzida por jaburetox foi observada, acompanhando o efeito antifúngico promovido pelo peptídeo (POSTAL et al., 2012). Utilizando-se espalhamento de luz e de raios X a baixo ângulo (SAXS), comprovou-se a capacidade do jaburetox de se inserir em membranas bilipídicas de lipossomas multilamelares mimético de plaquetas humanas, levando a alterações de parâmetros físico-químicos, como redução do número de lamelas, da distância interlamelar e da fluidez da membrana (Michelleto et al., 2016). A capacidade de interação do jaburetox com lipídeos e membranas também parece estar na base de seus efeitos neurotóxicos em insetos (BROLL, 2017).

Com relação a sua estrutura, o jaburetox foi caracterizado como um polipeptídeo intrinsecamente desordenado (LOPES et al., 2015). Proteínas intrinsecamente desordenadas não apresentam uma conformação nativa única, em contraste com as demais proteínas, as quais apresentam uma estrutura nativa bem definida. A natureza intrinsecamente desordenada do jaburetox foi evidenciada por meio de ferramentas de bioinformática (MARTINELLI et al., 2014) e confirmada experimentalmente por dicroísmo circular e ressonância magnética nuclear (RMN) (LOPES et al., 2015).

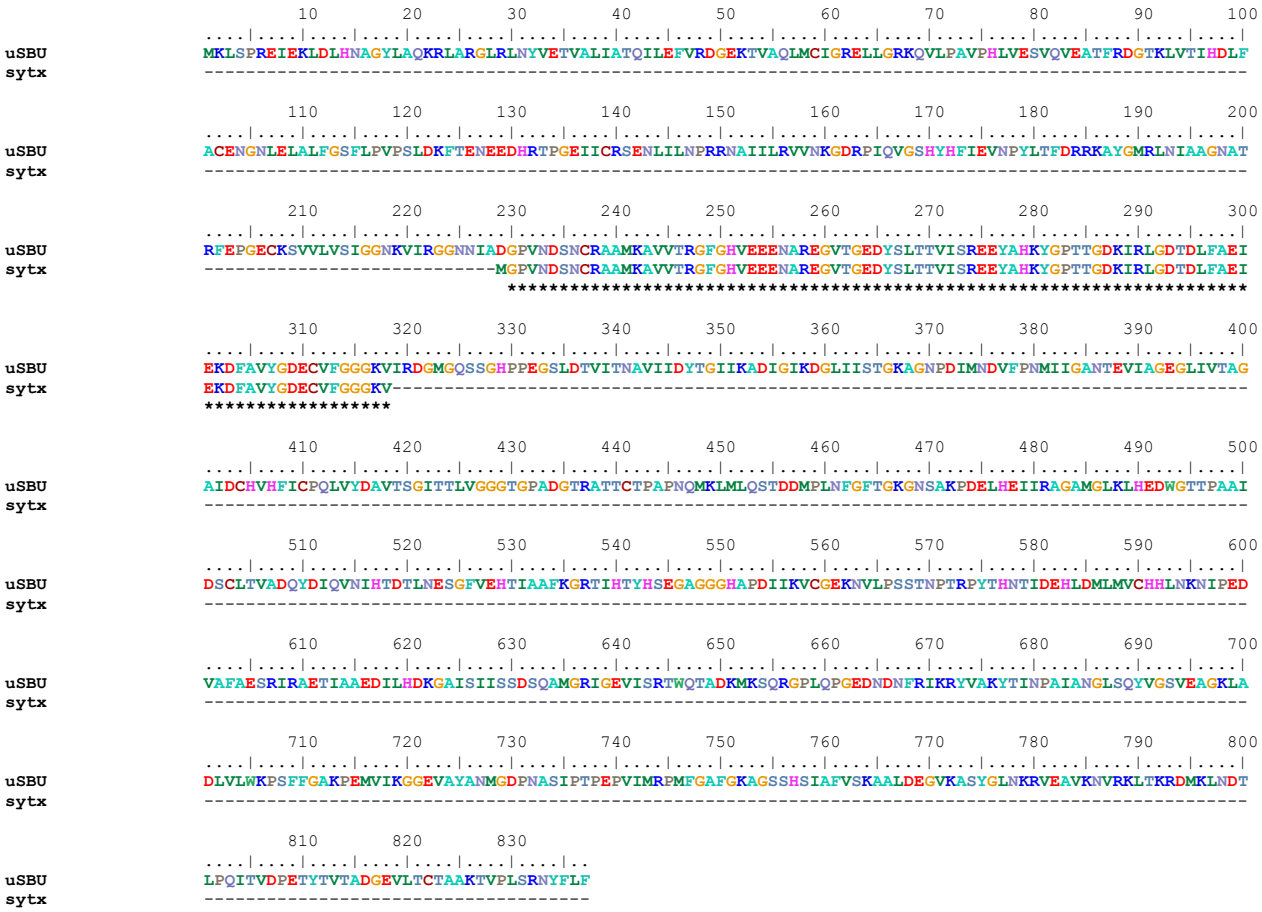
1.4.2 SOYURETOX

O soyuretox é um peptídeo equivalente ao jaburetox (derivado de *C. ensiformis*), porém derivado da urease ubíqua de soja (uSBU) (KAPPAUN, 2014). Para a localização da região homóloga correspondente ao jaburetox, a sequência de aminoácidos desse peptídeo foi alinhada com a sequência de aminoácidos correspondente da urease ubíqua. Esse novo peptídeo, agora derivado da urease ubíqua, foi clonado em vetor de expressão pET-23a para adicionar uma cauda de seis histidinas na região C-terminal do peptídeo. O peptídeo é constituído de 101 aminoácidos, tem massa molecular de 11.06 kDa e apresenta 72 % de identidade com o jaburetox.

A sequência de aminoácidos dos peptideos derivados de urease, jaburetox e a sequência de aminoácidos do soyuretox foram alinhados com as respectivas sequências das ureases, JBUREII para o jaburetox (Figura 2 A), urease ubíqua para o soyuretox (Figura 2 B), alinhamento das sequência de aminoácidos do Jaburetox e Soyuretox (Figura 2 C) e representação esquemática da estrutura primária mostrando a origem dos peptídeos nas respectivas ureases (Figura 2 D).

A

B


uSBU MKLSPREIEKLDLHNAGYLAQKRLARGLRLNYVETVALIATQILEFVRDGEKTVAQLMCIGRELLGRKQVLPAVPHLVESVQVEATFRDGTKLVTIHDLF
sytx
uSBU ACENGNLELALFGSFLPVPSLDKFTENEEDHRTPGEIICRSENLILNPRRNAIILRVVNKGDRPIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGNAT
sytx
uSBU RFEPGECKSVVLVSIGGNKVIRGGNNIADGPVNDSNCRAAMKAVVTRGFGHVEEENAREGVTGEDYSLTTVISREEYAHKYGPTTGDKIRLGDTDLFAEI
sytx -----------------------------MGPVNDSNCRAAMKAVVTRGFGHVEEENAREGVTGEDYSLTTVISREEYAHKYGPTTGDKIRLGDTDLFAEI
uSBU EKDFAVYGDECVFGGGKVIRDGMGQSSGHPPEGSLDTVITNAVIIDYTGIIKADIGIKDGLIISTGKAGNPDIMNDVFPNMIIGANTEVIAGEGLIVTAG
sytx EKDFAVYGDECVFGGGKV----------
uSBU AIDCHVHFICPQLVYDAVTSGITTLVGGGTGPADGTRATTCTPAPNQMKLMLQSTDDMPLNFGFTGKGNSAKPDELHEIIRAGAMGLKLHEDWGTTPAAI
sytx
uSBU DSCLTVADQYDIQVNIHTDTLNESGFVEHTIAAFKGRTIHTYHSEGAGGGHAPDIIKVCGEKNVLPSSTNPTRPYTHNTIDEHLDMLMVCHHLNKNIPED
sytx
uSBU VAFAESRIRAETIAAEDILHDKGAISIISSDSQAMGRIGEVISRTWQTADKMKSQRGPLQPGEDNDNFRIKRYVAKYTINPAIANGLSQYVGSVEAGKLA
sytx
uSBU DLVLWKPSFFGAKPEMVIKGGEVAYANMGDPNASIPTPEPVIMRPMFGAFGKAGSSHSIAFVSKAALDEGVKASYGLNKRVEAVKNVRKLTKRDMKLNDT
sytx
uSBU LPQITVDPETYTVTADGEVLTCTAAKTVPLSRNYFLF
sytx

C


jbtx MGPVNEANCKAAMEIVCRREFGHKEEEDASEGVTTGDPDCPFTKAIPREEYANKYGPTIGDKIRLGDTDLIAEIEKDFALYGDESVFGGGKV--ILEHHHHHH
sytx MGPVNDSNCRAAMKAVVTRGFGHVEEENAREGVTG--EDYSLTTVISREEYAHKYGPTTGDKIRLGDTDLFAEIEKDFAVYGDECVFGGGKVAAALEHHHHHH

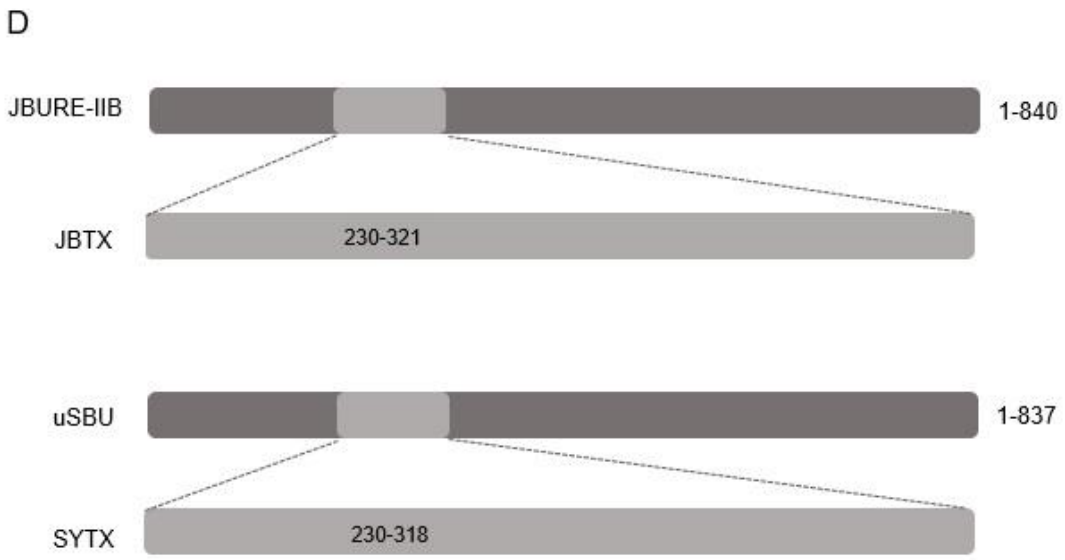


Figura 2. Alinhamento das ureases e seus peptídeos derivados usando CLUSTALW – posições marcadas com (*) para identidade, (:) para similaridade forte e (.) para fraca similaridade. A. Urease de *C. ensiformis* JBURE-IIB (GenBank: ACL14297.1) alinhada com o jbtx. B. uSBU (GenBank: AAO85883.1) alinhada com o sytx C. Alinhamento entre as sequências de aminoácidos do jbtx e sytx. D. Representação esquemática da estrutura primária das sequências da JBURE-IIB, JBTX, uSBU e SYTX, juntamente com o número de aminoácidos e localização dos peptídeos na urease correspondente (Modificado de PIOVESAN et al., 2014).

O peptídeo soyuretox mostrou ativiade antifúngica contra leveduras, comparáveis à do jaburetox, em concentrações micromolares (KAPPAUN, 2014). A superexpressão do soyuretox em raizes de soja da cultivar Williams 82 conferiu efeito protetor contra a infecção pelo nematóide da galha *Meloidogyne javanica*. As raízes expressando o soyuretox tiveram uma diminuição de 50 % da presença do nematóide e seus ovos, em relação às raízes que não expressavam o peptídeo (RECHENMACHER, 2016).

Considerando os resultados já obtidos que indicam o potencial biotecnológico como insecticidas dos peptídeos derivados de ureases, jaburetox e soyuretox, que são biodegradáveis, é imprescindível avaliar a biossegurança destas moléculas. Mulinari e colaboradores relataram que o jaburetox não mostrou toxicidade aguda,

quando injetado intraperitonealmente ou dado por via oral, para ratos e camundongos na dose de 10 mg/kg (MULINARI et al., 2007). No presente trabalho, uma avaliação toxicológica do soyuretox foi realizada em larvas de *zebrafish*.

1.5 *ZEBRAFISH*

Zebrafish (*Danio rerio*) é um pequeno teleósteo (3-4 cm) de água doce, utilizado como modelo biológico de estudo nas áreas de bioquímica (SIEBEL et al. 2011; ZIMMERMANN et al. 2016), farmacologia (COGNATO et al., 2012; BAGGIO et al., 2017), toxicologia (PEREIRA et al. 2012; SENGER et al. 2006) e neurociências (STEWART et al., 2014). Tais estudos são possíveis devido à similaridade de várias características dessa espécie com as de outros vertebrados. Cerca de 70 % dos genes de *zebrafish* apresentam homologia com genes humanos (HOWE et al., 2013). A comparação direta entre genes codificadores de proteínas em humanos e no *zebrafish* mostra que cerca de 82 % dos genes relacionados com doenças humanas têm ao menos um ortólogo nesse animal (HOWE et al., 2013). Além disso, o repertório comportamental e sistemas de sinalização presentes nesse organismo também podem ser comparados aos de outros grupos de vertebrados (RICO et al., 2011; STEWART et al., 2014).

O *zebrafish* possui uma série de características atrativas para o desenvolvimento de pesquisas, destacando o seu tamanho pequeno e o tempo de geração curto (KALUEFF, STEWART & GERLAI, 2014; STEWART et al., 2014). Além disso, esse animal possui um rápido metabolismo e uma grande sensibilidade a fármacos, absorvendo os compostos diretamente da água através das brânquias, e acumulando-os em diferentes tecidos, principalmente no sistema nervoso central (SNC) (GOLDSMITH, 2004).

O *zebrafish* apresenta fecundação externa, e os animais estão sexualmente maduros entre 3 e 4 meses de idade, com fêmeas produzindo centenas de ovos (LEGRADI et al., 2015; NERY et al., 2014). Os ovos são transparentes, sendo possível a observação das divisões celulares, e assim acompanhar a formação e o desenvolvimento do novo organismo (NERY et al., 2014)

Os animais apresentam um ciclo de desenvolvimento rápido, sendo que no primeiro dia (24 horas pós-fertilização, hpf), a maioria dos órgãos já está formada. O embrião se desenvolve externamente, sendo que durante os primeiros 2 a 3 dias de vida, eles estão protegidos por um córion transparente. Larvas também são transparentes, assim mudanças morfológicas podem ser facilmente monitoradas através de microscopia (KALUEFF, STEWART & GERLAI, 2014; STEWART et al., 2014). O *zebrafish* apresenta uma série de padrões de comportamento complexos, que são comparáveis aos de roedores e de humanos. Entre outros, eles demostram comportamento de medo (OGAWA, NATHAN & PARHAR, 2014), ansiedade (PARKER et al., 2014), agressividade (BONAN & NORTON, 2015), interação social (ZIMMERMANN et al., 2016) e memória (COGNATO et al., 2012; JIA, FERNANDES & GERLAI, 2014), que podem ser avaliados em diferentes estudos.

O *zebrafish* é um excelente modelo, com bastante confiabilidade, em estudos toxicológicos. Essa espécie tem sido utilizada para analisar a toxicidade de substâncias químicas (HILL et al., 2005), fármacos em estágios iniciais de desenvolvimento (BARROS et al., 2008) e estudos relativos a potenciais riscos ao meio-ambiente com o uso de pesticidas em plantações (SANCHES et al., 2018).

O conhecimento acumulado na neurotoxicologia sobre o desenvolvimento e transporte de compostos pela barreira hematoencefálica utilizou larvas de *zebrafish* como modelo de estudos (UMANS & TAYLOR, 2012). A barreira hematoencefálica do *zebrafish* é funcionalmente similar à de mamíferos, reforçando que este é um modelo confiável para estudos de biodisponibilidade de compostos químicos (UMANS & TAYLOR, 2012).

2. OBJETIVOS

Nessa tese tivemos os seguintes objetivos:

1) avaliar aspectos estruturais do soyuretox, um peptídeo recombinante derivado da urease de soja;

2) estudar propriedades biológicas do soyuretox;

3) estudar efeitos do peptídeo soyuretox no modelo *zebrafish.*

2.1 OBJETIVOS ESPECÍFICOS

Estudo estrutural do soyuretox:

* Otimizar a expressão do peptídeo em *Escherichia coli*;

* Estudar a estrutura secundária do soyuretox por dicroísmo circular;

* Estudar o soyuretox por ressonância magnética nuclear.

Estudo de propriedades biológicas do soyuretox

Em relação à atividade fungitóxica:

* Avaliar o estresse oxidativo e produção de ânions superóxidos na levedura *Candida albicans* após exposição ao soyuretox;

* Fazer a imunolocalização do soyuretox nas leveduras expostas ao peptídeo.

Em relação à atividade entomotóxica:

* Avaliar o impacto do soyuretox no sistema imune no barbeiro *Rhodnius prolixus,* através de sua capacidade de induzir agregação de hemócitos, em ensaios *in vitro e in vivo.*

Estudo de ureases e peptídeos derivados em larvas e adultos de *zebrafish*

*Analisar o comportamento em larvas expostas ao soyuretox: atividade locomotora e comportamento aversivo;

*Analisar a morfologia em larvas expostas ao soyuretox.

Capítulo II

Manuscrito submetido ao

Biochimica Biophysica Acta – General Subjects

Soyuretox, a recombinant peptide derived from soybean (*Glycine max*) ubiquitous urease: biological and structural properties, and toxicity screening in zebrafish larvae

Soyuretox, a recombinant peptide derived from soybean (*Glycine max*) ubiquitous urease: biological and structural properties, and toxicity screening in zebrafish larvae

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ABSTRACT

Background: Ureases, metalloenzymes that catalyze the hydrolysis of urea into ammonia and carbamate, display non-ureolytic properties contributing to defense mechanisms in plants. Previously we demonstrated the antifungal and insecticidal properties of jaburetox, a peptide derived from a jack bean urease. Here we studied soyuretox, a homologous peptide derived from the soybean (*Glycine max*) ubiquitous urease.

Methods: Soyuretox amplicon was cloned into pET23a and expressed in *E. coli*. Structural features of soyuretox were evaluated *in silico*, by NMR and circular dichroism (CD). Antifungal effects of soyuretox were analyzed on yeasts; measurement of reactive oxygen species and immunolocalization were performed in *Candida albicans*. Entomotoxic effects on *Rhodnius prolixus* were assessed using hemocyte aggregation assays. The toxicological profile of soyuretox was evaluated in *zebrafish* larvae.

Results: NMR and CD data revealed the intrinsically disordered nature of soyuretox and that it has a more ordered structure in presence of SDS micelles. Soyuretox is fungitoxic against yeasts in micromolar concentrations, inducing production of superoxide and binding to *C. albicans* cells. Soyuretox induced aggregation of *R. prolixus*' hemocytes *in vivo* and *in vitro*. No morphological changes were seen in soyuretox-treated (10-300 nM) zebrafish larvae, and behavioral alterations were seen only for the highest dose.

Conclusions: Soyuretox is an intrinsically disordered fungicidal and entomotoxic peptide with no relevant toxicity to *zebrafish* larvae. The data confirmed that the sequences encompassing soyuretox and the homologous jaburetox carry antifungal and entomotoxic domains of their parent ureases.

General significance: Soyuretox is a recombinant peptide with biotechnological potential for crop protection.

Keywords: intrinsically disordered protein, *Rhodnius prolixus,* molecular dynamics, circular dichroism, nuclear magnetic resonance, *Candida albicans.*

1. Introduction

Ureases (urea amidohydrolase, EC 3.5.1.5) are nickel-dependent enzymes that catalyze the hydrolysis of urea into ammonia and carbamate. These proteins are widely spread in bacteria, plants and fungi, but are not synthesized by animals (Ligabue-Braun et al., 2013; Krajewska, 2009). Regardless of their origin and quaternary organization, ureases are homologous proteins that exhibit at least 55 % identity at the amino acid sequence level. In addition to the enzymatic activity, these proteins present several other biological properties characterizing them as moonlighting proteins (Carlini and Ligabue-Braun, 2016).

We have previously shown that plant ureases display insecticidal properties, first discovered for the isoforms of jack bean (*Canavalia ensiformis*) ureases, CNTX and JBU, and then later found also for the embryo-specific soybean urease (Carlini et al., 1997; Follmer, Wassermann, and Carlini, 2004; Staniscuaski and Carlini, 2012). The insecticidal activity of *C. ensiformis'* ureases depends at least partially on the hydrolysis of the protein and release of entomotoxic peptides, upon the action of cathepsin-like digestive enzymes of the susceptible insects (Carlini et al., 1997; Ferreira-DaSilva et al., 2000; Piovesan et al., 2008; Real-Guerra, Carlini, and Staniscuaski, 2013; Defferrari et al., 2011). Based on these data, a recombinant peptide called jaburetox, representing an internal sequence of a jack bean urease, was cloned and expressed in *E. coli* (Mulinari et al., 2007; Postal et al., 2012). Given orally, jaburetox has a potent insecticidal effect against *Rhodnius prolixus,* a kissing bug vector of Chagas' disease, and to economically important insect pests, such as the cotton stainer bug *Dysdercus peruvianus,* and the fall armyworm *Spodoptera frugiperda,* the latter an insect not susceptible to plant ureases (Mulinari et al., 2007); reviewed in Kappaun et al., 2018).

Subsequently other biological activities were characterized for this peptide. Jaburetox is neurotoxic to *Triatoma infestans* and affects relevant enzymatic activities in the insect's central nervous system (Galvani et al., 2015). Fruttero and coworkers have demonstrated that jaburetox also interferes on *R. prolixus*' immune system,

rendering the insect more susceptible to bacterial infections. They found that jaburetox induces aggregation of *R. prolixus* hemocytes, cells involved in the insect's immune response, both *in vitro* and *in vivo* (Fruttero et al., 2016). Additionally, jaburetox has antifungal activity in yeasts, causing permeabilization of cell membranes, inhibition of the carbohydrate metabolism and morphology alterations with pseudo-hyphae formation (Postal et al., 2012).

Nuclear Magnetic Resonance (NMR) applied to solve 3D structure of Jaburetox revealed its intrinsically disordered nature (Lopes et al., 2015). Some intrinsically disordered proteins undergo structural changes when in contact with ligands which may drive them into a biologically active conformation. Jaburetox was found to interact with lipid vesicles and planar lipid bilayers (Martinelli et al., 2014; Barros et al., 2009; Michelleto et al., 2016; Broll et al., 2017) as well as with yeast and cockroaches natural membranes (Broll et al. 2017). Changes in the folding of jaburetox caused by these interactions were monitored using NMR, Circular Dichroism (CD) and fluorescence microscopy techniques.

