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Ureases de plantas e de bactérias: estudos funcionais e propriedades biológicas independentes da atividade enzimática.

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RESUMO

Ureases são enzimas altamente homólogas, encontradas em plantas, bactérias e fungos. A urease de feijão de porco, *Canavalia ensiformis*, foi a primeira enzima a ser cristalizada e também a primeira cuja presença de níquel foi demonstrada. Canatoxina, uma isoforma da urease de *C. ensiformis*, apresenta diversas atividades biológicas, além de sua atividade ureolítica: 1) atividade inseticida; 2) efeito secretagogo e pró-agregante em plaquetas de coelho; 3) ligação a glicoconjugados que contém ácido siálico; 4) atividade pró-inflamatória. Esses efeitos são independentes de sua atividade hidrolítica sobre a uréia, e envolvem ativação do metabolismo de eicosanóides e canais de cálcio. Outras ureases compartilham algumas atividades biológicas da canatoxina, como indução de agregação plaquetária por ureases de vegetais e de *Bacillus pasteurii*, e efeito inseticida, uma propriedade só encontrada para as ureases de plantas. Ureases microbianas contribuem para a patogênese de cálculos urinários, pielonefrites, incrustação de cateter, úlcera péptica e, possivelmente, tumores gástricos. *Helicobacter pylori*, uma bactéria Gram-negativa que coloniza a mucosa gástrica, causa úlcera péptica e carcinoma gástrico distal por um mecanismo ainda não completamente elucidado até o momento. Neste trabalho ureases de bactérias são estudadas, objetivando investigar se elas apresentam alguns dos efeitos biológicos descritos para a canatoxina. Foi demonstrado que as ureases recombinante de *H. pylori* e nativa de *B. pasteurii* induzem agregação de plaquetas de coelho por um mecanismo, similar ao da canatoxina, mediado por eicosanóides derivados da via da lipoxigenase. Os efeitos pró-inflamatórios da urease de *H. pylori* são também similares ao apresentado pela canatoxina e mediados pelo mesmo mecanismo de sinalização. Esses resultados contribuem para o entendimento da fisiopatologia de distúrbios causados por organismos produtores de urease.

ABSTRACT

Ureases are highly homologous enzymes found in plants, bacteria and fungi. Urease of the jackbean, *Canavalia ensiformis*, was the first enzyme ever crystallized and also the first enzyme shown to contain nickel. Canatoxin, an isoform of *C. ensiformis* urease, presents several other biological effects besides its ureolytic property: 1) insecticidal effect; 2) pro-aggregating activity in rabbit platelets; 3) binding to sialic acid-containing glycoconjugates; 4) pro-inflammatory activity. These effects are independent of urea hydrolysis and require activation of the eicosanoid metabolism and calcium channels. Other ureases have some of biological activities described for canatoxin, such as induction of platelet aggregation by jackbean and soybean ureases, and also *Bacillus pasteurii* urease, while insecticidal effects were seen only for plant-derived ureases. Microbial ureases play a role in the pathogenesis of urinary stones, pyelonephritis, urinary catheter incrustation, peptic ulceration and possibly in gastric tumors. *Helicobacter pylori*, a Gram-negative bacterium that colonizes the human stomach mucosa, causes gastric ulcers and cancer through a mechanism not yet fully elucidated. In this work we studied bacterial ureases to find out if they present some of the biological properties described for canatoxin. We demonstrate that a recombinant *Helicobacter pylori* urease and a native *Bacillus pasteurii* urease induce aggregation of rabbit platelets by a mechanism similar to that recruited by canatoxin, involving mediation by lipoxygenase-derived eicosanoids. *H. pylori* urease also induces pro-inflammatory effects similar to those presented by canatoxin and are mediated by the same signalling pathway. These findings may contribute to the enlightening of physiopathology of diseases promoted by urease-producing bacteria.