More recently we have obtained the expression of the soybean ubiquitous urease (uSBU) fused with glutathione S-transferase (GST), with allowed to study biological properties of this protein, found in very low concentrations in the soybean plant (Martinelli et al., 2017). The recombinant uSBU-GST was toxic against filamentous fungi and pathogenic yeasts, including *Candida albicans* and *C. tropicalis*. The protein uSBU-GST was also entomotoxic against *R. prolixus*, inducing aggregation of hemocytes both *in vivo* and *in vitro* (Martinelli et al., 2017).

In order to investigate whether an internal sequence of uSBU could be responsible for its entomotoxic and antifungal effects, as is the case of jaburetox and jack bean ureases, here we described the cloning and expression of a recombinant peptide, homologous to jaburetox, derived from uSBU. This recombinant peptide, called soyuretox, was structurally characterized using bioinformatics tools, CD and NMR techniques. Biological activities of soyuretox were screened on yeasts and *R. prolixus* hemocytes, and a toxicological analysis of the peptide was performed on zebrafish larvae.

2. Materials and methods

2.1 Cloning of soyuretox

Soybean ubiquitous urease previously cloned in pGEX-4T-2 (Martinelli et al, 2017) served as template in a PCR (polymerase chain reaction) to obtain the cDNA corresponding to the region homologous to jaburetox, which was designated as soyuretox. Primers were designed for amplification while introducing an initiation codon: the forward primer 5' CAACATATGGGTCCAGTTAATGATTCTAATTGC (*Nde*I site underlined) and reverse primer 3' CCAAGCGGCCGCGACTTTCCCACCTC (*Not*I site underline). The PCR reaction was performed in a final volume of 50 µL, containing 400 nM of each primer, 200 mM dNTPs, 1x pfu reaction buffer, 100 ng of the template and 2.5 U *Pfu* DNA polymerase (Thermo Scientific). Amplification was carried out under the following conditions: pre-denaturation at 95 °C for 2 min and 35 cycles, 95 °C denaturation for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. The PCR product was resolved on 1% agarose containing GelRed (Biotium). The vector pET23a and PCR product were digested with *Nde*I and *Not*I (Thermo Scientific) and the vector was dephosphorylated with FAST AP enzyme (Fermentas). The binding reaction was performed withT4 ligase enzyme (Ludwig). The pET 23a::soyuretox construction was sequenced by ABI PRISM 3100 automated sequencer.

2.2 Expression and purification

Soyuretox were expressed in *E. coli* BL21 (DE3) pLysS cells (Novagen, Madison, WI, USA) transformed by heat shock with the construct pET23a::soyuretox, which encoded a peptide with six His residues in its C-terminus.

Briefly, the pre-inoculum of soyuretox-transformed cells was maintained overnight at 37 °C and 150 rpm, in Luria Bertani (LB) medium supplemented with 1 % glucose, 100 µg/mL ampicillin and 40 µg/mL chloramphenicol. The cells were then inoculated in 1 L of LB plus 1 % glucose and grown at 37 °C under 200 rpm shaking,

until an OD = 0.7 was reached. IPTG at a final concentration of 0.23 mM was added to the culture, the temperature was cooled down to 21 °C and the cells were kept under stirring for more 24 h. The culture was harvested by centrifugation (15 min at 5,000 *g*) and the supernatant was discarded. The pelleted cells were suspended in 15 mL lysis buffer (50 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 7.5), sonicated (15 cycles of 1 min, 90 kHz) and centrifuged (12,000 *g*, 30 min) to obtain a peptide-rich supernatant. The purification was performed in two chromatographic steps. First, the supernatant was applied into a 5 mL Chelating Sepharose affinity column loaded with Ni^{2+} and equilibrated in the lysis buffer. The column was washed with 50 mM imidazole and then eluted with 200 mM imidazole in lysis buffer. In the second purification step, the peptide-enriched fractions were pooled and loaded onto a size exclusion column Superdex 200 16/60 (GE Healthcare) mounted on an ÄKTA purifier apparatus. The column was equilibrated in 50 mM sodium phosphate buffer containing 1 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM TCEP [tris(2-carboxyethyl)phosphine], pH 8.0, and eluted at 1 mL/min flow rate. Fractions containing soyuretox were pooled and protein concentration was measured by the Bradford method (Bradford, 1976) or by absorbance at 280 nm (considering a molar coefficient of 6085 M^{-1} cm^{-1}). The purification final yield was ~3 mg of soyuretox per liter of culture. A molecular mass of 11.06 kDa was determined for soyuretox using the ProtParam, available at ExPASy website (http://web.expasy.org/protparam/).

For circular dichroism and nuclear magnetic resonance experiments, production of soyuretox was made as follows: 50 mL of soyuretox pre-inoculum was maintained overnight at 37 °C and 150 rpm, with supplementation of 1 % glucose and 50 µg/mL carbenicillin in LB medium. The cells were inoculated into 1L of LB medium (plus 1 % glucose + carbenicillin) until an OD_{600} 0.6-0.7 was reached. The cells were collected by centrifugation at 4,500 g for 30 min and resuspended in M9 salt solution (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.246 g/L $MgSO_4$) and centrifuged again. The cell pellet was resuspended in M9 medium with supplementation of 1.25 g/L of $(NH_4)SO_4$ and 4 g/L of glucose. After 1 h, protein expression was induced by addition of 0.23 mM of IPTG plus 50 µg/mL carbenicillin. Labeled glucose and ammonium sulfate (^{13}C-glucose 99 % and ^{15}N-ammonium sulfate, Sigma Aldrich) were used when peptide was produced for NMR assays. At the end of the expression time, the cells were pelleted by centrifugation, lysed and purified following the two chromatographic steps described

above. The purity of the fractions was checked by SDS-PAGE (NuPAGENovex 12 %, Life Technologies) and gels were stained with ProBlue Stain (Giotto Biotech). The fractions containing the peptide were pooled and quantified by 280 nm.

Jaburetox was produced and purified as described in (Martinelli et al., 2014).

2.3 Circular dichroism (CD) spectroscopy

Soyuretox samples at 25 µM concentration were prepared in 50 mM sodium phosphate buffer at pH 6.5 or 8. To check if soyuretox undergoes structural changes when in contact with sodium dodecyl sulfate (SDS), solutions were prepared of the peptide in 50 mM sodium phosphate, 1 mM EDTA, 1 mM TCEP containing SDS in final concentrations of 0.1 mM, 1 mM, and 10 mM. Measurements were made on a Jasco 810 spectropolarimeter (Jasco Inc, Easton, US) in 0.1 cm optical path cuvettes in the λ range of 195 to 260 nm. Temperature stability was evaluated at 25 °C and 90 °C. The buffer's spectrum was subtracted from all measurements.

2.4 Nuclear magnetic resonance (NMR)

Samples of ^{13}C- and ^{15}N-labeled soyuretox (0.5 mM concentration) in 50 mM sodium phosphate buffer, 1 mM EDTA, 1 mM TCEP at pH 6.5 or 8.0, with addition of 90% H_2O/10% D_2O (v:v) were used for NMR spectroscopy. Soyuretox solutions were prepared in the absence and in presence of SDS at final concentrations of 0.1 mM, 1 mM, 1.5 mM and 10 mM. The critical micellar concentration of SDS was taken as 1.5 mM in buffer (Broll et al., 2017). The soyuretox spectra were acquired on a Brucker Avance700 Spectrometer (Bucker Corporation, Billerica, MA, USA). NMR data were analyzed with the TOPSPIN 3.2 program (BrukerBioSpin).

2.5 Molecular modeling and simulation

A structural model for soyuretox was built with MODELLER9v14 (Sanchez and Sali 2000) using the *C. ensiformis* major urease isoform structure (PDB ID: 3LA4) (Balasubramanian and Ponnuraj, 2010) as template. The best model (out of ten) was chosen based on stereochemical evaluation with PROCHECK (Laskowski et al., 1993) and theoretical validation of three-dimensional profiles with Verify3D (Lüthy, Bowie, and Eisenberg, 1992). The soyuretox peptide was subjected to molecular dynamics (MD) simulations following the protocol employed previously for the jaburetox peptide (Martinelli et al., 2014). Briefly, these simulations were carried out with GROMACS 4.5 suite (Hess et al. 2008) using GROMOS96 53a6 force field (Oostenbrink et al., 2004) for 500 ns. The systems were solvated in triclinic boxes using periodic boundary conditions, SPC water model (Berendsen, Grigera, and Straatsma, 1987), and counterions to neutralize the system. The Lincs method (Hess et al., 1997) 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 (Darden, York, and Pedersen, 1993). 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 (Berendsen et al., 1984), 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. 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 (Hess et al., 2008).

2.6 Western blotting

Western blot was done according to (Towbin, Staehelin, and Gordon 1979). Briefly, soyuretox was electrophoretically resolved and transferred by gravity to PVDF membranes (Millipore) in the running buffer (125 mM Tris, 960 mM glycine, 0.5 % SDS)

plus 20 % methanol. The membrane was blocked with a 5 % v/v nonfat milk solution in TBS (100 mM Tris, 1.5 M NaCl, pH 7.5) for 2 h. After washing the membrane was incubated for 2 h with rabbit anti-jaburetox polyclonal antibodies (1:7.500 dilution), followed by incubation with anti-rabbit IgG antibodies coupled to alkaline phosphatase (1:20.000; Zymed). The colorimetric reaction was developed with NBT (nitroblue tetrazolium) (Sigma-Aldrich) and BCIP (Sigma-Aldrich) (5-bromo-4-chloro-3-indolyl-phosphate). Bovine serum albumin (BSA; MP Biomedicals) was used as a negative control.

2.7 Yeast inhibition assay

The yeasts *Candida albicans* (CE022), *C. parapsilosis* (CE002) and *Saccharomyces cerevisiae* (1038) were kindly provided by Dr. Valdirene Gomes, Laboratory of Physiology and Biochemistry of Microorganisms (Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil). The growth assays were performed according to (Postal et al. 2012) with minor modifications. Yeasts were cultured in Sabouraud agar (Accumedia) plates for 48 h at 28 °C, then quantified in a Neubauer chamber.

Soyuretox samples (9 and 18 µM) previously dialyzed against 10 mM Tris pH 7.0 were incubated with cells (10^4 cells.mL^{-1}) in U-bottom microplates, in Sabouraud broth at 28 ºC, for 24 h. The dialysis buffer was used as negative control. For *C. albicans*, a dose-response curve was tested using soyuretox at 0.1, 0.5, 5.0, 10 and 15 µM. Growth was assessed after 24 h of incubation at 28 °C, by colony forming units (CFU.mL^{-1}): 20 µL of soyuretox-treated yeast cultures were serially diluted 10-fold in saline and plated on Sabouraud agar by drop plate method, and further incubated for 24 h at 28 °C. The experiments were performed in triplicate and the results are shown as means ± standard deviations (SD) of two independent bioassays.

2.8 Mechanism of inhibitory action on yeast

2.8.1 Oxidative stress: superoxide assay

The superoxide assay was performed as described by (Anderson and Greenwald 1985) with modifications. Aliquots of *C. albicans* suspensions (10^4 cells.mL^{-1}) were

incubated with 1 µM or 5 µM soyuretox and 10 µL of 10 % nitroblue tetrazolium (NBT; Sigma-Aldrich) for 24 h at 28 °C. After incubation, the supernatant was discarded and the plate was dried at room temperature. The production of superoxide anions by the soyuretox-treated cells was evaluated by the amount of formazan crystals formed. Formazan was solubilized in 2 M KOH (Sigma-Aldrich) and absolute dimethyl sulfoxide (DMSO, Sigma-Aldrich), and the absorbance was read at 620 nm (Reader 490 EZ-Biochrom).

2.8.2 Immunofluorescence

C. albicans cells were used for immunofluorescence assays. Yeast cells (10^5) were incubated with 1 µM soyuretox for 24 h in microplates. After transferring the content of each well to Eppendorf tubes, 8 % v/v formaldehyde was added at an 1:1 (v:v) ratio, followed by 1 h incubation at room temperature. Cells were washed with PBS by centrifugation (500 *g*), and then submitted to permeabilization-blocking treatment with 0.1 % Triton X100, 3 % BSA in 20 mM sodium phosphate, 150 mM NaCl, pH 7.0, for 30 min. After incubation with anti-jaburetox primary antibodies (1:750 dilution) for 1 h, the cells were washed 3 times and incubated with anti-rabbit IgG ALEXA 488 (green, Abcam) conjugated antibodies for 1 h. Controls were not incubated with either the primary or secondary antibodies. Finally, the cells were stained with 0.1 µg/mL DAPI (4',6-diamino-2-phenylindole; Sigma-Aldrich). For fluorescence microscopy, the cells were visualized in a Zeiss Axiovert 200 equipped with an AxiocamMRc camera, and images were captured using AxionVisionRel 4.8 software.

2.9 *In vivo* and *in vitro* aggregation of *Rhodnius prolixus* hemocytes

2.9.1 *In vivo* assays

R. prolixus were kindly supplied by Dr. Denise Feder (Universidade Federal Fluminense, Niteroi, RJ, Brazil). The *in vivo* hemocyte aggregation assays were performed administering soyuretox to the insects by injection or by feeding (Fruttero et al., 2016; Defferrari et al., 2014).

Fifth instar nymphs were injected into the hemocele with soyuretox (0.05 µg.mg^{-1} insect body weight) diluted in *R. prolixus* saline (150 mM NaCl, 8.6 mM KCl, 2 mM $CaCl_2$, 8.5 mM $MgCl_2$, 4 mM $NaHCO_3$, 34 mM glucose, 5 mM HEPES, pH 7; insect mean body weight ~50 mg). Saline alone was injected as negative control. After 6 h, the hemolymph of the insects was collected and diluted in an anticoagulant solution (10 mM EDTA, 100 mM glucose, 62 mM NaCl, 30 mM sodium citrate, 26 mM citric acid, pH 4,6). Free cells and aggregates were counted in a light field microscope hemocytometer. A cluster of 5 or more cells was considered an aggregate (Fruttero et al., 2016).

Fifth instar nymphs were artificially fed for 30 min on parafilm-coated acrylic plates containing the peptide diluted in saline plus 1 mM ATP, kept at 37 °C. The concentration of the peptide in the feed solution was calculated to give a dose of ~0.1 µg.mg^{-1} of insect body weight, considering the volume usually ingested by nymphs at this stage. Animals of the control group fed only in saline containing 1 mM ATP. The hemolymph of the insects was collected 18 h after the meal and diluted in anticoagulant solution. The number of free cells and cells was counted as described above (Fruttero et al., 2016).

2.9.2 *In vitro* assays

Hemolymph of fifth instar *R. prolixus* nymphs was collected with a micropipette after cutting one of the insect's legs. The pooled hemolymph was mixed with *R. prolixus* saline at an 1:1 (v:v) ratio. Soyuretox was added to the diluted hemolymph pool at final concentrations of 50, 100, 200 and 500 nM, followed by incubation for 1 h at room temperature under gentle agitation. Saline alone was used as negative control. The number of aggregates was counted as described above (Fruttero et al., 2016).

2.10 Zebrafish embryos and larvae

Zebrafish embryos were obtained by natural mating of wild-type adults zebrafish. Animals were from our own breeding colony, kept in recirculating water systems (Zebtec, Tecniplast, Italy), with osmosis-filtered water at controlled pH (7.0-7.5) and temperature (28 ºC ± 2 ºC). Nitrate, nitrite, ammonia and chloride levels were checked daily. Animals were maintained under day/night photoperiod cycle (14:10 hours) and

fed three times a day with commercial flakes (TetraMin, NC, USA) and supplemented with live brine shrimp.

The day before mating, two males and one female were placed in breeding tanks, separated overnight by a transparent barrier which was removed on the following morning. The fertilized eggs were collected for the experiments. All zebrafish protocols were approved by the institutional Animal Care Committee under the number 7659 (CEUA-PUCRS).

2.10.1 Eggs treatment

Eggs were placed in six-well plates (10 eggs/well) and exposed to soyuretox at concentrations of 10, 100 and 300 nM, or 5 mM sodium phosphate buffer (vehicle- pH 7.5) or water as controls groups (total volume 10 mL/well), for 4 hours. After the treatment, the eggs were washed with water and kept in Petri dishes in water until 5 days post-fertilization (dpf). The water was changed daily for all groups. Survival rate, determined by presence or lack of heart beat, was monitored daily.

2.10.2 Morphological evaluation

Morphological evaluation was performed in larvae at 5 dpf, under a stereomicroscope. Each animal was photographed individually, and body measurements were assessed by the NIS-Elements D software (Nikon Instruments Inc., Melville, USA). The body length was taken as the distance from the larval mouth to the pigmented tip of tail; the size of eyes was determined by measuring the surface area of the eyes; and the ocular distance was assumed as the distance between the inner edge of the two eyes (Lutte et al., 2015).

2.10.3 Exploratory behavior

The exploratory behavior of the larvae was evaluated at 5 dpf following the procedure described by (Colwill and Creton, 2011). Each larva was placed individually in a well of 24-well cell culture dishes, containing 2 mL of water. After one minute of habituation, five minute sessions were recorded for later analysis using the Ethovision software (version 11.5). Parameters evaluated were the distance traveled, mean speed, time in the central zone and latency.

2.10.4 Avoidance response

The method of Nery et al. (2014) was followed; five larvae were placed in one well of a 6-well plate placed over an LCD monitor, to assess cognitive ability and prevention response to a visual stimulus (a red ball with 1.35 cm diameter projected from the LCD monitor). The sessions were recorded for 5 min after 2 min of habituation. The path of the red ball was straight from left to right, traversing a distance of 2 cm in the middle of the well area, which the animals avoided by swimming on the other side of the well. In this assay, the aversive response to the stimulus was evaluated by the residence time on one side of the well.

2.11 Statistical analysis

Larval survival was analyzed by Kaplan-Meier analysis. Differences in locomotor and morphological parameters of larvae, considering time and concentration of soyuretox were evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons tests.

Yeast inhibition assay and oxidative stress were evaluated by one-way ANOVA, followed by Tukey's multiple comparisons test.

In vivo hemocyte aggregation was analyzed by one-way ANOVA followed by Student's t-test and *in vitro* aggregation results were evaluated by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test, and non-parametric ANOVA followed by Dunn's Multiple Comparison Test.

3. Results

3.1 Expression and purification of Soyuretox

The result of the two-step purification of soyuretox is shown in Figure 1A. Soyuretox was detected as an ~11 kDa band as shown in SDS-PAGE analysis, in agreement with the predicted molecular mass of 11.06 kDa.

The peptide immunoreacted with anti-jaburetox antibodies in Western blots (Figure 1B), as it could be expected considering the identity of 72 % between the two peptides. A faint band of a dimeric form of soyuretox is seen in the Western blot (indicated by an arrow).

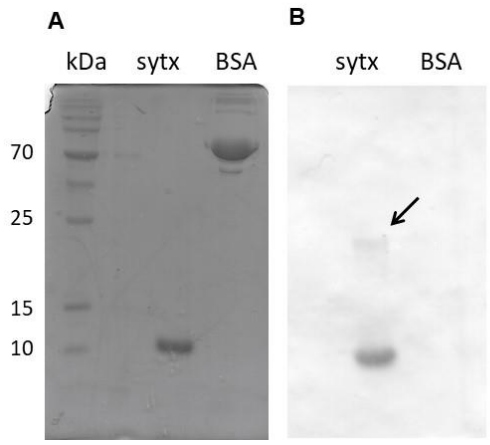

Figure 1. Purification and immunocharacterization of soyuretox. A. 12 % SDS-PAGE of soyuretox, after purification on a nickel affinity column followed by gel filtration. Lanes: sytx (soyuretox), purified soyuretox; BSA, bovine serum albumin (negative control). B. Western blot of the SDS-PAGE shown in panel A using polyclonal anti-jaburetox antibodies. Lanes: sytx, purified soyuretox; BSA, bovine serum albumin as negative control.

3.2 Molecular modeling and simulation

A structural model of soyuretox was subjected to molecular dynamics (MD) simulations following previous observations made with jaburetox (Martinelli et al. 2014). After 500 ns of MD simulation, soyuretox became more globular in solution (as observed for jaburetox) (Supplementary Figure 1) in an overall shape similar to the latter (Figure 2B). Soyuretox in solution showed marked changes of secondary

structure, with loss of helix turns and beta strands. The β-hairpin in C-terminal region of soyuretox disappears after 500 ns of unrestrained simulation (Figure 2C). For comparison, Figures D and E show the modeled structure of jaburetox before and after 500 ns MD, respectively.

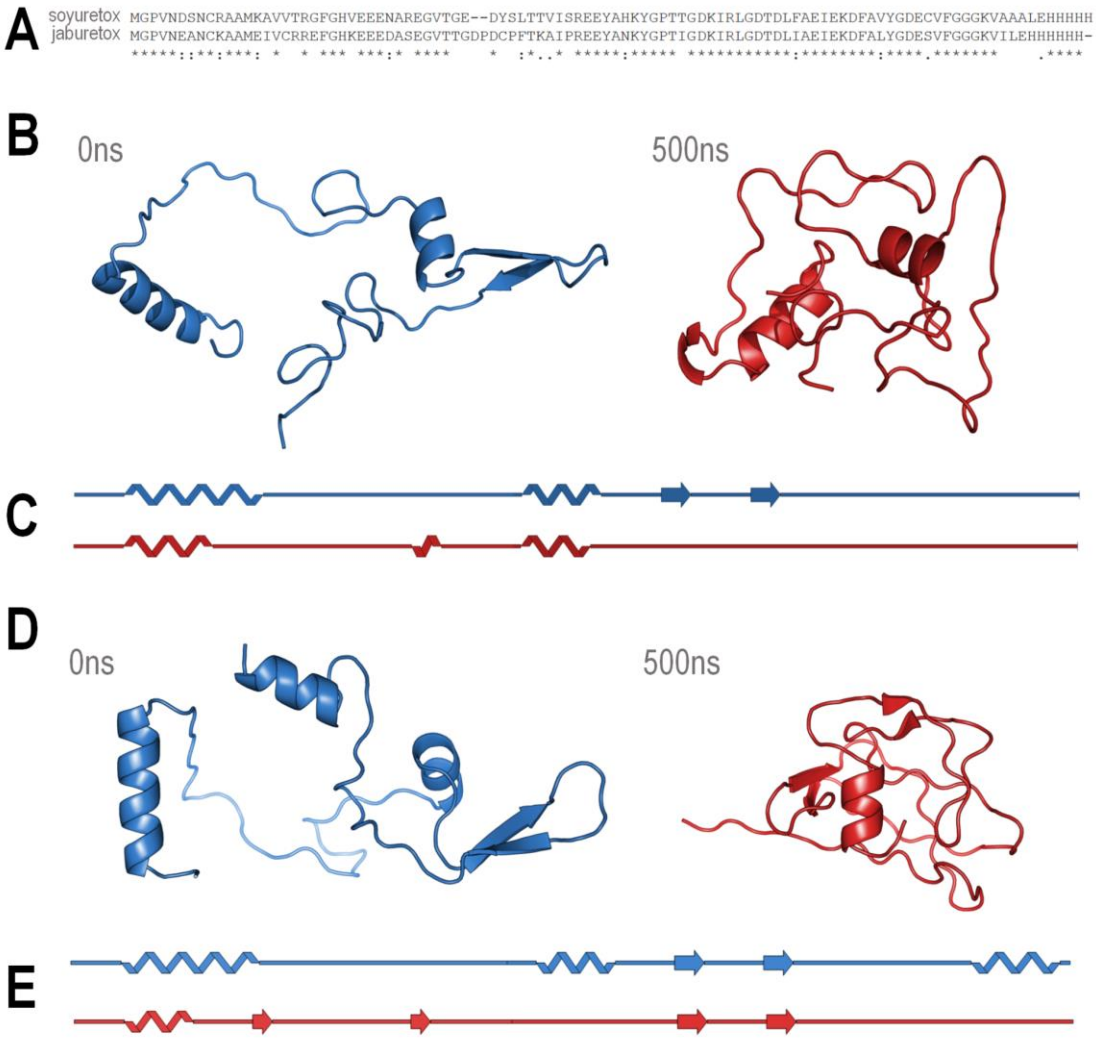

Figure 2. Conformational behavior of soyuretox and comparison with jaburetox. A. Alignment of amino acid sequences of soyuretox and jaburetox - positions marked (*) for identity (:) for strongly similar (.) for weakly similar; B. Three-dimensional representations of soyuretox before (0ns) and after (500ns) MD; C. Schematic representations of the secondary structure content of soyuretox as shown in B, respectively; D. Three-dimensional representation of jaburetox before (0ns) and after (500ns) MD; E. Schematic representations of the secondary structure content of jaburetox as shown in D, respectively. (D and E reproduced from Martinelli et al, 2014, with permission).

3.3 Circular dichroism (CD) spectroscopy

The secondary structure of soyuretox as analyzed by CD (Figure 3) indicated a typical spectrum of disordered state (random coil). Variation in temperature from 25 ºC to 90 ºC produced modest changes in the spectrum in the region of minimum signal (~200 nm), indicating an alteration in the peptide's folding. The absence of α-helix and β-sheet, denoted in the spectrum by strong negative signals in regions above 205 nm, was noted. Upon cooling down after the 90 ºC exposition, soyuretox regained most of its original folding.

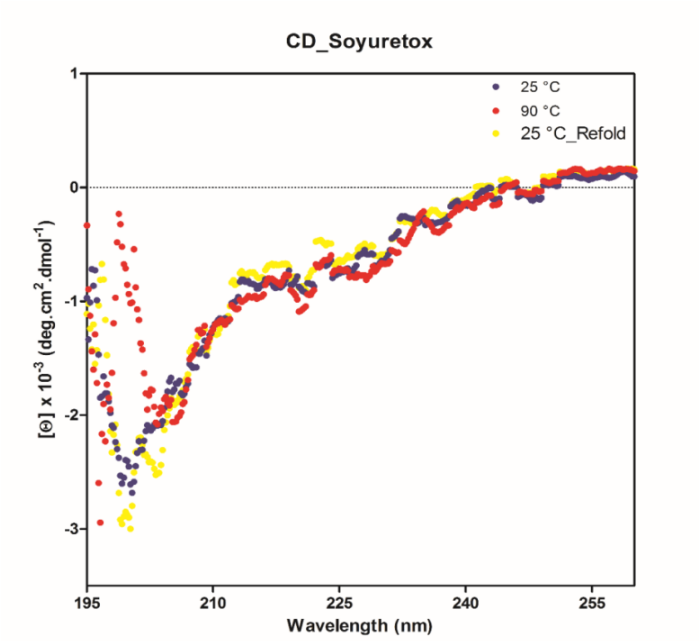

Figure 3. Circular dichroism of soyuretox (25 µM) in 50 mM sodium phosphate buffer, pH 6.5, at different temperatures. CD spectra at 25 °C (blue), 90 °C (red) and at 25 °C, after cooling (yellow). Typical results are shown.

To evaluate whether soyuretox binds to SDS and if this binding could induce folding (secondary structure) acquisition, CD spectra of the peptide in the presence of different concentrations of SDS were performed. For comparison, CD spectra of jaburetox in the same conditions were also obtained. Figure 4 shows the results,

indicating that there was an increase in the ordering of the peptide(s) in the presence of 10 mM SDS micelles, while no significant structural changes were observed at lower detergent concentrations (below the critical micellar concentration, CMC).

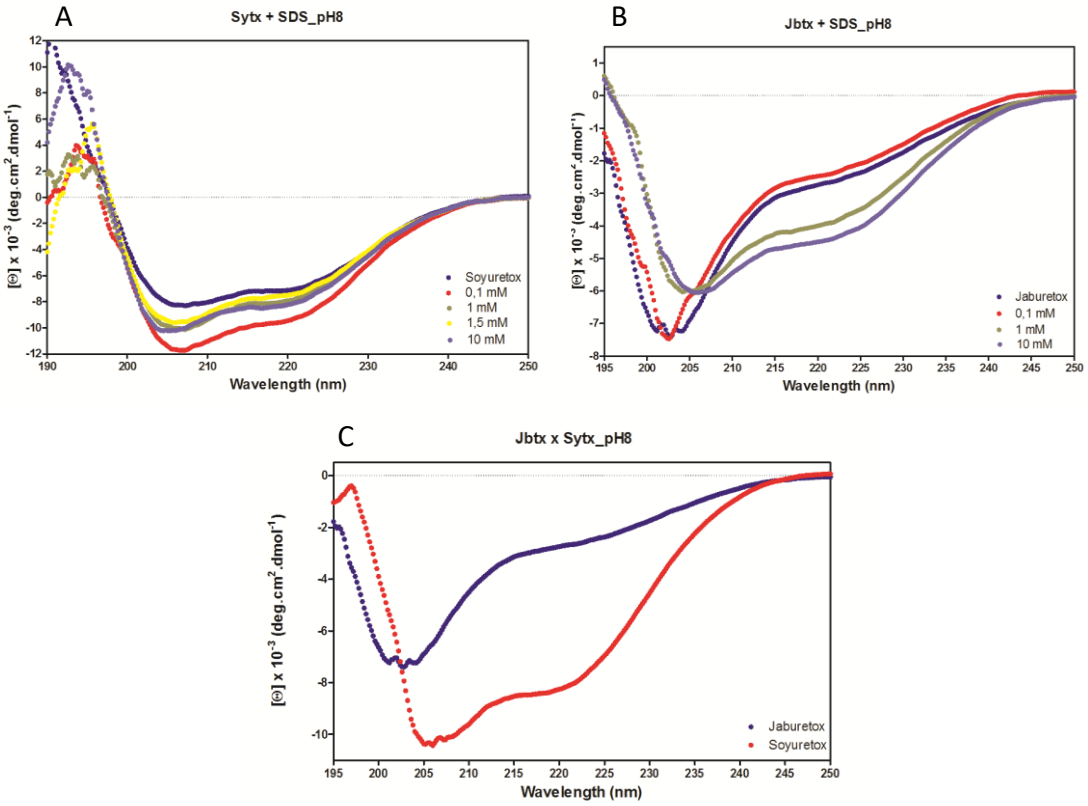

Figure 4. Circular dichroism (CD) of soyuretox and jaburetox, at 25 µM concentration, in the presence of SDS micelles. A. CD of soyuretox in the presence of 0.1 (red), 1 (gray), 1.5 (yellow), and 10 mM (purple) SDS or absence of SDS (blue). B. CD of Jaburetox CD in the presence of 0.1 (red), 1 (gray), and 10 mM (purple) SDS or absence of SDS (blue). C. CD spectra of soyuretox (red) and jaburetox (blue) in the absence of SDS were superimposed.

3.4 Nuclear magnetic resonance (NMR)

The NMR spectrum obtained for soyuretox is typical of intrinsically disordered proteins, which characteristically give low dispersion of signals in a proton nuclear magnetic resonance (Uversky, 2011). In Figure 5, the NMR spectra of the peptides

soyuretox (blue) and jaburetox (red) were superimposed to show that they are quite similar structurally.

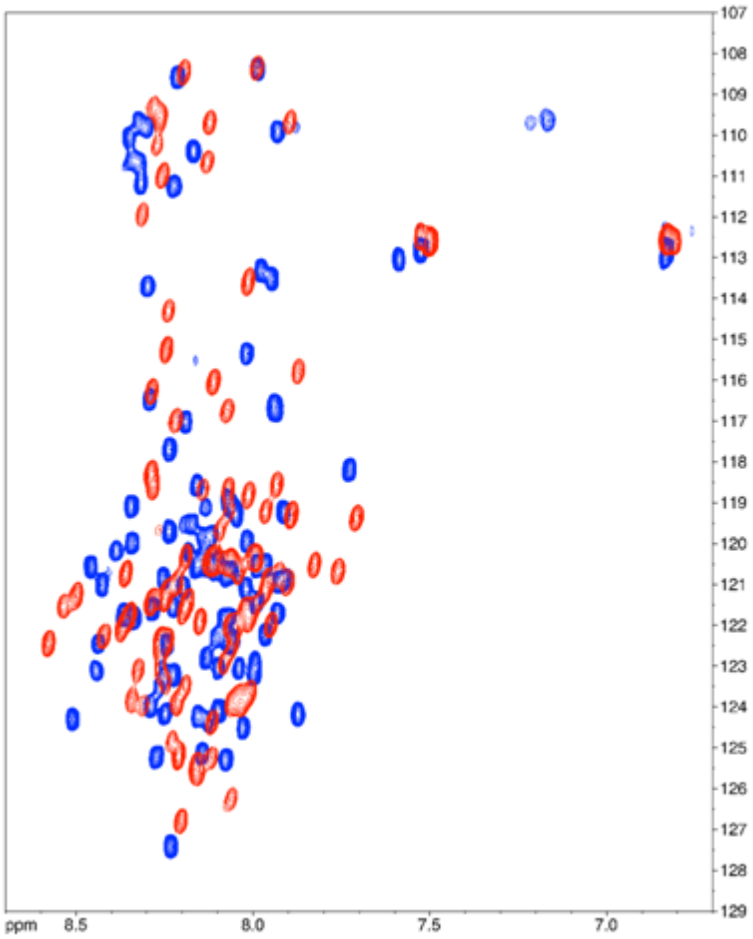

Figure 5. NMR superimposed spectra of jaburetox (red) and soyuretox (blue). Data were acquired for peptide (0.5 mM) solutions in 50 mM Na_2HPO_4, 1 mM EDTA, 1 mM TCEP, pH 6.5 at 280 nm.

To analyze whether binding to SDS could induce conformational changes in soyuretox, NMR spectra of the peptide were obtained in the presence of different concentrations of the detergent. Figure 6 shows these results. In the presence of 0.1 mM SDS (below CMC), there was no observable change in the spectrum; with 1 mM SDS (near the CMC), small modifications were observed. In contrast, the presence of 10 mM SDS (above CMC) resulted in significant changes in the spectrum, indicating that binding of soyuretox to SDS micelles led the peptide to acquire a more organized

structure. Nevertheless, the low dispersion of the NMR signals indicates that, even in the presence of SDS micelles, soyuretox still keeps its intrinsically disordered nature.

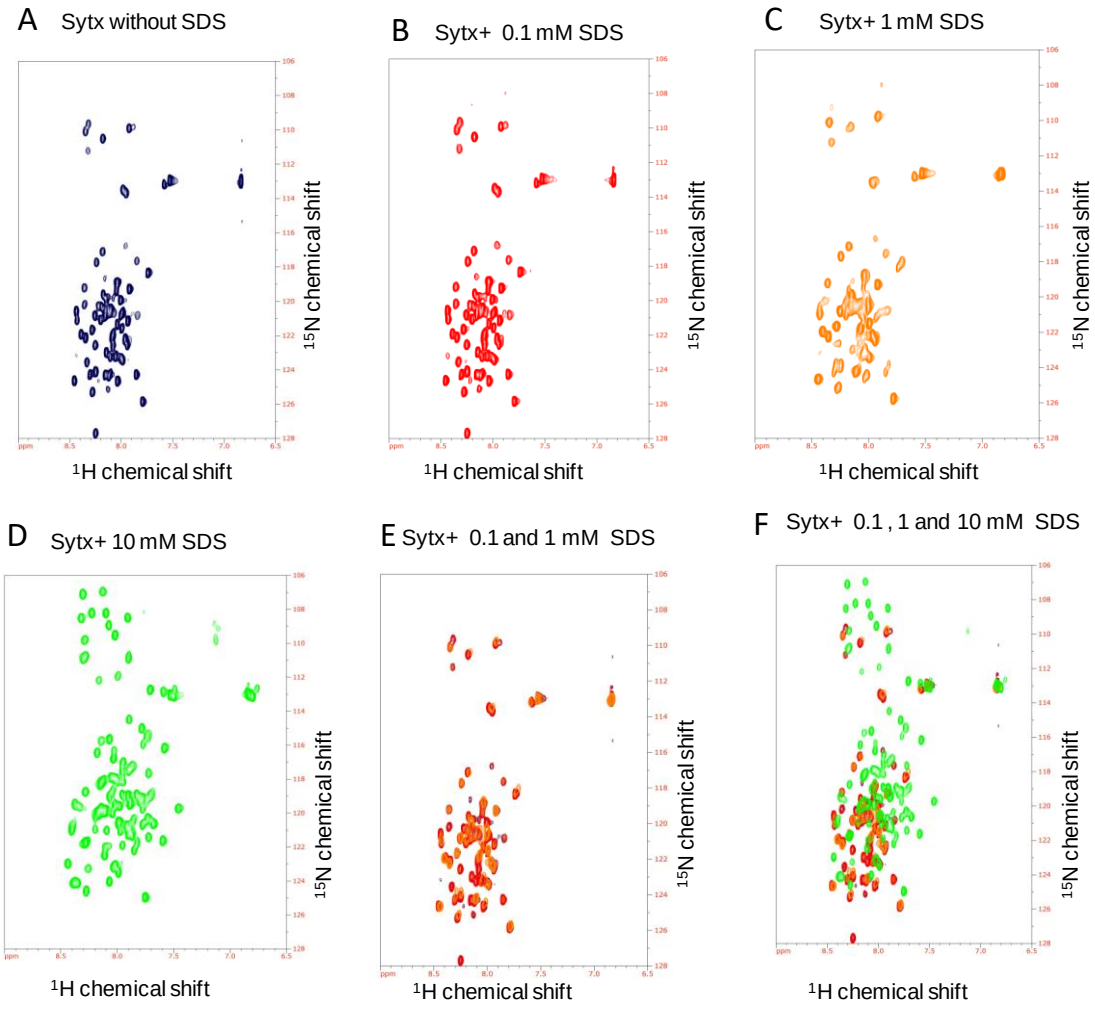

Figure 6 NMR of the soyuretox in the presence of SDS. A.soyuretox spectrum in the absence of SDS; B. soyuretox in the presence of 0.1 mM SDS (below CMC); C. with 1mM SDS (near CMC); D. with 10 mM SDS micelles (above CMC). E. superimposed spectra of soyuretox in the presence of 0.1 and 10 mM SDS. F. superimposed spectra of soyuretox in the presence of 0.1, 1 and 10 mM SDS.

The changes in folding seen for the SDS-bound soyuretox appear to be stable. After 10 days at room temperature, the spectrum of the 10 mM SDS-soyuretox remained the same, whereas that of SDS-free samples changed, probably indicating "degradation" or precipitation of the peptide under this condition (data not shown). Thus

the assignment of NMR spectra of soyuretox to solve its 3D structure was not possible because of the peptide's instability during the period of data acquisition.

3.5 Yeast inhibition assay

The fungitoxic activity of soyuretox was evaluated in three species of yeast, *Candida albicans*, *C. parapsilosis* and *Saccharomyces cerevisiae*. Soyuretox induced a significant inhibition of yeast multiplication at doses of 9 and 18 µM (Figure 7). A dose-response curve was obtained for *C. albicans*, with inhibition starting at 5 µM soyuretox. The antifungal effect of 15 µM soyuretox after a 24 h incubation with the yeasts resulted in a pronounced growth inhibition (Figure 7, panel D).

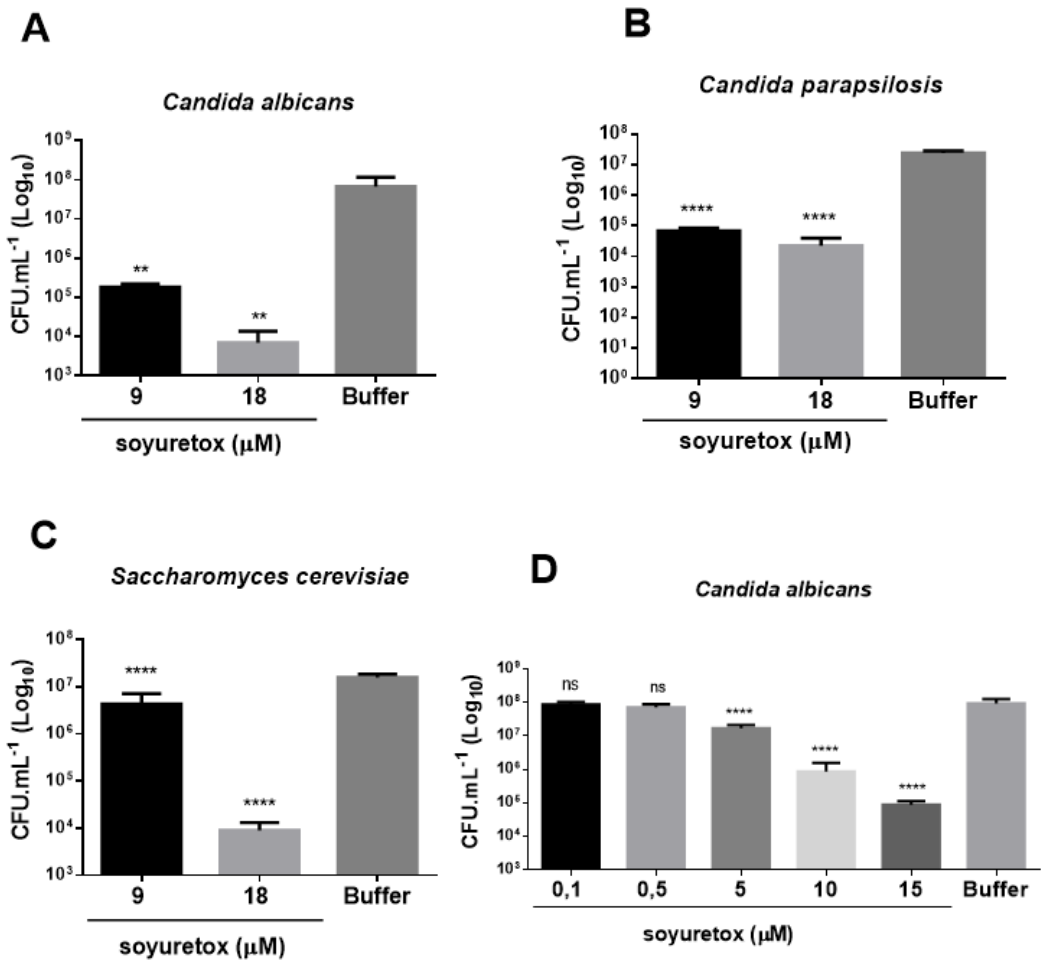

Figure 7. Antifungal effect in yeast of soyuretox. Yeast cells (10^4 cells.mL^{-1}) were exposed to soyuretox at 9 and 18 µM, for 24 h at 28 °C (A-C). *C. albicans* was treated with soyuretox in the 0.1-15 µM concentration range, for 24 h at 28°C (D). Negative controls were performed with 10 mM Tris pH 7 (buffer). After incubation with soyuretox, a colony forming unit (CFU) assay was carried out with the treated yeasts. Panels: (A) *C. albicans*; (B) C*. parapsilosis;* (C) *S. cerevisiae,* (D) dose-response curve of soyuretox in *C. albicans*. Values are mean ± SD. ** $p < 0.05$, **** $p < 0.0001$. ns: not significant.

3.6 Superoxide production assay

To elucidate a possible mechanism involved in the antifungal effect of soyuretox, the production of superoxide anions by *C. albicans* exposed to soyuretox was measured using the NBT assay (Figure 8). The reagent was maintained throughout the incubation time of *C. albicans* with soyuretox. The production of superoxide by cells

treated with 5 µM soyuretox for 24 h at 28 °C, indicated that oxidative stress contributes to the mechanism of antifungal action of the peptide in yeasts.

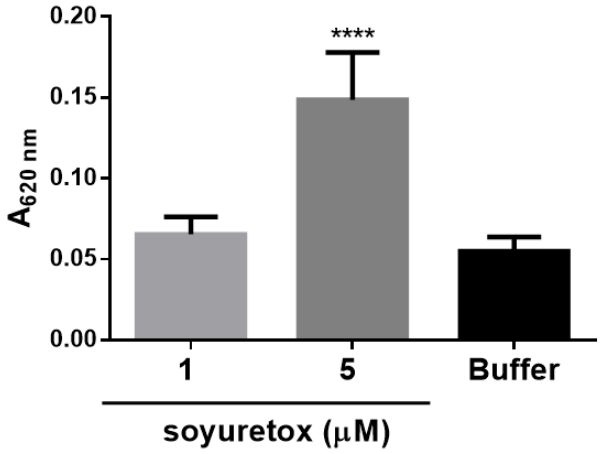

Figure 8. Soyuretox induces oxidative stress in *C. albicans.* Production of superoxide anions was measured for *C. albicans* incubated with soyuretox (1 and 5 µM) for 24 h at 28 °C, in the presence of nitroblue tetrazolium (NBT). The results are mean ± SD, averages of triplicates. * $p < 0.05$.

3.7 Immunofluorescence

The interaction of soyuretox with *C. albicans* was confirmed by immunofluorescence. Figure 9 shows that, after 24 h of incubation at 28 °C, soyuretox was found associated to the yeast cells, with little if any free peptide detected in the medium.