Lista de Abreviaturas

3D	tridimensional
ADP	Difosfato de adenosina
AMP	Monofosfato de adenosina
BPU	urease de *Bacillus pasteurii*
Cag A	Antígeno de Citotoxicidade A
CNTX	Canatoxina
DNA	Ácido desoxirribonucléico
DPA-Sepharose	Dipicolilamina-Sepharose
EC_{50}	Concentração efetiva para 50%
ED_{50}	Dose efetiva para 50%
HPU	Urease de *Helicobacter pylori*
IC_{50}	Concentração inibitória em 50%
IDA-Sepharose	Iminodiacetato-Sepharose
IMAC	Cromatografia de afinidade em metal imobilizado
iNOS	Óxido nítrico sintase indutível
JBU	Urease de *Canavalia ensiformis*
kDa	quilo daltons
Km	Constante de Michaelis-Menten
LD_{50}	Dose letal para 50%
PAF-Acéter	Fator de agregação plaquetária
PAI	Ilha de patogenicidade
PGE_2	Prostaglandina E_2
pH	Potencial hidrogeniônico
p-OHMB	p-hidroximercuribenzoato

SBU	Urease de *Gycine max*
SDS-PAGE	Eletroforese em gel de poliacrilamida com SDS
SDS	Dodecil sulfato de sódio
U/mg	Unidade de atividade enzimática por miligrama
Vac A	Citotoxina vacuolizante A
Vmáx.	Velocidade máxima da enzima
WEB2170	Bepafant; or 5-(2-chloro-phenyl)-3,4-dihydro-10-methyl-3-[(4-morphol- inyl) carbonyl]-2H, 7H-cyclopenta (4,5) thieno [3,2-*f*] [1,2,4 triazolo-[4,3-*a*] [1,4] diazepine])

1. INTRODUÇÃO

1.1. Ureases

Ureases (EC 3.5.1.5) são metalo-enzimas níquel dependentes que catalisam a reação de hidrólise de uréia à amônia e CO_2. A atividade catalítica das ureases está intimamente ligada à presença de dois átomos de Ni^{2+} no sítio ativo, que facilitam o ataque nucleofílico de uma molécula de água ao substrato, liberando a primeira molécula de amônia e carbamato, que hidrolisa-se espontaneamente em CO_2 e outra molécula de amônia (Figura 1). Esse mecanismo molecular foi descrito por Dixon et al., 1975, e revisto por Benini et al., 2001, para urease de *Bacillus pasteurii*, e por Callahan et al., 2005, para a urease de *Canavalia ensiformis*.

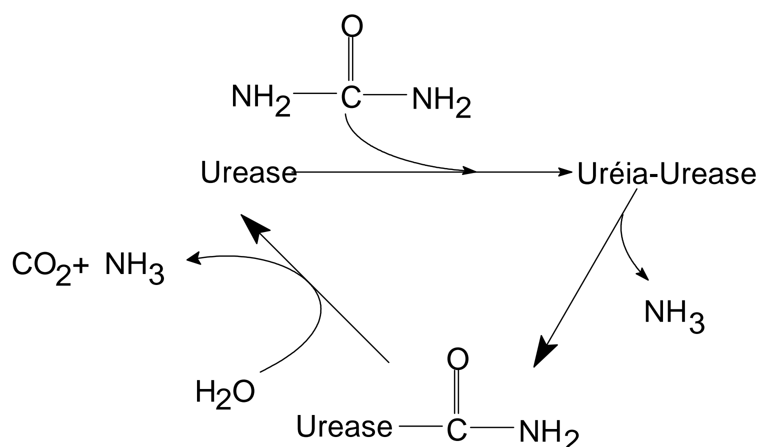

Figura 1. Hidrólise da uréia catalisada por ureases.

A urease da *C. ensiformis* (feijão de porco) é uma enzima de importância histórica, por ter sido a primeira enzima a ser cristalizada (Sumner, 1926), comprovando-se assim a natureza protéica das enzimas. Esta enzima foi também a primeira a ser identificada como metalo-enzima dependente de níquel (Dixon et al., 1975).