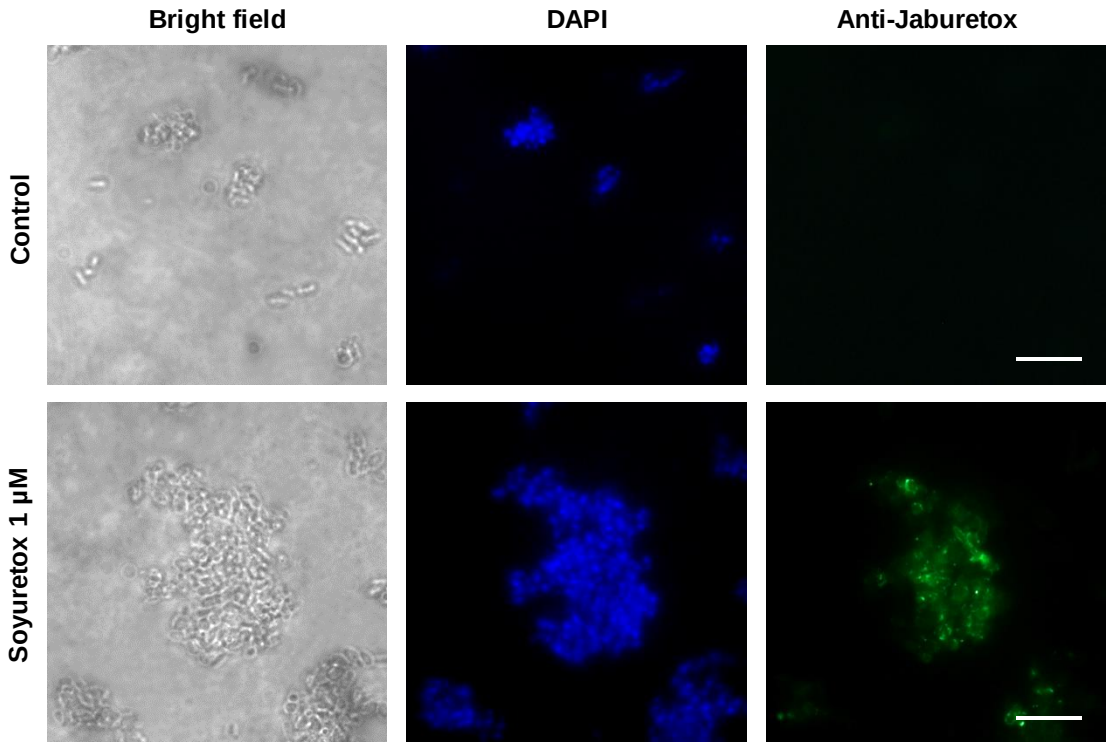

Figure 9. Soyuretox binds to *C. albicans*. Yeast cells were incubated with 1 µM soyuretox for 24 h at 28°C. Afterwards, the cells were fixed with formaldehyde 4 %, permeabilized and blocked with 5 % BSA - 0.1 % Triton X100 and incubated with rabbit polyclonal anti-jaburetox antibodies (1:750) followed by anti-rabbit IgG antibodies conjugated to Alexa 488 (green). Nuclei were stained with DAPI. Bars: 10 µm.

3.8 *In vivo* and *in vitro* aggregation of *Rhodnius prolixus* hemocytes

Aggregation of insect hemocytes induced *in vivo* by soyuretox was analyzed by injecting the peptide into the hemocoel (Figure 10) and by feeding (Figure 11). The results indicated that, although not affecting the number of free cells, soyuretox induced *in vivo* aggregation of *R. prolixus* hemocytes, either when injected into the insect's hemocoel or given by oral route.

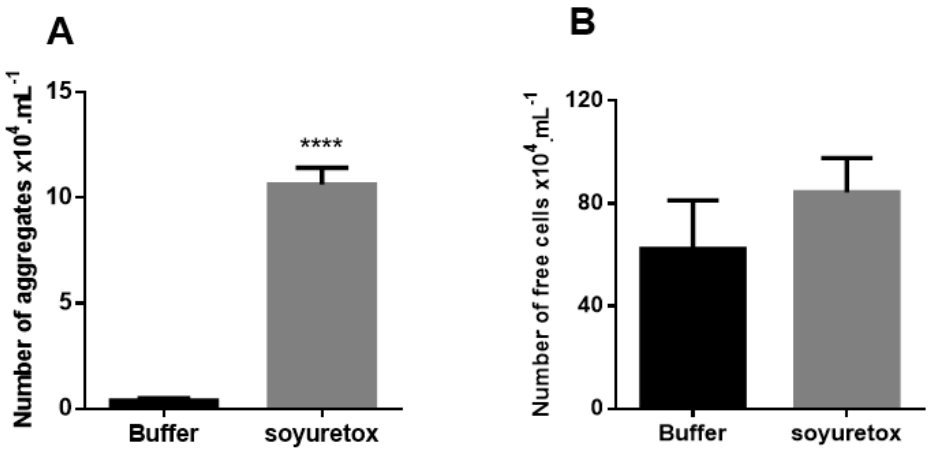

Figure 10. *In vivo* hemocyte aggregation induced by the injection of soyuretox into *R. prolixus*. Fifth instar nymphs were injected with buffer (control) or with soyuretox (0.05 µg.mg^{-1} insect body weight), 6 h afterwards the hemolymph was collected and diluted in anticoagulant solution. The number of hemocytes aggregates, defined as a cluster of at least 5 cells (A) and free cells (B) in hemolymph samples were counted using a hemocytometer. The values, expressed as the number of aggregates or free cells per mL of hemolymph, are mean ± SEM (n = 5).****$p < 0.0001$ compared to the control.

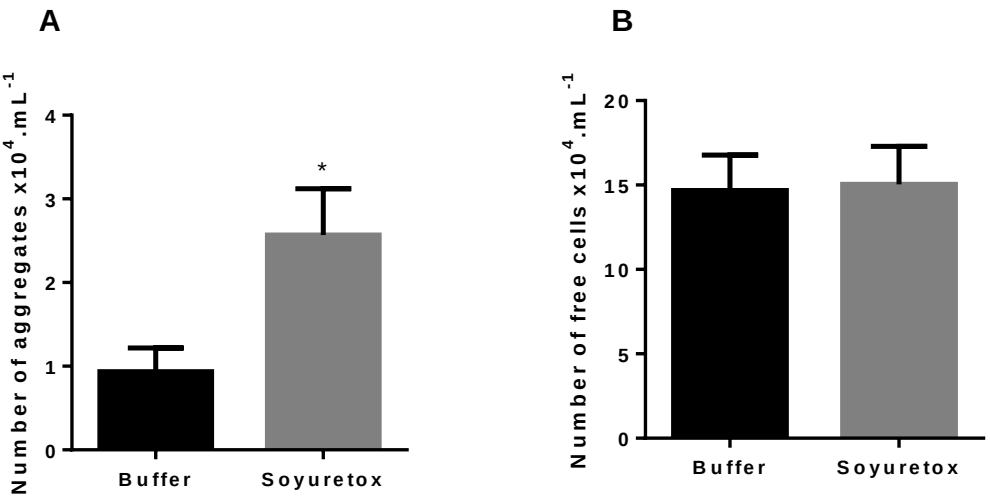

Figure 11. *In vivo* hemocyte aggregation induced by feeding *R. prolixus* with a soyuretox-containing solution. *Rhodnius prolixus* fifth instar nymphs were fed on buffer (control) or a soyuretox solution to give ~0.1 µg.mg^{-1} body weight dose. After 18 h, the hemolymph was collected, diluted in anticoagulant solution, and the number of aggregates (A) and of free cells (B) for each treatment was counted using a hemocytometer. The values are mean ± SEM (N = 8-9). *$p < 0.05$.

Soyuretox also induced aggregation of *R. prolixus* hemocytes in an *in vitro* assay. Figure 12 shows that incubation of hemolymph with 200 nM soyuretox led hemocytes to aggregate. Although the number of aggregates found in hemolymph samples treated with 500 nM soyuretox did not differ from control, the number of free cells decreased significantly, confirming the interaction of the peptide with insect cells.

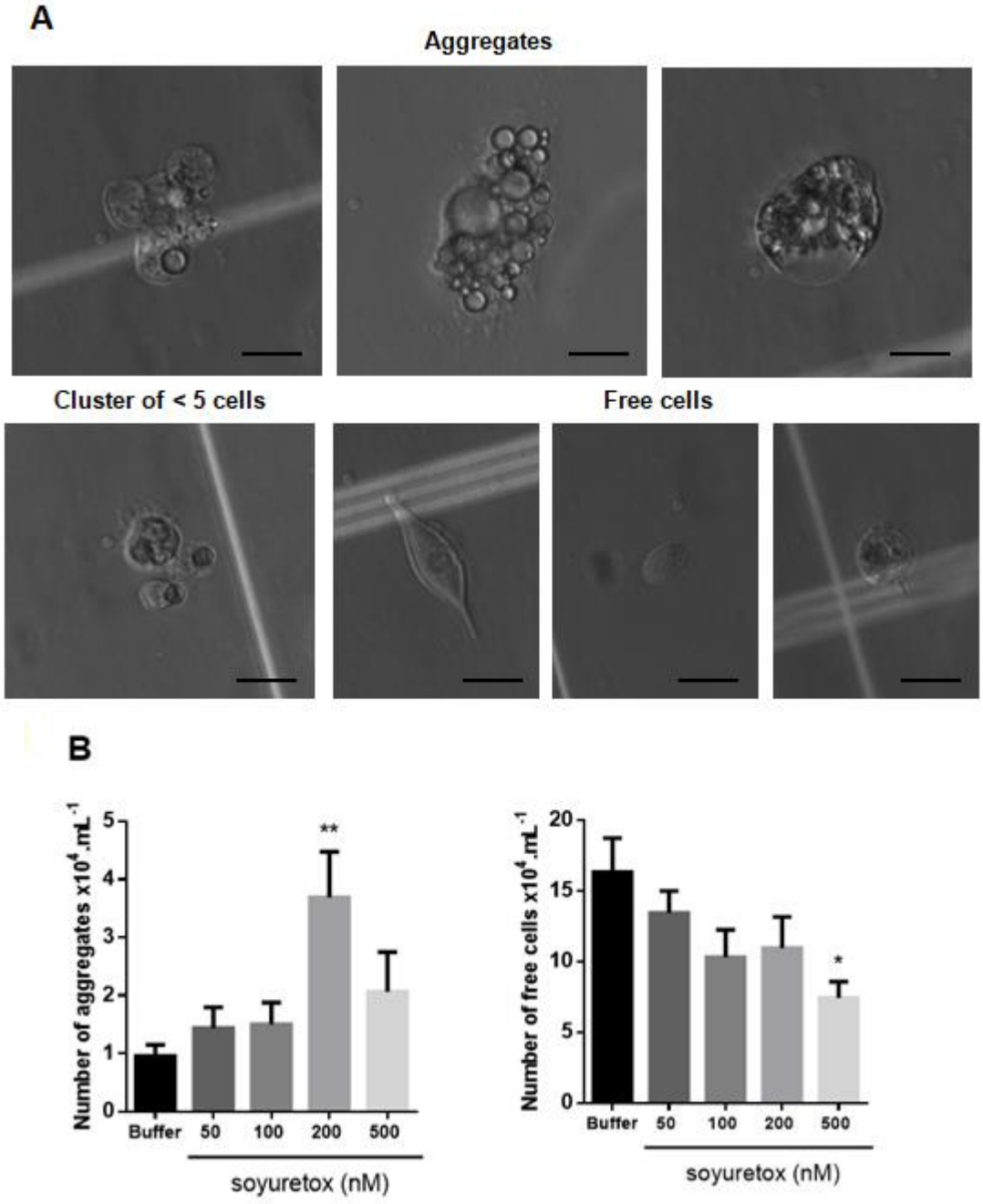

Figure 12. *In vitro* hemocyte aggregation induced by soyuretox. *R. prolixus* hemolymph samples were collected, diluted with *Rhodnius* saline, and incubated with saline (control) or with different concentrations of soyuretox for 1 h at room temperature. A. bright field images of representative hemocyte aggregates and free cells. Bars: 20 µm. B. the number of aggregates (five or more cells) and free cells for each treatment was counted using a hemocytometer. The values are mean ± SEM (n = 8-12). *$p < 0.05$; **$p < 0.03$.

3.9 Survival and development of zebrafish larvae

The potential embryotoxicity of soyuretox was assayed in zebrafish eggs exposed for 4 hours to 10, 100 or 300 nM of soyuretox. Compared to the survival rate of fishes in the control groups (sodium phosphate 5 mM pH 7 and water), the mortality rates found for the soyuretox-treated larvae (survival rates in percentages of 100, 95.4, 91.5 respectively) did not differ from controls groups. (Supplementary Figure 3).

3.9.1 Morphological evaluation

A potential teratogenic effect of soyuretox upon exposition of zebrafish eggs to the peptide for 4 hours was evaluated in the larvae on 5 dpf. There were no differences in body length, surface area of eyes and ocular distance among the controls (phosphate and water), and soyuretox-exposed groups at concentrations of 10, 100, 300 nM (Figure 13).

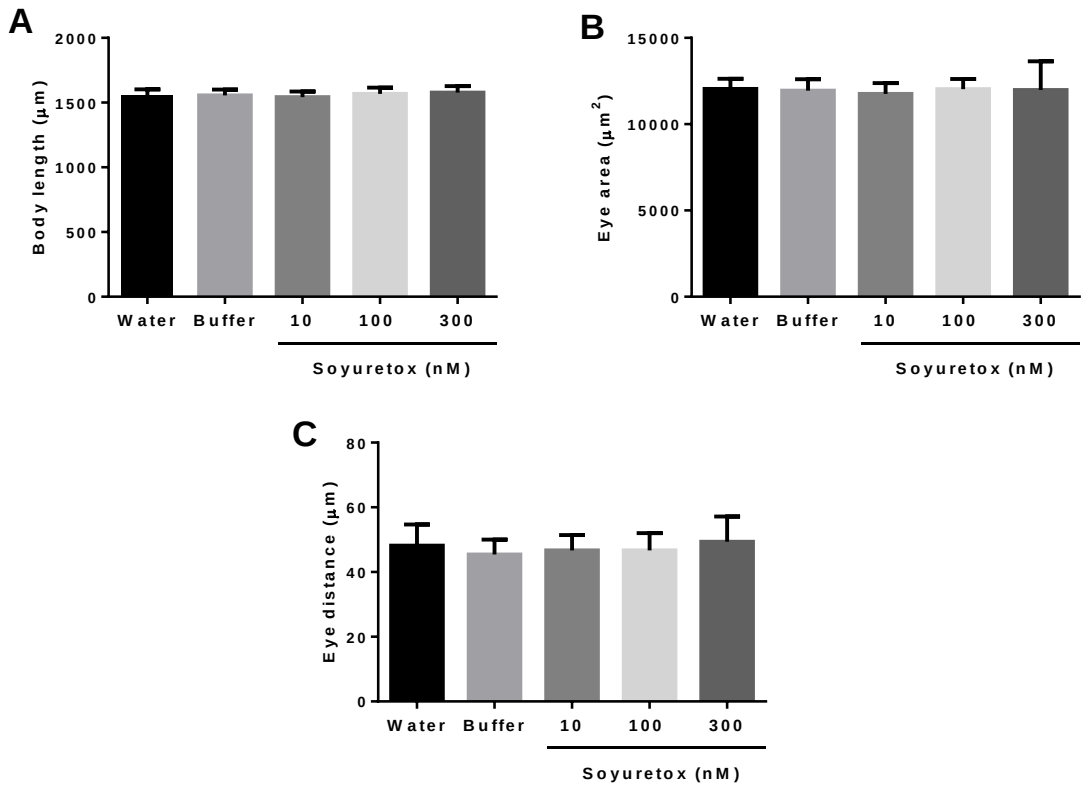

Figure 13. Effect of soyuretox in zebrafish. Morphological analysis of zebrafish larvae were conducted 5 dpf after a 4 h exposition to soyuretox or to sodium phosphate buffer or water (control groups) in the egg phase. A. Body size; B. Eye area; C. Eye distance. Data are means ± SD (N=24 per group). ANOVA followed by Tukey post-hoc test, with significance level of $p < 0.05$.

3.9.2 Behavioral Analysis in zebrafish larvae

The potential toxic effect of soyuretox on behavioral patterns of the zebrafish was assayed in larvae 5 days after a 4 h exposition of their eggs to 10, 100 or 300 nM soyuretox.

The exploratory behavior of larvae coming from eggs exposed to 10 and 100 nM soyuretox did not differ from control groups (Figure 14). On the other hand, animals exposed to 300 nM soyuretox displayed reduced travel distances and mean speed, increased time in central zone and increased escape response when compared to control groups.

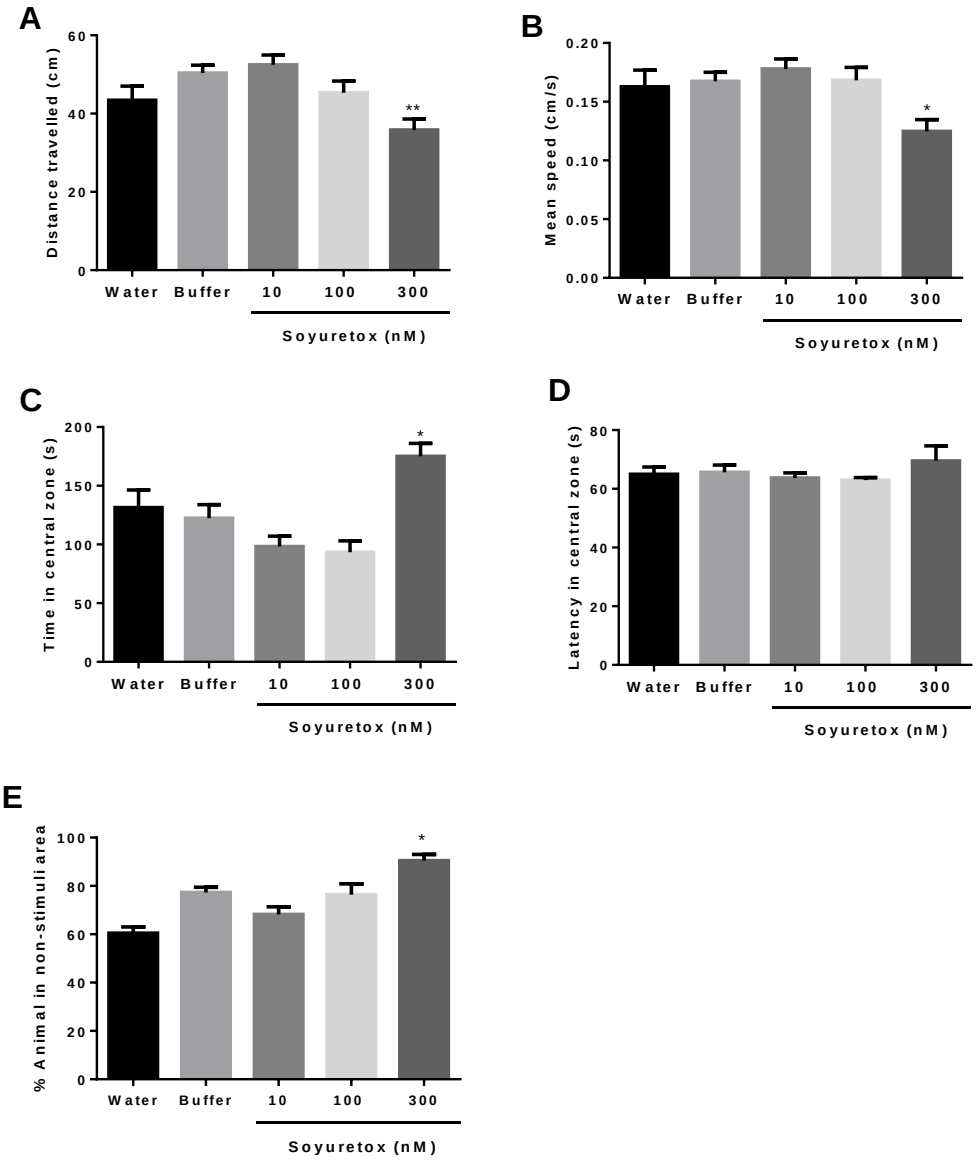

Figure 14. Exploratory and avoidance behaviors of soyuretox-exposed zebrafish larvae. Evaluation of the exploratory behavior of zebrafish larvae was conducted on 5 dpf after a 4 h exposition in the egg phase to soyuretox or to sodium phosphate buffer or water (control groups). A. Distance traveled; B. Mean speed; C. Time in the central zone; D. Latency in central zone; E. Avoidance behavior. Data were expressed as means ± SEM (N=23 per group). One-way ANOVA followed by Bonferroni's multiple comparisons tests with significance level of $*p < 0.05$

4. Discussion

In this work we reported the cloning, heterologous expression, structural characteristics and biological properties of soyuretox, a peptide derived from the soybean ubiquitous urease. This peptide is colinear to jaburetox, a homologous recombinant peptide derived from a *C. ensiformis* urease (Mulinari et al., 2007), which presents antifungal activity (Postal et al., 2012), and entomotoxic properties (Staniscuaski et al., 2009; Martinelli et al., 2014; Galvani et al., 2015; Fruttero et al., 2017; Fruttero et al., 2016). Our group has recently characterized the antifungal and entomotoxic activity of uSBU fused to GST (Martinelli et al., 2017). Here, the amino acid sequence of uSBU was examined to identify a sequence homologous to jaburetox, which was designated soyuretox, yielding an amino acid sequence with 72 % identity to the former (Figure 2A). Heterologous expression of soyuretox in *E. coli* BL21(DE3) pLysS cells was performed using the pET23a vector, which adds 6 His tail to the peptide C-terminus. Soyuretox was correctly expressed and purified in two steps, a Ni^{2+} affinity chromatography followed by a gel filtration column. The purified peptide presents a molecular mass of ~11 kDa. A faint band corresponding to a dimeric soyuretox appeared in Western blots (Figure 1B), a finding often seen also for jaburetox (Barros et al. 2009; Martinelli et al. 2014).

The intrinsically disordered nature of jaburetox has been reported, which was initially anticipated by bioinformatics tools (Martinelli et al., 2014) and later confirmed experimentally by CD and NMR analysis (Lopes et al., 2015). Here, soyuretox was characterized as an intrinsically disordered protein by computational tools and experimentally, by CD and NMR techniques. Contrasting to jaburetox's behavior in the molecular dynamics, the β-hairpin motif in the C-terminal domain of soyuretox was completely lost after 500 ns of unrestrained simulation (Figure 2E). Nevertheless, although both peptides behave as intrinsically disordered proteins, soyuretox kept a more ordered structure than did jaburetox in the molecular dynamics simulation. Despite the high similarity between jaburetox and soyuretox, the latter appeared more stable than is the former at pH 8 and within a 0.2–2.8 mg/mL (18-253 µM) concentration range (Supplementary Figure 2). The increased stability of soyuretox at pH 8.0 might reflect the presence of its two histidines in positions 23 and 51, contrasting with the

single His23 of jaburetox. Despite this, soyuretox was unstable in the concentrated solutions (50 mM) required for NMR studies, both at pH 6.5 and 8.0, not allowing assignments to solve its 3D structure.

CD and NMR data of soyuretox in the presence of SDS confirmed the binding of the peptide to the detergent in its micellar form. The data also demonstrated that, upon binding to the SDS micelles, soyuretox undergoes a folding transition to a more ordered structure, while remaining still largely unstructured. Although the 3D structure of soyuretox remains yet to be solved, our data revealed it has an increased content of secondary structure relative to jaburetox. Both peptides acquired a more ordered structure in contact with SDS micelles suggesting that binding to cell membrane lipids may drive the peptides toward a biologically active conformation.

The biological activities of soyuretox studied here were surprisingly similar to those known for jaburetox. Soyuretox dose-dependently inhibited the growth of all tested yeasts species (Figure 7) and this effect occurred in a similar micromolar concentration range as described for jaburetox (Postal et al., 2012), for JBU (Postal et al., 2012), and the recombinant uSBU-GST fused protein (Martinelli et al., 2017). Little is known so far about the mechanism(s) of antifungal action of ureases or urease-derived peptides. Postal et al., (2012) reported that submicromolar doses of jaburetox permeabilized the cell membrane of *S. cerevisiae* after a 24 h treatment. Formation of pseudo-hyphae, a stress-related response in yeast, and inhibition of carbohydrate metabolism were seen in jaburetox-treated yeast cells (Postal et al., 2012). Here, the immunofluorescence data showed that essentially all soyuretox bound to the yeast cells. At least in part, the inhibitory effect of soyuretox on *C. albicans* could be due to oxidative stress. Production of superoxide anions by soyuretox-treated yeasts paralleled the antifungal activity of the peptide, both seen with a minimum dose of 5 µM (Figure 8). It is known that various antimicrobial peptides active against *Candida* species share, as part of their antifungal mechanism, the production of ROS by the targeted yeasts. As examples, ROS production accompanied the fungitoxic effect of the *Phaseolus vulgaris* (L.) defensin PvD(1) against *C. albicans* at 16 µM concentration (Mello, 2011); the wasp-derived antimicrobial peptide protonectin after a 6 h treatment in doses of 128 and 256 µM, against *Candida glabrata* (Wang et al., 2015); the peptide arenicin-1, derived from a marine polychaeta, at 9 µM dose (Cho and Lee, 2011). *Rhesus* theta-defensin 1 (RTD-1) was shown effective against drug

sensitive and drug resistant clinical isolates of *C. albicans* and non-albicans *Candida* spp, in the dose range of 1.5- 3 µM. Fungal killing by theta-defensins occurred by intracellular accumulation ROS and cell permeabilization (Basso et al., 2018).

Martinelli et al., (2014) performed site-directed mutagenesis on the jaburetox molecule aiming to determine structure *versus* activity relationships, and demonstrated that the peptide's N-terminal half is endowed with insecticidal properties.Considering the 28 % difference in the amino acid sequences, and that the most divergent regions between jaburetox and soyuretox are located at the N-terminal halves, variations in the entomotoxic properties or even lack of them were expected for soyuretox (Figure 2A). Impairment of the insect immune response is an important component of the entomotoxic action of jaburetox, which can alone lead insects to death in case of infection by pathogenic microorganisms (Fruttero et al., 2016). Here we showed that soyuretox induced *R. prolixus* hemocytes to aggregate, both *in vivo*, either by injection or feeding the peptide to the insects, or in an *in vitro* assay. Thus, soyuretox may also affect the insect's immune system by recruiting hemocytes. The dose range in which the peptides induce aggregation of *R. prolixus* hemocytes are similar: ~200 mM in the *in vitro* assay and 2 µg/insect in the *in vivo* experiments (Fruttero et al., 2016). Two observations can be made from these data. First, the finding that soyuretox displays biological effects resembling those of jaburetox in an insect assay, points to a location of its entomotoxic and antifungal domains in one out of two small stretches of its amino acid sequence, Gly2-Met13 or Phe21-Thr35, that are the most similar when compared to jaburetox's primary sequence. Another plausible explanation for the equivalent entomotoxic effect of jaburetox and soyuretox was our choice of insect model. *R. prolixus* is a hematophagous insect, which relies on blood meals to survive and reproduce. In contrast to herbivorous insects, hematophagous insects had not evolved mechanisms of resistance to plant-derived insecticidal compounds. This fact could possibly explain why *R. prolixus* is equally sensitive to both peptides despite the differences in their N-terminal halves. To test the hypothesis of divergence within the entomotoxic domain(s) of jaburetox and soyuretox, it would be necessary to carry out assays comparing the effects of the peptides on herbivorous insects, preferentially with insects that are pests to soybean and non-soybean plants. Insects that feed on soybean plants have evolved mechanisms to evade the insecticidal effect of soybean ureases and to soyuretox, but probably not to jaburetox. In future studies with these

insects as models, it may be possible to identify which amino acids of urease-derived peptides integrate the core of their entomotoxic domain, and which amino acids confer specie-specific resistance to a given insect.

The data obtained with this study confirmed our hypothesis that the amino acid sequences encompassing soyuretox and jaburetox in their parental ureases are equivalent in terms of overall physicochemical and biological properties. As discussed in Mulinari et al., 2007, while the C-terminal half of this region is well conserved among ureases of distinct phylum, the N-terminal half diverged considerably in plants. Even though, considering the results shown here, antifungal and entomotoxic domain(s) within this region appear to have been conserved, at least among ureases in the Leguminosae family.

Considering the potent entomotoxic and antifungal properties we have demonstrated here for soyuretox, this recombinant peptide, as well as jaburetox, represent new candidates for the development of transgenic plants expressing these peptides aiming resistance or increased tolerance to phytophagous insects and phytopathogenic fungi. Preliminary studies indicated that transgenic maize expressing low levels of jaburetox showed increased resistance to the lepidopteran pest *Helicoverpa armigera* (Didoné, 2018). In this context, although biodegradable and ecofriendly, the biosafety profile of these peptides must be considered. Jaburetox was proven to be innocuous to mice and rats upon oral and intraperitoneal administration in a 10 mg/kg dose (Mulinari et al., 2007). Here we employed the zebrafish model to evaluate the toxicity profile of soyuretox. This fresh water teleost fish is a well accepted model for studies in areas covering biochemistry (Siebel et al., 2011; Zimmermann et al., 2016), pharmacology (Buske and Gerlai, 2011; Cognato et al., 2012), neurosciences (Stewart et al., 2014) and toxicology (Pereira et al., 2012; Senger et al., 2006). About 70 % of the zebrafish genes are homologous to human genes and 82 % of the human genes associated to diseases have at least one orthologue in this animal's genome (Howe et al., 2013). The zebrafish has signaling systems and an elaborated behavioral repertoire comparable to those of other groups of vertebrates. Among other behaviors, there are well established protocols to evaluate fear (Ogawa, Nathan, and Parhar, 2014), anxiety (Parker et al., 2014), aggressiveness (Bonan and Norton, 2015), social interaction (Zimmermann et al., 2016) and memory (Cognato et al., 2012; Jia, Fernandes, and Gerlai, 2014) using the zebrafish model. After a 4h

exposition of eggs to soyuretox at a maximal concentration of 300 nM, larval survival and development was followed up to 5 days post-fertilization. No lethality (Supplementary Figure 3) and no alterations in the parameters of larval morphology (Figure 13) were observed. On the other hand, the exposition of eggs to water containing the highest dose (300 nM or 3,318 µg per liter) of soyuretox altered the exploratory activity of 5 dpf larvae, reducing the travelled distance and the mean speed, increasing the time in the central zone, while increasing avoidance (Figure 14). The impaired exploration of a new environment seen in zebrafish exposed to 300 nM, in the absence of morphological changes, could suggest alterations in the dopaminergic system. It is well known that locomotor disorders associate to damage in the dopaminergic system in zebrafish (Bowton et al., 2010).

While it is difficult to empirically evaluate the dose of soyuretox taken up by the zebrafish eggs individually, the highest concentration of the peptide in water tested here does not represent a threat in the context of environmental safety. Massive amounts of transgenic plants decaying in the environment would be necessary to achieve a concentration of 3,318 µg soyuretox per liter of water. Insecticidal and antifungal proteins expressed in transgenic crops usually achieve levels of a few micrograms per gram of leaves. Examples are insect resistant maize and rice crops expressing Cry1 or Cry2 toxins from *Bacillus thuringiensis*, which are produced in the range of 0.46 – 139 µg per g of leaves (reviewed in (Liu et al., 2016). Although not in the market yet, transgenic plants expressing defensins (Sundaresha et al. 2016), chitinases (Zhang et al., 2016; Aghazadeh et al., 2016) or cystatins (Christova et al., 2018) also attained increased protection from herbivourous insects or fungal diseases accumulating micrograms of the heterologous protein.

In summary here obtained soyuretox, a new recombinant peptide derived from the soybean ubiquitous urease. *In silico* analyses and experimental determination of the structural features revealed the intrinsically disordered nature of soyuretox, and that the peptide acquires a more ordered secondary structure when in contact with SDS micelles. Soyuretox displays potent antifungal activity against pathogenic yeasts mediated at least in part by the production of reactive oxygen species. Soyuretox has entomotoxic activity, measured as induction of aggregation of *R. prolixus* hemocytes, both *in vivo* and *in vitro.* Altogether the data is consistent with the hypothesis that the sequence denoted by soyuretox represents antifungal and entomotoxic domains

evolutively conserved among Leguminosae ureases. Soyuretox can be regarded as safe to zebrafish larvae and in an environmental context regarding accumulation in water bodies. Overall our data bring into light the biotechnological potential of soyuretox as a promising molecule for the development of transgenic plants with increased resistance to insect herbivory and fungal disease.

Authors contributions:

KK conducted the experiments in all phases of this study and wrote the paper; AHSM helped to clone soyuretox and in antifungal assays; VB, BZ, ST conducted CD and RMN analyses; FCL helped in antifungal assays; RLB carried out molecular modeling and dynamics; LLF conducted immunofluorescence and *R. prolixus* assays; NRM helped in R. *prolixus* assays; CDB supervised the zebrafish assays; CRC wrote the paper and conceived this study.

Acknowledgements

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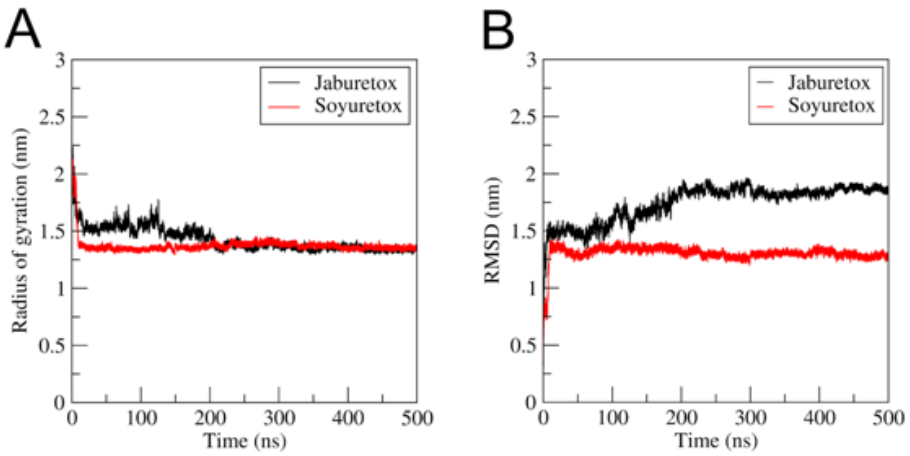
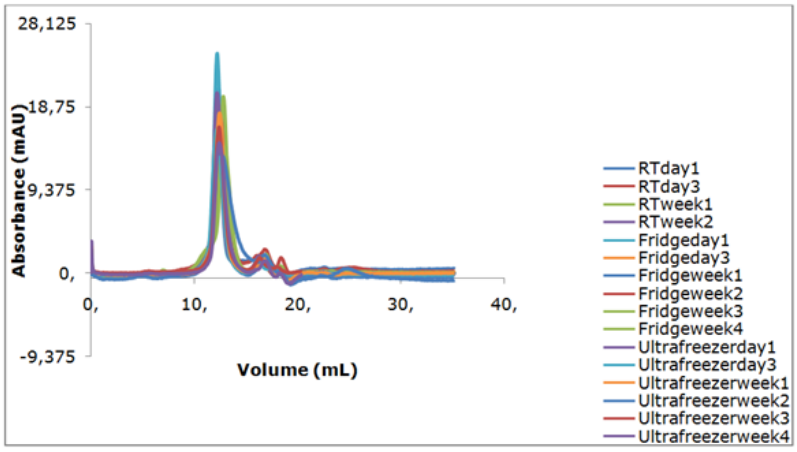
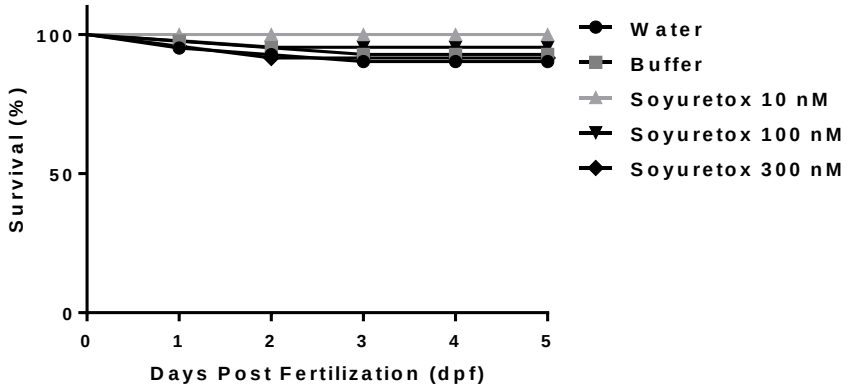
Supplementary Figures



Supplementary Figure 1.Time course of soyuretox conformational changes in aqueous solution. (A) Radius of gyration and (B) all-atom root mean square deviation (RMSD) of soyuretox molecular dynamic simulation during 500 ns (red), in comparison to jaburetox (black) (data from Martinelli et al., 2014).



Supplementary Figure 2. Stability analysis of soyuretox during for 4 weeks of storage at different temperatures. Soyuretox aliquots (2.8 mg/mL, 253 µM) in 50 mM sodium phosphate buffer, 1 mM EDTA, 1 mM TCEP, pH 8.0, were stored at room temperature (RT), in the fridge (4 °C) or in the ultrafreezer (-80 °C) for 1, 2, 3 and 4 weeks. At these time points, the aliquots were subjected to gel-filtration analysis in a Superdex 75 (16/60) column, and elution was followed by absorbance readings at 280 nm. Typical results are shown.



Supplementary Figure 3. Kaplan-Meier survival analysis of soyuretox-treated zebrafish larvae. Eggs were exposed to 10, 100 and 300 nM of soyuretox, or sodium phosphate 5 mM buffer or water (controls), and survival rate was followed daily until 5 dpf. No difference was found in the survival rates of soyuretox-treated zebrafish compared to the water group (Mantel-cox test $p < 0.05$). Data are means of 50 animals per group.

As referências do artigo estão compiladas ao final da tese.

Capítulo III

Considerações finais, conclusão e perspectivas

CONSIDERAÇÕES FINAIS

A clonagem e expressão heteróloga do gene codificador do peptídeo soyuretox, derivado da urease ubíqua de soja colinear ao jaburetox, foi realizada durante o mestrado (KAPPAUN, 2014). Naquele estudo, foram mantidas as mesmas condições de expressão utilizadas para o jaburetox, ou seja, a célula (BL21(DE3)-RIL), indução com 1 mM de IPTG e tempo de expressão de 3 h, a 37 ºC (Martinelli et al., 2014; POSTAL et al., 2012). Com o peptídeo soyuretox expresso dessa forma, obtivemos material suficiente para realizar os ensaios antifúngicos.

Porém, para estudar estruturalmente o soyuretox, a expressão do peptídeo precisou ser melhorada, já que para a realização da técnica de RMN é necessário grandes quantidades da proteína e expressão em meio pobre em nutrientes (Meio mínimo M9). No presente trabalho, as condições de expressão do soyuretox foram otimizadas através da troca de célula para *E. coli* BL21(DE3) pLYsS, diminuição da temperatura de expressão para 21 ºC, aumento do tempo de expressão para 24 h, e diminuição do IPTG para 0,23 mM. Adicionalmente, o inóculo passou a ser feito em duas etapas, conforme método estabelecido por (MARLEY, LU & BRACKEN, 2001). Nesse método, as células são multiplicadas em meio rico (como o LB) e em maior volume, seguido de lavagem e inoculação em meio M9, em menor volume. Os autores mostraram que a redução para 25 % do volume inicial, na passagem do primeiro para o segundo meio, leva a um aumento na expressão das proteínas, e isso é especialmente vantajoso no preparo de amostra para RMN, pela economia nos isótopos marcados (MARLEY, LU & BRACKEN, 2001). Por essa técnica obtivemos, após a modificação dos outros fatores, uma boa expressão, atingindo 3 mg de proteína por litro de cultura. As etapas de purificação foram mantidas as mesmas do jaburetox. Na última etapa, a cromatografia de gel filtração, foi inicialmente utilizado o tampão 50 mM Na_2HPO_4, 1 mM EDTA, 1 mM TCEP, pH 7,5, como realizado para o jaburetox. Após a gel filtração, o peptídeo é obtido tipicamente diluído, sendo necessária uma etapa de concentração, na qual o peptídeo precipitava extensamente. Diversos testes objetivando manter o peptídeo em solução concentrada foram necessários, como a adição de sais e variação de pH (dados não mostrados). O aumento de pH do tampão da gel filtração de 7,5 para 8,0 conferiu maior estabilidade ao soyuretox, sendo possível então atingir a concentração adequada para as medidas estruturais.

Para a realização da análise de RMN, o peptídeo deve ficar estável por pelo menos 7 dias, a uma temperatura de 25 ºC e em alta concentração. Apesar de que, em pH 8, a solução de soyuretox não formasse precipitados visíveis, esta solução não ficou estável o suficiente para a análise RMN, o que impediu a coleta de espectros de qualidade para a marcação (*assignment*), e cálculo de estrutura do peptídeo. No entanto, ainda assim, o espectro de RMN obtido para o soyuretox permitiu caracterizá-lo como sendo intrinsicamente desordenado. O peptídeo ficou estável em solução apenas quando em contado com micelas de SDS, permitindo a coleta de espectros.

Embora o jaburetox e soyuretox apresentem identidade de 72 % a nível de aminoácidos, a diferença de estabilidade entre os dois peptídeos é um ponto importante a ser destacado. A análise das sequência de aminoácidos dos peptídeos pelo ProtParam (https://web.expasy.org/protparam/) mostra que jaburetox e soyuretox apresentam pontos isoelétricos (pI) diferentes. O jaburetox apresenta pI de 5,0, enquanto o soyuretox apresenta ponto isoelétrico de 5,36. O fato do soyuretox ser mais estável em pH 8,0 pode refletir diferenças especialmente concentradas na região N-terminal dos peptídeos, em particular uma mudança no estado de ionização da cadeia lateral de duas histidinas, a His23 e His51 (pka ~6,0). O jaburetox contem uma His a menos do que o soyuretox, já que na posição equivalente à His51 ocorre uma Asn no jaburetox (Figura 2C).

Com relação ao possível uso do soyuretox como biopesticida, testes para avaliação da biossegurança do peptídeo foram realizados com o organismo modelo *zebrafish*. Nos anos 2000, foi mostrado pela primeira vez que *screenings* usando o *zebrafish* poderiam ser feitos em placas de 96 poços, adicionando os compostos diretamente na água, e minimizando as quantidades necessárias para os testes (PETERSON et al., 2000). O modelo *zebrafish* se tornou amplamente utilizado para análises toxicológicas e estudos de organogênese, por apresentar como vantagens o desenvolvimento externo (o que elimina a toxicidade relacionada a mãe), desenvolvimento rápido do sistema nervoso, transparência dos ovos e estágios larvais, além de manutenção com custo mais baixo e de possibilitar *screenings* de alto rendimento, quando comparados com modelos de roedores. O animal apresenta ~70 % de genes homólogos a humanos (HOWE et al., 2013), e já tem o genoma totalmente sequenciado.

He e colaboradores, 2014, revisaram o uso do *zebrafish* como animal modelo para a descoberta de fármacos e o acompanhamento de segurança e toxicidade, frisando que os diversos estudos disponíveis têm confirmado que o *zebrafish* e mamíferos são similares em seu desenvolvimento e fisiologia básica. Além do mais, há vários estudos mostrando que o modelo *zebrafish* é adequado para testes de segurança para protótipos de fármacos, e tem o seu uso aprovado pelo FDA – *Food and Drug Administration*, dos Estados Unidos da América (HE et al., 2014).

No nosso estudo, com a exposição dos ovos de *zebrafish* ao soyuretox, não foram observadas alterações comportamentais e morfológicas nas doses 10 e 100 nM. Foram ainda realizados ensaios preliminares com ureases de *C. ensiformis*, JBU e CNTX, que são proteínas inseticidas e antifúgicas, em larvas e adultos de *zebrafish*. Os animais expostos a JBU não apresentaram alterações comportamentais e morfológicas em larvas quando expostas no 5 dpf por 24 h na dose de 300 nM, bem como em adultos injetados intraperitonealmente nas doses de 10 e 30 mg/kg (dados não mostrados). A JBU também não mostrou atividade tóxica quando administrada por via intraperitoneal em murinos (FOLLMER et al., 2001).

Em relação ao soyuretox, observamos efeitos comportamentais, mas sem estar acompanhado de alterações morfológicas, para os ovos de *zebrafish* tratados com 300 nM do peptídeo. Esta é uma concentração muitíssimo elevada se for comparado com os níveis de superexpressão de uma proteína inseticida já obtidos em plantas transgênicas (revisado em (LIU et al., 2016). Por exemplo, plantas de milho e de arroz resistentes a insetos, transgênicas para toxinas Bt e já liberadas para comércio, tipicamente expressam poucas microgramas de toxinas por g de folha. Outras plantas transgênicas que expressam diferentes peptídeos antimicrobianos e/ou inseticida, acumulados nos tecidos vegetais também na escala de microcrogramas por g, estão em fase de teste (revisado em LIU et al., 2016). Já foram obtidas raízes de soja que superexpressam o soyuretox e estas mostraram proteção contra a infecção pelo nematóide da galha *M. javanica* (RECHEMACHER, 2016). O estado de transgenia dessas plantas foi confirmado por análise da expressão gênica e não foi possível mensurar a quantidade de proteína soyuretox acumulada na planta, provavelmente por estar abaixo do limite de detecção do método empregado. Porém, baseado na literatura disponível, não se espera que a expressão do soyuretox nessas plantas seja maior do que a verificada nas plantas transgênicas já estudadas. Uma planta de soja

transformada com o soyuretox, mais resistente a doenças fúngicas e insetos praga, seria a primeira cultivar transgênica superexpressando o peptídeo que poderia chegar ao mercado consumidor.