Ureases são sintetizadas por vários organismos como bactérias, fungos e plantas (Mobley & Hausinger, 1989). Ureases de plantas e fungos são trímeros ou

hexâmeros de uma única cadeia polipeptídica, já ureases de bactérias apresentam mais de uma subunidade, sendo a proteína nativa formada por um heterotrímero de três cadeias, $(\alpha\beta\gamma)_3$, para as enzimas de *B. pasteurii* e *Klebsiella aerogenes* e outras já descritas (Mobley & Hausinger, 1989), ou um heterotrímero $(\alpha\beta)_3$ de duas cadeias, em espécies do gênero *Helicobacter* (Mobley et al., 1995) .

Apesar de terem sido os primeiros, os cristais já obtidos da urease de *C. ensiformis* apresentaram baixa difração frente a raio-X, de modo que sua estrutura não pode ser determinada por este método (Jabri et al., 1992, Jabri et al., 1995). Por outro lado, as estruturas cristalográficas de três isoenzimas de origem bacteriana já foram elucidadas: *K. aerogenes* (Jabri et al., 1995), *B. pasteurii* (Benini et al., 2000) e *Helicobacter pylori* (Ha et al., 2001).

Análises de seqüências de aminoácidos das isoenzimas de diferentes origens mostram uma alta similaridade na estrutura primária das proteínas, indicando que as ureases são variantes da mesma enzima ancestral e provavelmente possuem o mesmo tipo geral de estrutura terciária. Essas seqüências, no entanto, não demonstram claramente uma divisão filogenética entre ureases de plantas e bactérias ou entre bactérias gram-negativas e gram-positivas. Portanto, ainda há questionamentos quanto a origem do(s) gene(s) que codifica(m) a urease e o mecanismo de sua dispersão (Mobley et al., 1995).

Apesar das diferenças na estrutura quaternária entre as ureases, a identidade de seqüência primária é maior que 50% entre bactérias e plantas, e com similaridades maior que 70% dentro de cada grupo (Mobley et al., 1995). O motivo estrutural envolvido no mecanismo de catálise destas enzimas parece ser bem conservado, principalmente no que diz respeito às histidinas que complexam os átomos de níquel, que são críticos para a atividade enzimática (Figura 2).

Canavalia ensiformis	DCH$_{407}$VH$_{409}$YI...GLK$_{490}$IH...NIH$_{519}$TD... TYH$_{545}$SE...MVC$_{592}$HHL... TID$_{633}$SQ
Solanum tuberosum	DCH$_{401}$VH$_{403}$FI...GLK$_{484}$LH...NIH$_{513}$TD...TYH$_{539}$SE...MVC$_{586}$HHL...SSD$_{627}$SQ
Glycine max	DCH$_{405}$VH$_{407}$FI...GLK$_{488}$LH...NIH$_{517}$TD...TYH$_{543}$SE...MVC$_{590}$HHL...SSD$_{631}$SQ
Arabidopsis thaliana	DCH$_{405}$VH$_{407}$FI...GLK$_{488}$LH...NIH$_{517}$TD...TYH$_{543}$SE...MVC$_{590}$HHL...SSD$_{631}$SQ
Oryza sativa	DCH$_{405}$VH$_{407}$FI...GLK$_{488}$LH...NIH$_{517}$TD...TYH$_{543}$SE...MVC$_{590}$HHL...SSD$_{631}$SQ
Bacillus sp.	DAH$_{136}$I H$_{138}$FI...GLK$_{219}$LH... AIH$_{248}$TD...TYH$_{274}$TE...MVC$_{321}$HHL... SSD$_{362}$SQ
Proteus vulgaris	DTH$_{134}$I H$_{136}$FI...GLK$_{217}$IH... AIH$_{246}$SD...VFH$_{272}$TE...MVC$_{320}$HHL... SSD$_{360}$SQ
Helicobacter pylor	DTH$_{136}$I H$_{138}$FI...GFK$_{219}$IH... AIH$_{248}$TD...TFH$_{274}$TE...MVC$_{321}$HHL... SSD$_{362}$SQ
M. tuberculosis	DCH$_{141}$VH$_{143}$LI...GFK$_{224}$LH...ALH$_{253}$SD...AYH$_{279}$TE...MVC$_{326}$HHL...GSD$_{367}$SQ
Klebsiella aerogenes	DTH$_{133}$I H$_{135}$WI...GLK$_{216}$IH... ALH$_{245}$SD...TFH$_{271}$TE...MVC$_{318}$HHL...SSD$_{359}$SQ