CONCLUSÃO

Nessa tese, analisamos aspectos estruturais e atividades biológicas do peptídeo soyuretox, concluindo que:

- Alterações das condições de expressão do peptídeo em *E. coli* possibilitaram obter um maior rendimento do soyuretox, atingindo 3 mg de proteína por litro;
- Tampão Fosfato de sódio contendo 1 mM de EDTA e 1 mM TCEP em pH 8.0 é o mais adequado para a manutenção do peptídeo na forma solúvel;
- Estudos estruturais do peptídeo soyuretox por dicroísmo circular (CD) e ressonância magnética nuclear (RMN), mostraram sua natureza intrinsecamente desordenada, confirmando análises de bioinformática;
- O soyuretox liga-se a micelas de SDS, e essa interação desencadeia alterações conformacionais que levam o peptídeo a um estado mais ordenado, sem contudo perder a característica de ser intrinsecamente desordenado. Neste aspecto, o soyuretox se diferencia do jaburetox;
- O mecanismo de inibição do soyuretox em *C. albicans* é, pelo menos em parte, resultante da produção de espécies reativas de oxigênio;
- O peptídeo foi imunolocalizado na superfície da levedura;
- O peptídeo apresenta atividade entomotóxica em *R. prolixus*, verificada por ensaio de agregação de hemócitos *in vivo* e *ex vivo*;
- Em ensaios realizados pela primeira vez com um peptídeo derivado de urease, demonstramos que o soyuretox é seguro para ovos/larvas de *zebrafish* até a concentração de 100 nM, sem causar letalidade, alterações morfológica ou no comportamento dos animais;
- O peptídeo não causou letalidade ou mudanças morfológicas em *zebrafish* expostos a dose de 300 nM. Todavia, expostos a essa dose, os animais apresentaram alterações no comportamento exploratório. Conforme a literatura

mostra, esta seria uma concentração muito alta do peptídeo para ser expressa em planta transgênica;

- Soyuretox é um peptídeo com potencial uso biotecnológico, porém mais testes são necessários para ratificar a sua segurança.

PERSPECTIVAS

Para a continuidade e aprofundamento do presente estudo, levantamos como perspectivas:

- Realizar estudos de RMN 4D do soyuretox para determinar a estrutura do peptídeo na ausência e na presença de micelas de SDS e lipídeos derivados de leveduras e/ou de insetos;
- Completar a análise de biossegurança e avaliação toxicológica do soyuretox no *zebrafish* em outros esquemas experimentais como tempo de exposição e realizar ensaios em adultos de *zebrafish* após injeção de soyuretox;
- Caso seja detectada alguma toxicidade do soyuretox no *zebrafish*, realizar análise de qPCR com genes candidatos. Candidatos de genes a serem analisados incluem aqueles envolvidos na alteração de comportamento exploratório observado no animais tratados com a maior dose do soyuretox, em especial aqueles ligados ao sistema dopaminérgico;
- Finalização do ensaio de análise de toxicidade de larvas e adultos de *zebrafish* expostos às ureases de *C. ensiformis*.

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ANEXOS

S I P E S Q

Sistema de Pesquisas da PUCRS

Código SIPESQ: 7659 Porto Alegre, 20 de dezembro de 2016.

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou o Subprojeto de Pesquisa "Estudo do efeito das ureases, peptídeos derivados e moduladores em zebrafish (Dario rerio)" coordenado por CELIA REGINA R. DA S. CARLINI, vinculado ao Projeto Guarda-Chuva "5789 - Multifuncionalidade de Ureases: fator de virulência de microrganismos e papel na defesa de plantas" coordenado por CELIA REGINA R. DA S. CARLINI.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
null	null	20/12/2016 - 20/12/2020

Atenciosamente,

Comissão de Ética no Uso de Animais (CEUA)


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Soybean ubiquitous urease with purification facilitator: An addition to the moonlighting studies toolbox

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ABSTRACT

Ureases are nickel-dependent enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide. In soybean (*Glycine max*), the embryo-specific urease (eSBU), the ubiquitous urease (uSBU), and a third isoform (SBU-III) are synthesized. Our group has previously demonstrated that eSBU, purified from seeds, has antifungal properties against phytopathogenic fungi, entomotoxicity against *Dysdercus peruvianus*, the ability to induce blood platelet aggregation, and these properties are independent of its enzymatic activity. Here we describe the biological properties of apo-uSBU fused to glutathione S-transferase (GST) produced in *Escherichia coli*. Removal of GST affected apo-uSBU stability. We performed a Response Surface Methodology to optimize GST-uSBU production to 5 mg per liter and then bioassays were carried out. The recombinant protein exhibited inhibitory effects on filamentous fungi and affected fungal secondary metabolism. *Candida albicans* and *C. tropicalis* were also susceptible to GST-uSBU and formed pseudo-hyphae. The fusion protein was toxic against *Rhodnius prolixus,* with the toxicity being accompanied by *in vivo* and *in vitro* hemocyte aggregation. Rabbit platelet also aggregated in the presence of GST-uSBU. Thus, uSBU displayed similar biological properties as previously described for eSBU even when fused to GST, reinforcing the proposed role of ureases in plant defense.

1. Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase), metalloenzymes that catalyze the hydrolysis of urea to produce ammonia and carbon dioxide, are produced by plants, fungi and bacteria but not by animals [1,2]. Ureases contain two catalytically important atoms of nickel [3] that require a set of accessory proteins to place them in the active site of the apoenzymes [4,5]. In plants, ureases are abundant mainly in seeds of some members of the Fabaceae and Curcubitacea families [1]. It has been postulated that these enzymes are not only involved in nitrogen bioavailability, but also in plant defense processes [6]. Over the last two decades our group has demonstrated that ureases from different organisms are multifunctional proteins, displaying a number of biological properties unrelated to their enzyme nature, including platelet aggregation, insecticidal, and antifungal activities [6–10].

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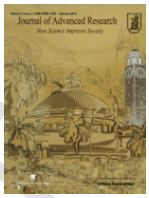

Review

Ureases: Historical aspects, catalytic and non-catalytic properties. A review

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ABSTRACT

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-containing enzyme produced by plants, fungi, and bacteria that catalyzes the hydrolysis of urea into ammonia and carbamate. Urease is of historical importance in Biochemistry as it was the first enzyme ever to be crystallized (1926). Finding nickel in urease's active site (1975) was the first indication of a biological role for this metal. In this review, historical and structural features, kinetics aspects, activation of the metallocenter and inhibitors of the urea hydrolyzing activity of ureases are discussed. The review also deals with the non-enzymatic biological properties, whose discovery 40 years ago started a new chapter in the study of ureases. Well recognized as virulence factors due to the production of ammonia and alkalinization in diseases by urease-positive microorganisms, ureases have pro-inflammatory, endocytosis-inducing and neurotoxic activities that do not require ureolysis. Particularly relevant in plants, ureases exert insecticidal and fungitoxic effects. Data on the jack bean urease and on jaburetox, a recombinant urease-derived peptide, have indicated that interactions with cell membrane lipids may be the basis of the non-enzymatic biological properties of ureases. Altogether, with this review we wanted to invite the readers to take a second look at ureases, very versatile proteins that happen also to catalyze the breakdown of urea into ammonia and carbamate.

History and molecular features of ureases

Ureases (urea amidohydrolase, EC 3.5.1.5) are ubiquitous metalloenzymes, produced by plants, fungi and bacteria, but not by animals. The most proficient enzymes known to date, ureases catalyze the hydrolysis of urea into ammonia and carbamate (which then decomposes into another ammonia molecule and carbon dioxide), accelerating the rate of this reaction by a factor of at least 10^{14} when compared to the urea decomposition by elimination reaction [1–4].

Computational modeling of urease proficiency led to the proposal of a value up to 10^{32} times the theoretical rate of uncatalyzed urea hydrolysis [5]. However, one can argue that, in solution, this value is not realistic due to limits imposed by the diffusion of the substrate in water.

Urea, the natural substrate of ureases, was first isolated from human urine by Rouelle in 1773 and about a half century later, Wöhler achieved the synthesis of urea, the first organic molecule to be obtained from inorganic ones [6]. The first ureolytic microorganism, *Micrococcus ureae*, was isolated by van Tiehem in 1864, and the first enzyme with ureolytic activity was isolated from putrid urine by Musculus in 1874. The name "urease" was proposed in 1890 by Miquel [4]. Urease contributed two historical landmarks in Biochemistry. First, the crystallization of urease isolated from jack bean (*Canavalia ensiformis*) seeds by James B. Sumner, in 1926, demonstrated the proteinaceous nature of enzymes [7], a discovery laureated with the Nobel Prize in Chemistry in 1946. Second, the biological significance of nickel was recognized in 1975, after studies of Zerner's group revealing the presence of nickel ions in the active site of the jack bean urease (JBU), obligatory for its catalytic activity [8]. The identification of a plant toxin as a urease in 2001 can be considered as a third breakthrough involving ureases, as it led to the discovery of non-catalytic properties of these enzymes [9]. This finding widened our knowledge on the array of functions performed by these proteins, besides their role in nitrogen metabolism [10].

Ureases are members of the superfamily of amidohydrolases and phosphotriesterases, which display catalytically active metal(s) in their active sites. With a few exceptions reported [11,12], ureases carry two Ni^{2+} ions in their active sites [4,13]. Ureases from different sources share about 55% identity in their primary sequences suggesting divergence from a common ancestral protein. X-ray crystallography studies revealed that plant and bacterial ureases share a common basic "trimeric" structure [4,14]. The number of polypeptide chains

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that form the "monomer" or functional unit varies according to the source of urease. For plant and fungal ureases this functional unit is a single polypeptide chain (α). The functional unit of bacterial ureases is formed by two subunits (α and β, so far found only in the genus *Helicobacter*) or three (α, β and γ) types of polypeptide chains. The most abundant structure of plant ureases is a dimer of trimers $(\alpha_3)_2$ although a few dimeric/trimeric/tetrameric plant and also fungi ureases have been described. Bacterial ureases are trimers $([\alpha\beta\gamma]_3)$ while *Helicobacter pylori*'s urease has been crystalized as a tetramer of trimers of dimers $([\alpha\beta]_3)_4$ (reviewed in [10,14]). The amino acid sequences of smaller subunits of prokaryotic ureases are collinear to the corresponding region in the single chain of eukaryotic ureases [4].

Fig. 1 illustrates the structural features of ureases.

The primitive state of these proteins – single- or three-chained – is one of the unanswered questions regarding ureases. Using phylogenetic inference and two algorithms applied to three different datasets, a 3-to-1 transition in the number of urease's subunits was observed, implying a three-chained ancestral urease from which all the present enzymes derived. In that scenario, the two-chained ureases in the genus *Helicobacter* are not evolutionary intermediates of the eukaryotic single-chained ureases [15].

Table 1 presents an updated list of ureases for which molecular and kinetics characteristics are known.

Activation and catalytic properties of ureases

The active site of ureases consists, besides the two nickel atoms, of one carbamylated lysine, four histidines and one aspartate residue. The crystal structures of bacterial ureases from *Klebsiella aerogenes* [50] and *Sporosarcina* (former *Bacillus*) *pasteurii* [56] first revealed the architecture of the enzymes' active site. These two ureases have nearly superimposable active sites, very similar to those of other ureases characterized afterwards, implying that this architecture is representative of all ureases. In the active site, the carbamylated lysine bridges the two nickel atoms, with Ni(1) further coordinated by two histidines and Ni(2) by the other two histidines and by an aspartate residue. Additionally, a hydroxide ion bridges the two Ni atoms, which along with other three terminal water molecules (W1, W2, W3), forms an H-bonded water tetrahedral cluster in the active site (Fig. 2) [4,14,2].

Besides the amino acid residues that compose the active site itself other residues, including a conserved cysteine, form the "mobile flap", which works as a gate for the substrate. This flap is composed by a helix-turn-helix motif and is responsible for substrate influx and product efflux in ureases, especially via motion control of a conserved histidine residue [2]. In the catalysis, amino acid residues of the mobile flap participate in the substrate binding, mainly through H bonds, thereby stabilizing the catalytic transition state and accelerating the reaction [2,4,14].

The mechanism for urea hydrolysis catalyzed by urease (Fig. 2) has been a hotly debated subject (see [64,65]). Currently, it seems to be an agreement on the mechanism, strongly supported by studies with urease inhibitors [14,66–68]. After taking the place of water molecules W1-W3 (Fig. 2A) in the urease active site, urea binds to Ni(1) ion through the carbonyl oxygen, making the urea carbon more electrophilic and, thus, more susceptible to nucleophilic attack (Fig. 2B). Then urea binds to Ni(2), through one of its amino nitrogen atoms, establishing a bidentate bond with urease (Fig. 2C). This bond is believed to facilitate the water nucleophilic attack on the carbonyl carbon resulting in a tetrahedral intermediate (Fig. 2D), from which

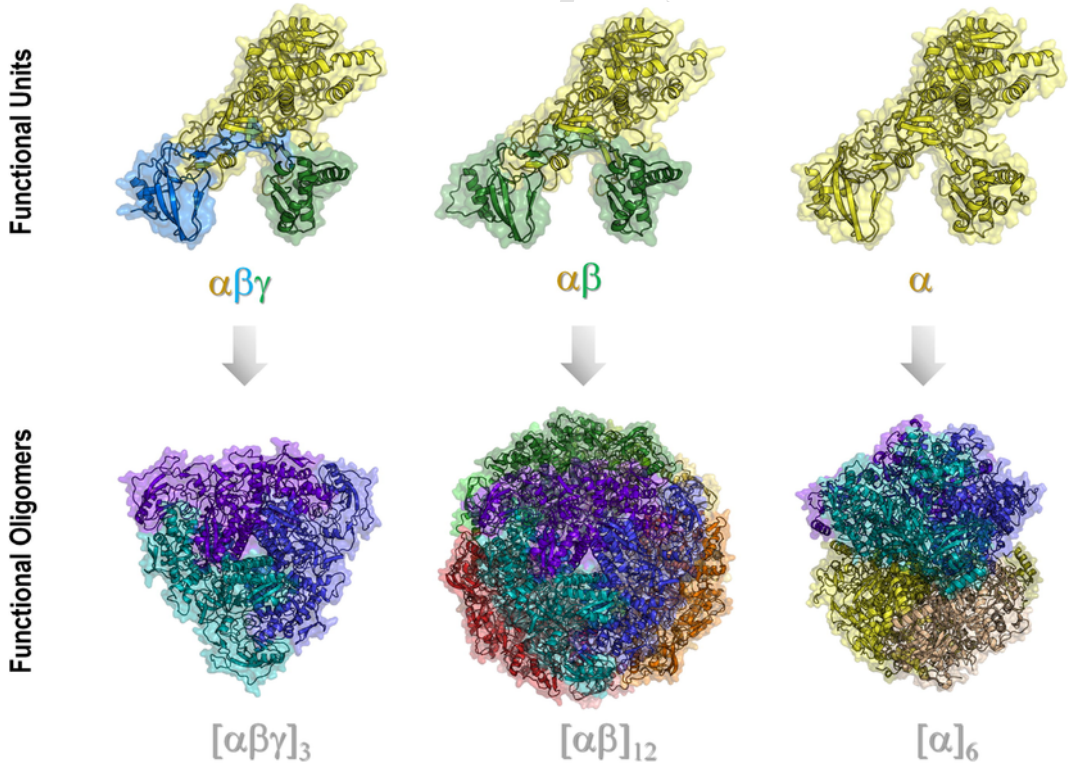

Fig. 1. Urease structural conservation. A functional unit can be formed by a heterotrimer (as in *Sporosarcina pasteurii*, PDB id 2UBP), a heterodimer (as in *Helicobacter pylori*, PDB id 1E9Z) or by a single unit (as in *Canavalia ensiformis*, PDB id 3LA4). These functional units (or monomers) form larger complexes, such as trimers, hexamers or dodecamers.

Table 1
Biochemical and structural data on selected ureases of plants, bacteria and fungi.

	Source Isoform GenBank identifier	Native M_r Oligomeric state	Number of residues – M_r subunit(s)[a]	p*I*	km for urea (mM)	Optimal pH	3D structure (PDB id)	Refs
PLANTS	*Arabidopsis thaliana* AT1G67550		838 aa					[16]
	Canavalia ensiformis JBU M65260.1	540 kDa α_6	840 aa 90.8 kDa	5.0–5.1	2.9–3.6	7.0–7.5	3LA4	[17–23]
	Canavalia ensiformis CNTX	180 kDa α_2	n.a. 95 kDa	n.a.	2–3	n.a.	n.a.	[9]
	Cajanus cajan JN107804.1	540 kDa- α_6	840 aa 90 kDa	n.a.-	3.0	7.3	4G7E	[24,25]
	Glycine max Embryo-specific AY230157	α_6	840 aa 93.5 kDa	n.a.	0.2–0.6	7.0	n.a.	[26,27]
	Glycine max Ubiquitous AY276866	345 kDa α_3	837 aa	n.a.	0.8	5.25 8.75	n.a.	[26,28,29]
	Gossypium hirsutum	α_6	98.3 kDa	n.a.	0.12–0.15	8.0	n.a.	[30]
	Morus alba AB479106.1	175 kDa α_2	90.5 kDa	n.a.	0.16	9.0	n.a.	[31]
FUNGI	*Aspergillus nidulans*	540 kDa α_6	840 aa 90 kDa	n.a.	1.33	8.5	n.a.	[32]
	Aspergillus niger XM_001388748.2	540 kDa α_6	837 aa 90 kDa	n.a.-	3.0	8.0	n.a.	[33]
	Cryptococcus gattii CPC735_069440	180 kDa α_2	840 aa 90 kDa	n.a.	2.0	8.0	n.a.	[34,35]
	Cryptococcus neoformans CNAG_05540	α_2	832 aa 90 kDa	n.a.	n.a.	n.a.	n.a.	[36]
	Coccidioides posadasii CPC735_069440	540 kDa- α_6	840 aa 90 kDa	n.a.	n.a.	n.a.	n.a.	[37]
	Coccidioides immitis U81509	α_4	839 aa 91.5 kDa	5.5	4.1	8.0	n.a.	[38]
	Schizosaccharomyces pombe	α_2	835 aa 91.2 kDa	n.a.	1.03	8.2	n.a.	[39]
BACTERIA	*Aerobacter aerogenes* PRL-R3			n.a.	2.8	7.5	n.a.	[40]
	Arthrobacter oxydans	242 kDa		4.3–4.7	12.5	7.6	n.a.	[41]
	Brevibacterium ammoniagenes	200 kDa $(\alpha\beta\gamma)_3$	α 67 kDa	4.1	32	7.0	n.a.	[42]
	Brucella suis Two operons	$(\alpha\beta\gamma)_3$		5	5.6	7.0	n.a.	[43,44]
	Helicobacter pylori M60398	1.06 MDa $([\alpha\beta]_{3)\,4}$	β 238 aa 30 kDa α 569 aa 62 kDa	5.9	0.2–0.8	8.0–8.2	1E9Z	[45–48]
	Klebsiella aerogenes M36068	$(\alpha\beta\gamma)_3$	γ 100 aa 11.1 kDa β 106 aa 11.7 kDa α 567 aa 60.3 kDa	n.a.	2.8	7.75	1FWJ	[49,50]
	Morganella morganii	590 kDa $(\alpha\beta\gamma)_3$	63 kDa 15 kDa 6 kDa	0.7				[51,52]
	Providencia stuartii	230 kDa $(\gamma2\beta2\alpha)_2$	γ 9 kDa β 10 kDa α 73	5.4	9.3	n.a.	n.a.	[53]

Table 1 (*Continued*)

Source Isoform GenBank identifier	Native M_r Oligomeric state	Number of residues – M_r subunit(s)[a]	p*I*	km for urea (mM)	Optimal pH	3D structure (PDB id)	Refs
Proteus mirabilis M31834	252 kDa $(\alpha\beta\gamma)_3$	γ 100 aa 11 kDa β 109 aa 12.2 kDa α 567 aa 61 kDa	5.2–5.9	13	7.5	n.a.	[52,54]
Selenomonas ruminantium	360 kDa		n.a.	n.a.	2.2	8.0	[55]
Sporosarcina pasteurii KR133628	260 kDa $(\alpha\beta\gamma)_3$	γ 101 aa 11.1 kDa β 122 aa 14 kDa α 570 aa 61.4 kDa	4.6	17.3	8.0	4CEU	[56,57]
Staphylococcus leei	480 kDa $[(\gamma\beta\alpha)5$	γ 12 kDa β 21 kDa α 65 kDa	n.a.	1.66	n.a.	n.a.	[58]
Staphylococcus saprophyticus	427 kDa $(\gamma\beta\alpha)_4$	γ 13.9 kDa β 20.4 kDa α 72.4 kDa	4.7	9.5	6.0–7.0	n.a.	[59]
Staphylococcus xylosus X74600	300 kDa $(\alpha\beta\gamma)_3$	γ 16.3 kDa β 17.8 kDa α 64 kDa	4–5	n.a.	7.2	n.a.	[60]
Ureaplasma ureolyticum L40490	274 kDa $(\alpha\beta\gamma)_3$	γ 102 aa 11.2 kDa β 121 aa 13.6 kDa α 614 aa 66.6 kDa	5.0–5.2; 4.6	2.5	6.9–7.5	n.a.	[61–63]

[a] Regardless of the names given to urease's subunits in the initial or original reports, here the subunits were designated according to their homologous protein domains.

NH_3 and carbamate are released (Fig. 2E). The main controversy point was that while Benini et al., 1999 [65] proposed that the nucleophilic attack is performed by the bridging hydroxide which provides protons to the NH_3 group, Karplus et al., 1997 [64] argued that it is a His residue from the active site mobile flap that acts as a general acid for this protonation. As an alternative, Karplus et al., 1997 [64] also considered the monodentate binding of urea to Ni(1) with Ni(2) providing the water molecule as a nucleophile for the carbonyl carbon of urea. In addition to these two hypothesis, Estiu and Merz, 2007, based on simplified computer models for the active site, proposed that hydrolysis and elimination could occur competitively in ureases, in which a "protein-assisted elimination" would be favored [69].

To achieve full ureolytic activity, the active site of ureases needs prior insertion of nickel ions and also carbamylation of its lysine residue. In bacteria, four accessory proteins (UreD, UreF, UreG, and UreE) are involved in the assembly of urease's active metallocenter. For reviews on this topic see [13,70–73]. In bacteria, the urease genes *Ure*A, *Ure*B, and *Ure*C encoding the enzyme's subunits are grouped with genes for the accessory proteins *Ure*D, *Ure*E, *Ure*F, and *UreG*. In the case of *K. aerogenes*, these genes are organized in an *Ure*DABCEFG operon. Knockout and complementation studies of each accessory protein separately have shown that, *Ure*E as an exception, *Ure*D, *Ure*F and *Ure*G are crucial for the production of a fully activated "mature" urease [70,74,75].

The traditional model for urease activation starts with UreD, the first protein that binds to the apo-urease oligomer, and serves as a scaffold for the formation of the activation complex. Then UreF binds $(UreABC–UreD)_3$, and acts as a GTPase-activating protein, since its binding to $(UreABC–UreDF)_3$ correlates to the GTPase activity when further binding of UreG completes the activation complex. UreG, the first intrinsically disordered enzyme to be described [76,77], acts as a GTPase delivering energy for the urease maturation process. As GTP is hydrolyzed, the nickel-binding chaperone UreE delivers the metal ions to the $(UreABC–UreDFG)_3$ oligomer [76,77].