Figura 2. Alinhamento das sequências de aminoácidos das regiões que participam diretamente do sítio catalítico, ou estão próximas a este, da urease da *C. ensiformis* e ureases bacterianas. Em vermelho estão assinalados os resíduos críticos para a atividade ureásica. A região em azul está próxima do sítio catalítico da enzima, mas não participa da reação de catálise. É na cisteína desta região que se liga o p-hidroxi-mercuribenzoato, resultando na inibição da reação por impedimento estéreo (dados coletados do Protein Data Bank). Retirado de Follmer, 2004.

1.2 Ureases microbianas

Ureases microbianas são importantes na transformação de certos compostos nitrogenados no meio ambiente, no metabolismo de ruminantes e na patogênese de algumas doenças em seres humanos (Mobley & Hausinger, 1989).

A atividade ureásica está amplamente distribuída em microorganismos no solo e no ambiente aquático, onde exerce um importante papel no metabolismo do nitrogênio, como em processos degradativos envolvendo o "turnover" de proteínas e nucleotídios, e mais importante, na fixação do nitrogênio a partir da uréia. A reciclagem de uréia para formação de outros compostos nitrogenados (como

proteínas e nucleotídios) é de grande importância no balanço de nitrogênio em ruminantes (Mobley & Hausinger, 1989).

Ureases bacterianas estão envolvidas na patogênese de diversas condições clínicas, estando diretamente implicadas, por exemplo, na formação de cálculos urinários, pielonefrite, encefalopatia hepática (podendo evoluir para coma hepático, devido à produção de amônia por organismos ureolíticos) e, principalmente, úlcera péptica causada por *H. pylori*. Cálculos urinários e pielonefrite têm como agente etiológico, em cerca de 15-20% dos casos, a bactéria *Proteus mirabilis.* O aumento no pH, decorrente da hidrólise da uréia catalisada pela urease bacteriana, leva à precipitação de íons normalmente solúveis na urina, causando a urolitíase (Heimer & Mobley, 2001, Mobley & Hausinger, 1989).

1.2.1. *Helicobacter pylori*

O *H. pylori* é uma espiroqueta, gram negativa, microaerófila, com 2,5 a 5,0 µm de comprimento e 0,5 a 1,0 µm de largura, que possui de 4 a 6 flagelos com aproximadamente 30 µm de comprimento (Goodwin & Armstrong, 1990). Em 1982, Marshall e Warren isolaram pela primeira vez esse microrganismo, conduzindo a microbiologia gástrica à uma nova era (Warren & Marshall, 1983; Marshall & Warren, 1984). Atualmente, o *H. pylori* é reconhecido como o principal agente etiológico de gastrite crônica, úlcera péptica e possivelmente no desenvolvimento de adenocarcinoma gástrico distal (NIH Consensus Conference, 1994). Estima-se que esse microrganismo pode ser encontrado em cerca de 50% da população mundial, e em países subdesenvolvidos, mais de 70% das pessoas estariam infectadas (Queiroz & Luzza, 2006).

O *H. pylori* coloniza a mucosa gástrica e áreas de metaplasia gástrica localizadas no intestino. A infecção é acompanhada de processo inflamatório caracterizado pela infiltração de células inflamatórias mono e polimorfonucleares no epitélio (Harris et al., 1996). A intensidade da inflamação pode ser altamente variável, desde uma infiltração mínima da lâmina própria com arquitetura glandular intacta até uma inflamação severa, com formação de micro abscessos (Genta & Graham, 1994). Em geral, há degeneração das células epiteliais, com depleção de mucina, vacuolização citoplasmática e desorganização de glândulas da mucosa (Figueiredo et al., 2005, Kalia & Bardhan, 2003).

Sugere-se que a transmissão desse microrganismo possa ocorrer a partir de três rotas. A primeira, e menos comum, está relacionada ao contato de pacientes com instrumentos endoscópicos contaminados. A transmissão direta do contato entre humanos, via fecal-oral, é talvez a mais importante (Queiroz & Luzza 2006). Apesar de *H. pylori* ter sido isolado das fezes de crianças infectadas, o isolamento desse microrganismo das fezes não é comum, o que sugere que deva ocorrer descamação gástrica intermitente. Água contaminada por material fecal pode ser também uma fonte importante de transmissão. Por último, a via de transmissão oral-oral foi identificada em casos na África, onde em algumas tribos, as mães pré-mastigam o alimento dos filhos (Megraud 1994).

Muitos fatores de virulência da bactéria estão envolvidos na patogênese de *H. pylori*, incluindo várias enzimas (urease, catalase, lipase e algumas proteases) e toxinas, como a citotoxina vacuolizante, codificada pelo gene *vacA*, e a proteína imunogênica Cag A, codificada pelo gene *cagA*, que esta localizado em uma ilha de patogenicidade (Cag-PAI). A Cag-PAI contém também diversos outros genes responsáveis pela virulência, expressão de citocinas pró-inflamatórias (IL-8) em

células epiteliais, e expressão de um conjunto de proteínas formadoras de um sistema de secreção do tipo IV (T4SS) para o transporte da proteína CagA para células eucarióticas (Censini et al., 1996); (Figueiredo et al., 2005)

1.2.2 A urease de *Helicobacter pylori*

A urease é uma enzima altamente expressa por *H. pylori*, podendo compor de 10% a 15% das proteínas totais dessa bactéria. A urease nativa de *H. pylori* possui massa molecular de aproximadamente 540 kDa, é uma metaloenzima níquel dependente e hexamérica. O monômero é composto por duas cadeias polipeptídicas (UreA [30kDa] e UreB [62kDa]) em proporção 1:1 (Dunn et al., 1990, Hu & Mobley, 1990). A afinidade dessa enzima pelo substrato uréia, com um Km ~ 0,3 mM, a torna cataliticamente eficiente até mesmo nas concentrações submillimolares de uréia presentes nos fluídos humanos (Dunn et al., 1990, Hu & Mobley, 1990).

Pelo menos sete genes estão envolvidos na produção da urease de *H. pylori*. Os genes *ureA* e *B* codificam as duas subunidades que compõem a enzima, enquanto que os genes *ureE, F, G, H* codificam proteínas acessórias responsáveis pela incorporação do níquel no centro ativo da urease. O gene *ureI* codifica uma proteína que funciona como um canal na membrana externa, atuando na internalização da uréia. Além desses genes, uma proteína transportadora de níquel é expressa a partir do gene *nixA* (Mobley et al., 1995).

A urease de *H. pylori* é considerada um fator de virulência, sendo a sua atividade um marcador para diagnóstico utilizado amplamente (Krogfelt et al., 2005). Mutantes de *H. pylori* urease negativos são incapazes de colonizar leitões gnotobióticos e, tampouco, camundongos "*nude*" (Eaton et al., 1991, Tsuda et al., 1994). Supõe-se que a principal função dessa enzima esteja relacionada com a