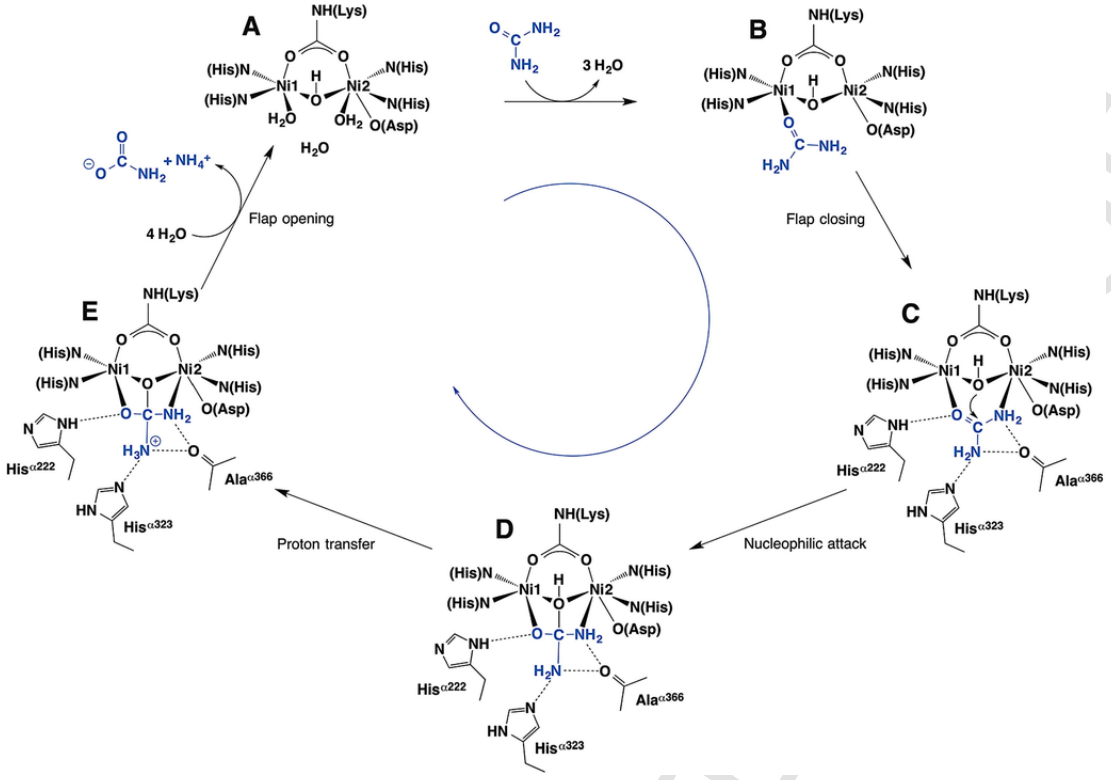

Fig. 2. Catalytic mechanism of ureases. Structure-based urease catalytic mechanism of the enzymatic hydrolysis of urea. The *Sporosarcina pasteurii* urease residue-numbering scheme is used. Please refer to the text for a stepwise description of the mechanism. Note that Ni(1) and Ni(2) are labeled Ni1 and Ni2 in this figure. Reproduced from [14] under permission from the Royal Chemical Society.

This model has been further refined with the increasing amount of structural information on individual urease accessory proteins [14]. In this new activation proposal, Ni^{2+}-bound UreE binds apo-UreG, facilitating GTP uptake by UreG (presence of Mg^{2+} ions is required), with Ni^{2+} ions being translocated from UreE to UreG. Then, the $(UreDF)_2$ complex competes with UreE for the Ni^{2+}-UreG to form the supercomplex apo-urease/Ni^{2+}-$(UreDFG)_2$. In the final step, $KHCO_3/NH_4HCO_3$ catalyzes GTP hydrolysis by UreG, thus completing urease activation. All urease accessory proteins are taken as metallochaperones that bind and/or transport nickel ions while driving the apo-urease into its fully active conformation. In plants and fungi, the functions of the bacterial UreG and UreE chaperones appear to be combined in a single UreG protein, which carries a histidine-rich domain with metal binding properties in its N-terminal segment [78,79]. The reason why eukaryotes lack UreE is still unknown [79].

The role of each accessory protein in the activation process has been a research hot topic in the last decade and there are some questions yet to be answered, mostly on the sequence of events and oligomerization state of each protein in the activation complex. The description at low resolution by small-angle X-ray scattering of the *K. aerogenes* $(UreABC–UreD)_3$ and $(UreABC–UreDF)_3$ oligomers started to uncover what the activation complex looks like [80]. Computational studies provided models of the activation complex [81]. The crystal structure of *H. pylori's* UreD-UreF-UreG complex revealed the presence of tunnels that span the entire length of both UreF and UreD, through which the delivery of nickel ions from UreG to the apo-urease could possibly occur [73,82].

Ureases inhibitors

Studies on urease's inhibitors have been carried out both to provide molecular insights on how the catalytic site machinery works as well as searching for effective inhibitors to counterbalance urease's catalyzed urea hydrolysis in a number of situations [83,84]. Urease inhibitors are a topic of intense investigation. The substrate urea, urea analogues and ammonium ions (products of urea hydrolysis), are weak inhibitors of urease [4]. Searching the Web-of-Sciences database (March 6th, 2018) for articles with "urease" in the title retrieved 4509 documents, 920 were found using "urease" and "inhibit∗" of which 413 were published since 2010. Please refer to the next section, "Biological roles of ureases", for more information on the importance of ammonia release by ureases.

An extensive and detailed review on the different classes of urease inhibitors can be found in [14]. Other articles on this special issue of *Journal of Advanced Research* deal in more details with urease inhibitors.

Sulfur compounds

Thiols, particularly β-mercaptoethanol, are of historic importance as urease inhibitors that, back in 1980, provided to B. Zerner's group crucial information on the active site of JBU [85]. Thiolate anions ($R\text{-}S^-$) inhibit ureases in a competitive manner. X-ray analysis of *S. pasteurii* urease complexed with β-mercaptoethanol (PDB code 1UBP) revealed its thiolate anion bridging the two Ni^{2+} ions in the active site and the hydroxy group further chelating the metallocenter [56]. Sulfite also acts as competitive pH-dependent inhibitor of urease [86].

Hydroxamic acids

Acetohydroxamic acid, the most studied derivative of this group of metal-binding compounds, acts as a urease slow-binding competitive inhibitor. It has been found interacting with the two nickel ions in the active sites of *S.(B.) pasteurii* (PDB code 4UBP), *H. pylori* (PDB code 1E9Y) and a mutated form of *K. aerogenes* (PDB code 1FWE) ureases [86]. So far, acetohydroxamic acid is the only urease inhibitor with therapeutic application to treat hyperammonemia in cirrhosis of *H. pylori* positive-patients [87] and it has been used to reduce urinary stones and treat urinary infections due to *Proteus mirabilis* infections [84,88]. However, this compound induces severe side effects, including teratogenesis, psychoneurological and muscular symptoms [89], which limit its use and caused its withdraw from the general market [84].

Phosphorous compounds

Amide and esters of phosphoric/thiophosphoric acids

Studies on phosphorus-based compounds as urease inhibitors started in the 1970s after the observation that some organophosphate-based insecticides inhibit soil urease [4,90]. In 1980, Dixon et al. described that phophoramidate inhibited JBU through its binding to the two nickels in the enzyme's active site [65,85]. Derivatives of phosphoric and thiophosphoric acid are potent inhibitors of urease [4]. A great number of derivatives have been developed and patented for potential application in infections by urease-producing pathogenic microorganisms [83] and in agriculture to avoid hydrolysis of urea used as fertilizer [84]. For all the derivatives of this class of inhibitors, the initial enzymatic hydrolysis of the molecule generates diamidophosphate, which is believed to be the actual urease inhibitor [14]. The main issue involving organophosphate inhibitors of urease is related to their low stability in acidic pH. To overcome this problem non-hydrolysable aminophosphinic acids have been developed [91,92].

Phosphate

Phosphate is a pH-dependent urease competitive inhibitor in the pH range 5.0–8.0, but negligible at pH higher than 7.5–8.0 [23,93]. X-ray diffraction structural data on phosphate-inhibited *S.(B.) pasteurii* urease inhibited with phosphate elucidated that the binding mode involves the formation of four coordinated bonds with both Ni ions in the enzyme's molecule [93]. It is a weak inhibitor compared to its amides (phosphoramidates) that rank among the most active urease inhibitors.

Fluoride

The mode of inhibitory action of fluoride, explored mostly using *S.(B.) pasteurii* urease, was described as a pH-sensitive mixed inhibition, which varies from a weak competitive mode in acidic medium to a stronger uncompetitive mechanism in alkaline conditions [57]. Five crystal structures of the enzyme in its fluoride-inhibited state were analyzed to establish that one fluoride ion binds to Ni(1) of the active site, while the nickel bridging hydroxide is replaced by another fluoride ion [57]

Quinones

Ubiquitous in the nature, quinones have bactericidal and antifungal activities, and participate of biologically relevant redox mechanisms. Quinones were described as urease inhibitors in the 1970s in studies of Bremner's group, pointing to 1,4-benzoquinone as a promising inhibitor of soil urease [94]. More recently, Krajewska's group reported on the kinetics of the inhibition of JBU by quinones, demonstrating a general slow-binding concentration-dependent mechanism indicative of a covalent modification of the conserved cysteine residue in the mobile flap of the active site. In addition to the covalent modification, quinones might inhibit urease through arylation and oxidation of its thiol groups [95].

Polyphenols

Catechol, the simplest molecule with a polyphenol scaffold, was shown to inhibit soil urease by Bremner and Douglas early in 1970s [94]. Plants are rich sources of polyphenolic compounds with antioxidant and bactericidal properties, generally regarded as beneficial for human health. For instance, polyphenols present in the green tea and other herbal beverages inhibited *H. pylori* urease (HPU) *in vitro* and reduced infection by *H. pylori* in Mongolian gerbils [96,97]. The mechanism of inhibition of urease by catechol is not yet fully understood. Current hypothesis are that inhibition by catechols could be due to a time dependent oxidation to ortho-benzoquinone which acts as the actual inhibitor by modifying protein's functional groups [98,99], and/or that polyphenols can coordinate with transition metals forming catechol–metal complexes, inactivating urease's metallocenter [100].

Other urease inhibitors

Although in most cases detailed structural data are not available, other classes of urease inhibitors are known, including boron-containing acids, citrates, and heavy metals. For a review on these topics see [4,14]. Heavy metals such as Hg, Ag, and Cu are slow reacting inhibitors of ureases [101,102]. Bismuth (Bi^{3+}) was shown to inactivate HPU by interacting with the cysteine residue of the mobile flap [103]. Due to the bactericidal activity, bismuth compounds have been widely used to treat gastric ulcers associated to *H. pylori* infection [104,105].

Biological roles of ureases that require ureolytic activity

Urease activity enables microorganisms to use urea as their sole nitrogen source. Urease synthesis may be constitutive or synthesized as a stress-related response of bacteria to counteract low environmental pH [106]. Ureolytic activity of the human gut microbiota hydrolyzes up to 30% of all urea produced in our bodies [107]. Microbial ureases are important also in dental health [108]. The production of alkali subsequent to salivary urea cleavage by oral microbiota urease was shown to inhibit dental cavities and plaque formation [109]. In ruminants, animal-derived urea is cleaved by bacterial ureases in the forestomach, releasing ammonia as nitrogen source for the rumen microbiota, which in turn serves as biomass to feed the animals [110,111].

Pathogenesis of many clinical conditions in humans and other animals are related directly to the ureolytic activity of bacterial or fungal enzymes [112,113]. Some examples are as follows. *Proteus mirabilis* is the most common organism that causes urinary stones in humans, due to urine alkalization promoted by its urease, contributing to the pathogenesis of pyelonephritis and catheter encrustation. Precipitation of urinary salts in the alkalinized urine results in struvite and carbonate apatite crystallization [114]. The bacterium *H. pylori* colonizes the stomach mucosa of half of the world's population, significantly increasing the risk of gastric ulcers and cancer [113,115,116].

HPU, which constitutes about 10% of the total cell protein, enables bacterial survival in the stomach by neutralizing the acidic medium [117]. Ureolytic organisms in the digestive or urinary tract potentially contribute to hepatic encephalopathy and coma resulting in hyperammonemia and brain intoxication [118]. Reduction of the ureolytic bacteria load and the use of acetohydroxamic acid as a urease inhibitor are considered therapeutic approaches under these conditions [119–121]. Other pathogens also produce urease to acquire acid resistance and enable colonization, among which are Shiga-toxin producing *Escherichia coli* [122], *Yersinia enterocolitica* [123], *K. pneumoniae* [124], *Brucella abortus* [125], and *Haemophilus influenza* [126]. Fungal ureases are involved in the pathogenesis of human cryptococcosis by *Cryptococcus neoformans* [127,128], and *Cryptococcus gattii* [35], and of coccidiodomycosis (San Joaquin Valley fever) by *Coccidioides immitis* and *Coccidioides posadasii* [37]. However, the role of microbial ureases as virulence factors has a still largely ignored contribution of non-enzymatic properties of these proteins, a subject that will be covered in the following section.

Urease is ubiquitous in plants and can be found in all vegetal tissues [129,130]. Nitrogen is a limiting element for plant growth, second only to carbon. Worldwide used as a soil fertilizer, urea is a relevant N source for plants, and dedicated urea transporters actively import this compound from the soil [131]. Urea hydrolysis to release ammonia and carbon dioxide is the main physiological role attributed to ureases in plants [130,132]. Urease is abundant in the soil, both in living bacteria and as extracellular urease, bound to clays and humic substances [133,134]. Ureolysis by cell-free ureases alkalinizes the soil inducing calcium carbonate precipitation and affecting the availability of minerals [135,136]. In addition to that, high levels of soil urease reduce the efficiency of urea fertilization leading to loss of ammonia into the atmosphere and ammonia-induced phytotoxicity [90,137]. The search for urease inhibitors with agricultural applicability to optimize urea fertilization is an intense field of investigation. These topics are broadly covered in other articles of this thematic issue of the *Journal of Advanced Research*.

Biological properties of ureases independent of ureolysis

Table 2 lists the biological properties of ureases found not to require ureolysis, either because urea is not available or its concentration is negligible, or the study employed ureases that were enzymatically incompetent (either with blocked active sites or in the inactive, nickel-deprived, apo-urease form).

Ureases play a role in cell-to-cell or organism-to-organism communication. Arginases with lectin properties from the lichens *Evernia prunastri* and *Xanthoria parietina* were shown to bind to a glycosylated urease in the cell wall of the homologous algae. The polygalactosylated urease is produced only in the season when the algal cells divide assuring recognition of the phycobiont by its fungal partner in the mutualistic association of these lichens [138,139].

Ureases were evaluated for a role in soybean nodulation by the diazotrophic bacterium *Bradyrhizobium japonicum* [140]. Soybean and jack bean ureases were characterized as chemotactic factors recognized by the bacterial cells *in vitro*. Independent of the urease status of the nodulating bacteria, urease-deficient mutant soybean plants had fewer but larger nodules when compared to the wild-type plant. Leghemoglobin production in wild-type plants was higher and peaked earlier than in urease-deficient plants, indicating a less efficient process of nitrogen fixation. Inhibition of urease activity in wild-type plants did not reproduce the results seen in mutated plants. These data made clear that soybean urease(s), but not the bacterial enzyme, participate(s) somehow of the plant-diazotrophic bacteria symbiosis. This role of the soybean urease does not require ureolysis and is relevant for biological nitrogen fixation by the plant [140].

Among microbial ureases that play a role as virulence factors, much attention is given to HPU because of its crucial role in the pathogenesis of gastric diseases. Production of urease proved to be essential to allow stomach colonization by *H. pylori*, however studies carried out in the early 1990s have shown that neutralization of gastric acidity is not the only function of the protein [141,142]. Following the steps of our previous observations made on ureases from jack bean (*C. ensiformis)* and from *S.(B.) pasteurii* (reviewed in [10] – see next sections), we have reported several other biological properties of the purified recombinant HPU, observed in the 10^{-6}–10^{-8} M range of protein concentration. These properties include induction of lipoxygenase-dependent activation and aggregation of rabbit [143] and human platelets [144]; induction of lipoxygenase-dependent chemotaxis and ROS production in human neutrophils [145]; delaying apoptosis in human neutrophils [145] and in gastric epithelial cells [146]; increase of the lipoxygenase content in neutrophils [145]; induction in

Table 2
Ureolysis-independent biological properties of selected ureases and urease-derived peptides.

Ureases and derived-peptide	Entomotoxic properties	Antifungal activity	Mammal neurotoxicity	Exocytosis in platelets	Eicosanoid signaling	Chemotactic activity
PLANTS						
CNTX	✓	✓	✓	✓	✓	✓
JBU	✓	✓	✓	✓	✓	✓
eSBU	✓	✓	n.d	✓	n.d	✓
uSBU**	✓	✓	n.d	✓	n.d	n.d.
GHU	n.d	✓	n.d	n.d.	n.d	n.d.
PPU	✓	✓	n.d	n.d.	n.d	n.d.
BACTERIA						
SPU	?	n.d	?	✓	✓	n.d.
HPU**	?	✓	✓	✓	✓	✓
PMU**#	✓	✓	n.d	✓	n.d.	n.d.
UREASE-DERIVED PEPTIDES						
JBTX**	✓	✓	?	?	✓	n.d.
SYTX**#	✓	✓	?	n.d.	n.d.	n.d.

CNTX, canatoxin (*C. ensiformis*); JBU, jackbean urease (*C. ensiformis*); eSBU, embryo-specific soybean urease (*G.* max); uSBU, ubiquitous soybean urease; GHU, *Gossypium hirsutum* (cotton) urease; PPU, pipeon pig urease (*C. cajan*); SPU, *Sporosarcinapasteurii* urease; HPU, *H. pylori* urease; PMU, *Proteus mirabilis* urease; BJU, *B. japonicum* urease; JBTX, jaburetox; SYTX, soyuretox.
✓ presence of biological activity; ? absence of biological activity; ** Recombinant protein; n.d. not determined; # unpublished result.

platelets of the production of lipoxygenase-derived eicosanoids [143]; promotion of angiogenesis in human umbilical endothelial cells and in the chicken embryo chorioallantoic membrane model [146]; and induction of processing of pre-mRNA encoding pro-inflammatory cytokines in human platelets [144]. Most of these effects are also displayed by an enzyme-inhibited HPU, while some are induced by one of its isolated subunits alone [144], indicating that these biological effects do not require urea hydrolysis. Other groups also reported biological roles of HPU that are carried out by one of its subunits, implying absence of ureolysis. HPU's subunit B was shown to bind to Th17 lymphocytes [147] and to CD74 on gastric epithelial cells thereby eliciting production of IL-8 [148]. HPU's subunit A contains a nuclear localization signal (sequence $_{21}$KKRKEK$_{26}$), and it was found in the nuclei of COS-7 cells [149,150] and AGS gastric epithelial cells, inducing alterations in the cells' morphology [150].

Altogether these non-enzymatic biological effects of HPU point out to a relevant contribution (yet mostly ignored) of this protein to the inflammatory process that underlies the gastric diseases caused by *H. pylori*. Because HPU activates non-gastric cells such as platelets, neutrophils, endothelial cells, among others, it may contribute as well to the pathogenesis of extragastric illnesses, in particular cardiovascular diseases. Probably none of the future urease inhibitors that are being conceived or are presently under development will have any use to counteract HPU's pro-inflammatory effects or other unwanted contributions of this protein that are not due to its ureolytic activity. Thus, there is an urgent need to understand the structural basis of the non-enzymatic biological properties of HPU, and of other microbial ureases with relevant roles as virulence factors, aiming the design of drugs that could specifically block these other activities. Such new urease inhibitors could be used alone or together with ureolysis inhibitors, to target all the noxious effect of ureases involved in pathogenesis.

Neurotoxicity of ureases

The discovery of the non-enzymatic properties of ureases is closely related to the study of their neurotoxicity, both in rodents and in insects. Canatoxin (CNTX) is an isoform of *C. ensiformis* urease, first isolated from the plant seeds as a neurotoxic protein causing convulsions and death of rats and mice, with an $LD_{50} \sim 2$ mg/kg, given by intraperitoneal route [151]. Two decades after the isolation of CNTX, it became evident that the neurotoxic protein is actually an isoform of the most abundant urease (JBU) found in the same seeds [9]. Canatoxin is a non-covalent dimer of ~95 kDa subunits with one zinc and one nickel atom per subunit [9,12] what probably explains its lower ureolytic activity. CNTX and JBU differ in one order of magnitude in their sensitivity to the irreversible inhibitor *p*-hydroxy-mercurybenzoate (*p*HMB), an oxidant of thiol groups [9] and in their metal-binding affinities [152].

Studies on CNTX have indicated that its primary mechanism of action at the cellular level is to induce exocytosis, triggering a signaling pathway that characteristically involves eicosanoids derived from the lipoxygenases pathway (reviewed in [10]). This biological property of CNTX was reported in a number of mammalian models, both *in vivo* and *in vitro*, among which are blood platelets and rat brain synaptosomes. The aggregating activity of CNTX in rabbit, rat, guinea pig or human platelets occurs in the nanomolar range [153]. CNTX-activated platelets recruit a lipoxygenase-mediated pathway that leads to influx of external Ca^{2+} through opening of voltage-gated Ca^{2+} channels and without release of intracellular [Ca^{2+}] pools. The increased cytoplasmic [Ca^{2+}] triggers exocytosis of platelet granules that contain ADP, which in turn induces the aggregation response [153,154]. Later the ability to induce platelet aggregation was reported for JBU [9], the embryo-specific [155] and the ubiquitous [156] isoforms of soybean ureases, *B.*(*S.*) *pasteurii* urease [155,157], and HPU [143], thus it is a property common to one-, two-, and three-chained ureases.

The observations that *p*HMB-treated CNTX, in which the ureolytic activity is irreversibly blocked, was still lethal to mice and still able to promote platelet aggregation set the ground for the discovery of the non-enzymatic biological properties of ureases [9]. In the following two decades, a lot more of ureolysis-unrelated effects were described for *C. ensiformis* ureases as well as for ureases from other sources (reviewed in [10]).

The exocytosis inducing effect of CNTX was later characterized in rat brain synaptosomes, which responded dose-dependently to the neurotoxin by releasing neurotransmitter vesicles previously loaded with radiolabeled serotonin or dopamine. At 500 nM CNTX, the amount of neurotransmitter released from the synaptosomes was similar to that obtained by depolarization with 50 mM KCl [158]. The ability of CNTX to promote secretion in synaptosomes correlates with the neurotoxicity it induces *in vivo* in mice and rats. The medullar origin of CNTX-induced seizures and other CNS-related effects were described in rodents [159].

More recent data have shown that JBU (10–100 nM) induces Ca^{2+} events in cultured rat hippocampal neurons, an effect also observed for HPU (Piovesan, A.R., unpublished results). In patch clamp experiments, it was observed that JBU increases the frequency of spontaneous firing action potentials in cultured rat hippocampus neurons, rising the amplitude of sodium currents, and apparently not affecting potassium currents. A higher frequency of spontaneous excitatory post synaptic currents was also seen, consistent with a seizure-like activity (Dal Belo, C. A., unpublished data). Studies using microPET (Positron Emission Tomography) indicated an increase of ~30% in the uptake of ^{18}Fluor-desoxy-glucose in the brain of CNTX-treated anaesthetized rats, particularly affecting the hippocampus, a typical finding for seizure-inducing drugs (De Almeida, C.G.M., unpublished results).

Similar to our observations, JBU had been previously reported to be lethal and to produce seizures in mice and rabbits after intravenous administration [160]. Likewise, purified HPU was shown to kill mice upon intraperitoneal injection, producing hypothermia, convulsions and death [161]. In both studies, the neurotoxicity of the ureases was attributed to the high levels of ammonia found in the animal's blood. Although hyperammonemia probably contributes to the neurotoxic effects induced by CNTX in mice and rats, surely it does not tell the whole story, considering that *p*HMB-treated CNTX still caused neurotoxic symptoms and seizures leading to death of the animals [9].

Contributions of ureases to plant defense against predators and pathogens

The first description of the insecticidal effect of a urease was published in 1997 showing that ingestion of CNTX killed insects [162]. The susceptibility of the insects to CNTX's lethal effect depended on the type of their digestive enzymes. Insects with acidic midguts and cathepsin-like proteinases, like the cowpea weaver *Callosobruchus maculatus* (Bruchidae) and the kissing bug *Rhodnius prolixus* (Hemiptera), were susceptible to CNTX while insects with alkaline midguts and trypsin-like enzymes were not. These data were interpreted as evidence for the need of proteolytic activation of CNTX that, once ingested, is hydrolyzed by insect cathepsin-like enzyme(s) releasing an internal peptide(s) with insecticidal activity. In fact preventing CNTX hydrolysis by adding a cathepsin B inhibitor simulta-

neously with the toxin in the insects' diet protected them against the lethal effect [162]. In the following years we described that JBU/CNTX and the embryo specific soybean urease were insecticidal against the hemipterans *Nezara viridula* [163], *Dysdercus peruvianus* [155,164], *Oncopeltus fasciatus* [165], and K. Ponnuraj's group in India reported the insecticidal effect of the pigeon pea urease (*Cajanus cajan*) against *Callosobruchus chinensis* [25].

The proteolytic activation of CNTX by insect cathepsin-like enzymes was further investigated. Insecticidal peptides were isolated from CNTX's fragments after digestion with *C. maculatus* enzymes [166]. The most active peptide, pepcanatox, with a molecular mass of ~10 kDa had its N-terminal sequence determined and, based on this information, a recombinant peptide named jaburetox was obtained by heterologous expression in *E. coli* [167]. Cathepsin D-like enzymes from *D. peruvianus* midgut that were able to perform hydrolysis of CNTX/JBU and release the insecticidal peptide were characterized [164,168,169]. A similar study was performed with JBU and the milkweed bug *Oncopeltus fasciatus,* identifying a cathepsin L that hydrolyzed the urease to release a ~10 kDa entomotoxic peptide [165].

The recombinant peptide jaburetox was cloned using as template the cDNA of JBURE-II, a third isoform of urease found in *C. ensiformis* [170,171]. Based on jaburetox's sequence, a recombinant insecticidal peptide called soyuretox was produced [172] having as template the cDNA of the ubiquitous soybean urease which, like the embryo-specific urease, also kills *R. prolixus* [156]. Interestingly, the region that encompasses the jaburetox/soyuretox sequence, comprising about 90 amino acid residues, displays a lower similarity when compared to that of the complete sequence of different ureases, suggesting less evolutionary pressure to conserve this entomotoxic "domain" of plant ureases [15,167].

But the proteolytic release of entomotoxic peptides does not tell the whole story of urease's entomotoxicity. Evidences showing that the entire urease molecule is entomotoxic *per se* started to add up with studies on the anti-diuretic effect of *C. ensiformis* ureases. In Carlini et al., 1997 [162], we showed that CNTX produced an important anti-diuretic effect in *R. prolixus* that peaked about 4 h after the insects received the "meal" containing the toxin, disappearing after 24 h. However, the hydrolysis of CNTX in the insect midgut was not detected before 18 h, suggesting that the anti-diuretic effect was produced by the entire protein. Later, JBU and the jaburetox peptide were shown to cause anti-diuresis in *R. prolixus'* isolated Malpighian tubules in the concentration range of 10^{-10} and 10^{-15} M, respectively [173]. Surprisingly, although both molecules induced antidiuretic effects, JBU and jaburetox triggered different signaling pathways leading to antidiuresis [173]. In the following years other papers were published by our group describing a list of entomotoxic effects of JBU, some of which are not shared with jaburetox, such as alteration in water transport and of the contractility in the crop of *R. prolixus* [174]. Similar to the data indicating recruitment by ureases of eicosanoid-mediated pathways in mammalian systems (reviewed in [10]), JBU effects in insects required a phospholipase A_2 type XII [175] and prostaglandins [176]. JBU and jaburetox targeted the immune system of *R. prolixus*, inducing an eicosanoid-dependent aggregation of hemocytes and alterations in cell morphology [176,177] that render the insect more susceptible to entomopathogenic bacteria [177].

Both JBU and jaburetox are neurotoxic to insects from different orders. Jaburetox was immunolocalized in the brain of *Triatoma infestans* (Hemiptera) and neurotoxic symptoms preceded death of the insects injected with the peptide [178]. JBU-induced effects were studied in the cockroach *Nauphoeta cinerea* (Blatodea) revealing that both, the central and the peripheral nervous systems are targeted by the urease, with alterations of the cholinergic, octopaminergic and GABA-ergic pathways as part of its entomotoxic mode of action [179]. The effects of JBU were also investigated on neuromuscular junctions of *Locusta migratoria* (Orthoptera) and of *Drosophila melanogaster* (Diptera), and the resulting data pointed to interference of JBU on neurotransmitter release, probably by disruption of the calcium machinery in the pre-synaptic region of insect neurons [180].

Previous studies with *B.*(*S.*) *pasteurii* urease suggested lack of insecticidal properties for microbial ureases, which was attributed to the absence of part of jaburetox's sequence in those proteins [155]. However, later reports on insecticidal activity of ureases of bacteria from *Photorhabdus* and *Xenorhabdus* genera [181], *Yersinia pseudotuberculosis* [182] and *Proteus mirabilis* (Broll, V. et al., unpublished results) indicated that bacterial ureases are indeed entomotoxic and insecticidal, in agreement to the fact that ureases contain other entomotoxic domains besides the sequence corresponding to jaburetox.

Ureases are toxic against filamentous fungi and yeasts [183]. The fungitoxic activity of CNTX was the first reported showing that the protein at 2% concentration caused growth inhibition of the phytopathogenic filamentous fungi *Macrophomina phaseolina, Colletotrichum gloesporioides* and *Sclerotium rolfsii* [184]. Becker-Ritt et al., 2007, reported that JBU and the soybean embryo-specific ureases inhibited growth and/or spore germination of seven other species of filamentous fungi at sub-micromolar concentrations and caused damage to cell wall, even after blockage of their ureolytic active sites. In this same study, the two-chained HPU also inhibited fungal growth although with less efficiency [185]. The native ureases of cotton seeds (*Gossypum hirsutum*) [30] and of pigeon pea [25], and the recombinant non-ureolytic apoureases, JBURE-IIb [171] and a ubiquitous soybean urease fused to glutathione transferase [156], were also shown to be detrimental to filamentous fungi.

In Postal et al., 2012, JBU was tested in the 10^{-6}–10^{-7} M range against different yeast species and caused inhibition of proliferation and of glucose metabolism, morphological alterations with pseudohyphae formation, and cell membrane permeabilization, eventually leading to cell death [186]. Jaburetox induced similar effects against the yeasts but at one to two orders of magnitude higher doses. Studies with peptides from a papain-hydrolyzed JBU indicated the presence of other fungitoxic domains in the protein, besides jaburetox [186]. Soyuretox, a peptide derived from the soybean ubiquitous urease, is also fungitoxic in the same concentration range as observed for jaburetox [172]. Detached leaves of "urease-null" soybean transgenic plants, due to co-suppression of ureases genes, and infected with uredospores of the Asian rust fungus *Phakopsora pachyrhizi* developed more lesions and pustules when compared to leaves of wild plants with normal levels of ureases, suggesting a protective role of ureases against fungal diseases in the wild plants [187].

Interestingly, a non-catalytical urease was identified in the soybean genome. This urease lacks critical features of the enzyme's active site, but it is expressed in various plant tissues [188], reinforcing the multifunctional characteristics of the protein, especially when related to plant defense. It is tempting to predict that more of these non-catalytical ureases will be found as more plant genomes are decoded. Altogether these data suggests that urease-overexpressing plants or transgenic plants jaburetox/soyuretox may represent alternatives to achieve resistance to insect herbivory and/or fungal disease in agriculture. In this context it is important to mention that ureases can be generally regarded as biosafe proteins, which are present in relatively large quantities in most edible plants and are particularly abundant in seeds of legumes and in fruits such as tomatoes, melon, and watermelon, that are eaten in raw state [129,132]. Although more studies

are needed to ascertain the biosafety of urease-derived peptides, no acute toxicity was detected for jaburetox given in high doses either injected or by oral route to mice and neonate rats [167]. Preliminary data obtained for soyuretox in the zebra fish (*Danio rerio*) model indicated toxicity only in the highest tested doses (Kappaun, K. et al., unpublished results).

Structural aspects of jaburetox

Models of the tridimensional structure of jaburetox [167,189] indicated the existence in the C-terminal half of the peptide of a prominent β-hairpin motif, a feature that could be related to a pore-forming activity eventually leading its neurotoxicity. A β-hairpin in the region of JBU corresponding to jaburetox was found in its crystallographic structure [22]. Aiming to carry out structure versus activity studies on jaburetox, three mutants corresponding to truncated versions of the peptide were obtained: Jbtx Δ−β, which lacked the β-hairpin motif (residues 61–74 deleted); Jbtx N-ter (residues 1–44), corresponding to the N-terminal half; and Jbtx C-ter (residues 45–93), corresponding to the C-terminal half of jaburetox [190]. In insect bioassays, the Jbtx Δ−β peptide kept the entomotoxic properties of the whole peptide, clearly indicating that the β-hairpin motif is not required for the insecticidal effect. On the other hand, while Jbtx N-ter remained entomotoxic, the Jbtx C-ter peptide, which contains the β-hairpin motif, was less active or inactive when tested on two different insect models. The data support the conclusion that the N-terminal half of jaburetox carries its most important entomotoxic domain [190].

Molecular dynamics studies employing long simulations of jaburetox in aqueous medium suggested that the peptide becomes largely unstructured after 500 ns, more accentuated in its N-terminal domain, while the initial structure observed for its moiety in JBU's crystals is completely lost [190]. Subsequently light scattering, circular dichroism and nuclear magnetic resonance spectroscopy studies of jaburetox in solution determined that it is an intrinsically disordered polypeptide [191]. Regions of jaburetox which exhibited tendency to form one small alpha-helix close to the N terminus, and two turn-like motifs, in the central portion and close to the C terminus, respectively, were predicted as sites of potential interaction with other proteins or lipids, suggesting that upon such interactions structural changes could be triggered to drive the peptide into a biologically active conformation [191]. The solution structure of soyuretox was determined using the same methodologies and revealed its intrinsically disordered nature, although with more secondary structure elements when compared to jaburetox (Kappaun, K. et al., unpublished results).

Interaction of ureases and urease-derived peptides with lipids and membranes

The interaction of jaburetox with lipid membranes was first reported by Barros et al., 2009 [189]. In this study, jaburetox was shown to cause leakage of carboxyfluorescein entrapped inside large unilamellar vesicles, without lysis of the liposomes. The leakage was greater in vesicles composed by acidic lipids and depended on the state of aggregation of jaburetox. Molecular dynamics applied to jaburetox suggested that its β-hairpin motif could anchor at polar/non-polar interfaces [189]. However, as mentioned earlier, even if the β-hairpin does interact with insect membranes, it is not essential for the entomotoxic properties of jaburetox. Moreover all three truncated versions of jaburetox developed by Martinelli et al., 2014, disrupted liposomes, revealing the presence of more than one lipid interacting domain in the peptide [190].

In another study, JBU, jaburetox and its mutated peptides were tested for an ion channel forming activity in planar lipid bilayers [192]. All proteins formed well resolved, highly cation-selective channels exhibiting two conducting states (7–18 pS and 32–79 pS, respectively). Urease (20 nM) and Jbtx N-ter (1 μM) were more active at negative potentials, while the channels formed by the other peptides were not voltage-dependent. This study was the first direct demonstration of the capacity of *C. ensiformis* urease and jaburetox to permeabilize membranes through an ion channel-based mechanism, which may be the basis of their diverse biological activities. Molecular models of JBU showed that the moiety corresponding to jaburetox is well exposed at the protein's surface, from where it can probably "enforce" the interaction of the entire urease with lipid bilayers, a hypothesis formulated to explain why the polypeptides share many, although not identical, biological properties [192].

To elucidate whether an interaction with lipids could induce conformational changes in the intrinsically disordered molecule of jaburetox, the structural behavior of the peptide was probed using nuclear magnetic resonance and circular dichroism spectroscopies when in contact with membranes models [193]. The interaction of jaburetox with SDS micelles increased its content of secondary and tertiary structure elements. When exposed to large unilamellar vesicles and bicelles prepared with phospholipids, conformational changes were observed mostly in N-terminal regions, but without significant acquisition of secondary structure motifs. Fluorescence microscopy was used to demonstrate that the lipid vesicles could displace the interaction of jaburetox with lipid-rich membranes of the cockroach nervous chord. These data suggested that contacts of the N-terminal moiety of jaburetox with membrane phospholipids lead to its anchorage to cell membranes and promote conformation changes of jaburetox into a more ordered structure that could facilitate its interaction with membrane-bound target proteins [193].

Further studies aiming to elucidate the mechanism of interaction of JBU and jaburetox with lipid membranes were carried out using multilamellar liposomes with a lipid composition simulating that of human platelets, subjected to dynamic light scattering and small angle X-rays scattering (SAXS) analyses [194]. Results were obtained indicating that both JBU and jaburetox are able to insert themselves into the lipid bilayers, reducing the hydrodynamic radius of the vesicles, altering the lamellar repeat distance, the number of lamellae, and decreasing the membrane's fluidity. The interaction of jaburetox affected the vesicle's internal bilayers and caused more drastic effect on the multilamellar organization of the liposomes than did JBU. In the same study, the interaction of JBU with giant unilamellar vesicles (GUVs) made of fluorescent phospholipids showed that JBU caused membrane perturbation with formation of tethers. The date reinforced the idea that JBU can interact with multilamellar liposomes, probably by inserting its jaburetox "domain" into the vesicle's external membrane [194].

Concluding remarks

While the history of research on urease as an enzyme is almost 150 years old, dating back to the 1870s, the knowledge that ureases perform other biological roles unrelated to ureolysis is considerably younger, not 50 years yet. An unbiased view of these molecules as more than enzymes is needed to allow discovery of yet unsuspected biological properties of ureases. Finding ureases in sources little explored so far, deciphering the structural characterization of a broad range of ureases including non-ureolytic proteins, and to investigate a potential synergy between catalytic and non-catalytic properties of

ureases, are only a few of the open fields in the study of these enzymes.

There are many proposed technological application of ureases [195–200] all of which explore exclusively the enzyme's catalytic activity. There are though certainly much more to be explored with these proteins, starting with the biotechnological use of ureases (and of urease-derived peptides) as transgenes to protect crops against insect herbivory and disease-causing fungi, or as ecofriendly insecticides to control insect borne diseases. The comprehension of ureases as virulence factors not only as an ammonia-producing and alkalinizing agent but acting in a much more complex way, endowed of exocytosis-inducing and pro-inflammatory activities, and recruiting the participation of eicosanoids, may through lights to ways of finding new pharmacological approaches to many pathologies.

Finally, with this review, apart from historical and structural aspects of ureases, we wanted to encourage the readers to take a second look at ureases, very versatile proteins that happen also to catalyze the breakdown of urea into ammonia and carbamate.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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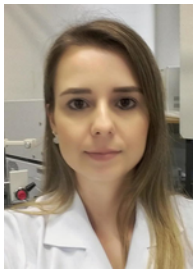

Karine Kappaun is currently PhD student at Laboratory of Neurotoxins at Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). She has a bachelor degree in Pharmacy at Universidade Federal do Rio Grande do Sul (2011) and Master degree in Cellular and Molecular Biology (2014), in the same University. During her graduate program she studied the properties and structural properties of the urease-derived peptides jaburetox and soyuretox, having been abroad at Universitá di Bologna at Bologna, Itália (2015–2016). Nowadays, she is focused on the understanding the neurotoxic effects of ureases in zebrafish.

Angela Regina Piovesan is presently a postdoctoral fellow of the Biotechnology Center, Federal University of Rio Grande do Sul (UFRGS) and also works at Laboratory of Neurotoxins of the Brain Institute, Pontifical Catholic University of Rio Grande do Sul, both located in Porto Alegre, Brazil. She has a bachelor degree in Biology and experience on biochemical characterization of proteins and enzymes. Nowadays, she is involved in the study of neurotoxic effects of ureases applying several approaches, such as neuronal calcium images and electrophysiological recordings. Besides that, she also manages some undergraduate students in their laboratory tasks.

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Rodrigo Ligabue-Braun is currently a research assistant at the Biotechnology Center of Universidade Federal do Rio Grande do Sul UFRGS), Brazil. Employing a wide range of computational approaches, his research topics are chiefly focused on neglected toxins, moonlighting properties of proteins, and the effect of unstructured or metastable regions of polypeptides on the structure-function paradigm. His work on venomous mammals has attracted readers both from the toxinology community and the general public alike. Aside from research, Dr. Ligabue-Braun has published chapters of textbooks in Brazil and also coordinated science outreach programs aiming low-income students from local communities.

Curriculum Vitae resumido

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Formação acadêmica/titulação

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Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2012 - 2014 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Clonagem e expressão da urease da bactéria diazotrófica*Azospirillum brasilense* FP2 e do peptídeo soyuretox, derivado da urease ubíqua de soja (*Glycinemax*), Ano de obtenção: 2014
Orientador: Célia Regina Ribeiro Da Silva Carlini
Co-orientador: Irene Silveira Schrank
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2006 - 2011 Graduação em Farmácia.

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Estudos com o Jaburetox: efeito tóxico de *E. coli* liofilizadas carregadas com o peptídeo e análise da influencia do epitopo V5 na formação de agregados
Orientador: Célia Regina Ribeiro da Silva Carlini
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Formação complementar

2015 - 2015 Curso de curta duração em Biomolecular NMR Spectroscopy. (Carga horária: 20h).
Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Porto Alegre, Brasil

2015 - 2015 Curso de curta duração em Biological Roles ofmetals. (Carga horária: 20h).
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Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

2012 - 2014 Vínculo: Bolsista, Enquadramento funcional: Mestrado, Regime: Dedicação exclusiva

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Outras informações:

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Produção bibliográfica

Artigos completos publicados em periódicos

1. **KAPPAUN, K.**; PIOVESAN, A. R.; LIGABUE-BRAUN, R.; CARLINI, CÉLIA R. Ureases: Historical aspects, catalitic and non-catalitic properties: A review. JOURNAL OF ADVANCED RESEARCH. , 2018.

2. MARTINELLI, ANNE H.S.; LOPES, FERNANDA C.; BROLL, VALQUIRIA; DEFFERRARI, MARINA S.; LIGABUE-BRAUN, R.; **KAPPAUN, K**.; TICHOTA, DEISE M.; FRUTTERO, LEONARDO L.; MOYETTA, NATALIA R.; DEMARTINI, DIOGO R.; POSTAL, MELISSA; SILVA, MONICA M.; Becker-Ritt, Arlete Beatriz; PASQUALI, GIANCARLO; CARLINI, CÉLIA R.
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3. MARTINELLI, ANNE H.S.; **KAPPAUN, KARINE**; LIGABUE-BRAUN, RODRIGO; DEFFERRARI, MARINA S.; PIOVESAN, ANGELA R.; STANISÇUASKI, FERNANDA; DEMARTINI, DIOGO R.; DAL BELO, CHARISTON A.; ALMEIDA, CARLOS G.M.; FOLLMER, CRISTIAN; VERLI, HUGO; CARLINI, CELIA R.; PASQUALI, GIANCARLO Structure-function studies on jaburetox, a recombinant insecticidal peptide derived from jack bean (Canavalia ensiformis) urease. Biochimica et Biophysica Acta. G,

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2. LOPES, F. C.; MARTINELLI, A. H. S.; POSTAL, M.; KAPPAUN, K.; TICHOTA, D. M.; Pasquali, G.; BRANDELLI, A.; Carlini, C.R.
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XIV Reunião Anual do Programa de Pós-Graduacao em Biologia Celular e Molecular. , 2012.

3. KAPPAUN, K.; MABONI, F.; SCHRANK, I. S.; Carlini, C.R.
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4. MARTINELLI, A. H. S.; KAPPAUN, K.; POSTAL, M.; PIOVESAN, A. R.; Pasquali, G.; Carlini, C.R.
Jaburetox-2Ec, peptídeo recombinante derivado da urese: estudo de mutagênese sitio-dirigida In: XIV Reunião Anual do Programa de Pós-Graduacao em Biologia Celular e Molecular, 2012, Porto Alegre, RS.
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Estudo da estabilidade dos peptideos derivados da urease de C*anavalia ensiformis*: Jaburetox-2Ec e Jaburetox-V5 In: XXIII Salão de Iniciação Científica UFRGS, 2011, Porto Alegre, RS.
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7. KAPPAUN, K.; MARTINELLI, A. H. S.; Pasquali, G.; Carlini, C.R.
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9. Becker-Ritt, A.B.; MARTINELLI, A. H. S.; KAPPAUN, K.; Carlini, C.R.
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Expressão de ureaseubiqua de soja em *Escherichia coli* In: XX Salão de Iniciação Científica UFRGS, 2008, Porto Alegre.
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Expression ofsoybeanubiquitousurease in *Escherichia coli* In: XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular,, 2008, Águas de Lindóia. **Livro de resumos**. , 2008.

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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.
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Orientações e supervisões em andamento

Iniciação científica

1. Lucas Emanuel Stein. **Estudo de ureases em zebrafish**. 2017. Iniciação científica (Ciências Biológicas) - Pontifícia Universidade Católica do Rio Grande do Sul

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