formação de um microclima neutro para o microrganismo no lúmen gástrico, possibilitando sobrevivência das bactérias em ambientes de pH desfavorável. Apesar de inibidores de urease terem sido utilizados no tratamento de algumas destas patologias, seu uso foi descontinuado pelo fato de muitos pacientes apresentarem reações colaterais adversas. O uso clínico de ácido acetohidroxâmico, um quelante de níquel inibidor de urease, causa depressão na síntese de DNA, afetando a medula óssea, além de ser teratogênico em doses elevadas (Figueiredo et al., 2005, Montecucco et al., 1999). Além do efeito neutralizador da acidez gástrica, a urease de *H. pylori* está envolvida na ativação de fagócitos e na produção de citocinas inflamatórias (Cinque et al., 2006, Harris et al., 1996). *H. pylori* induz em macrófagos *in vitro* aumento da óxido nítrico sintase induzível (iNOS) (Wilson et al., 1996, Gobert et al., 2002), resultando em uma grande liberação de oxido nítrico, o qual está associado à ativação de células do sistema imune no tecido lesado. A ativação de monócitos, leucócitos e recrutamento de células inflamatórias, resultam em dano indireto para o epitélio gástrico (Mobley et al., 1995, Fan et al., 2000). A degradação de uréia e, conseqüentemente, a liberação de amônia também resulta em danos celulares (Barer et al., 1988) e contribui na indução de vacuolização das células epiteliais gástricas, em conjunto com a toxina VacA (Montecucco et al., 1999). Esses dados sugerem que a urease seja importante também no desenvolvimento de lesões gástricas, e não só na manutenção da bactéria em pH desfavorável.

1.3 Ureases em Plantas

Apesar da abundância de ureases em tecidos vegetais, principalmente sementes de Curcubitáceas e de Leguminosas, pouco se conhece sobre a função dessa enzima em plantas, uma vez que a uréia não é um metabólito majoritário em

tecidos vegetais onde esta enzima está presente (Polacco & Holland, 1993, Sirko & Brodzik, 2000). O caso mais bem estudado é o da soja (*Glycine max*) que possui duas isoformas de urease: uma enzima ubíqua, presente em todos os tecidos examinados, e uma segunda isoenzima, designada embrião-específica, expressa no embrião em desenvolvimento e preservada na semente madura, onde sua atividade é cerca de 1000 vezes maior que a urease ubíqua em todos os tecidos (Polacco & Sparks, 1982, Polacco & Winkler, 1984). Plantas mutantes, sem a urease embrião-específica, não apresentam nenhuma das anormalidades associadas à perda da atividade da urease ubíqua, como necrose foliar, acúmulo de uréia nas folhas e sementes, e retardo na germinação, levando os autores desse estudo a sugerirem que essa isoenzima não teria uma função fisiológica essencial (Stebbins et al., 1991, Polacco & Holland, 1993).

Traçando um paralelo com os efeitos patogênicos de ureases bacterianas, foi sugerido que a urease embrião-específica não teria um papel importante na assimilação de uréia, e sim como metabólito secundário, atuando na defesa química do vegetal em função da amônia liberada, juntamente com fatores antinutricionais, como inibidores de proteases e lectinas, proteínas inativadoras de ribosomos, entre outros. Reforça essa hipótese a indução da expressão de urease em resposta à injúria microbiana, ou por ataque de insetos (Polacco & Holland, 1993).

1.3.1 Ureases de *Canavalia ensiformis*

As leguminosas do gênero *Canavalia* são nativas do Novo Mundo, com 48 espécies de distribuição pan-tropical, tendo sido cultivadas em épocas pré-colombianas, como alimento humano e de animais domésticos (Sauer & Kaplan 1969, Vargas et al., 1993). A planta é excepcionalmente resistente ao ataque de

insetos, que se atribuí ser devido à presença de metabólitos secundários, como a canavanina, um análogo de arginina (Rosenthal & Dahlman 1986). A semente de *Canavalia ensiformis*, o feijão-de-porco, é fonte de várias proteínas de interesse, como a urease (Sumner, 1926) e a lectina concanavalina A (Sumner & Howell, 1936), e a canatoxina (Carlini & Guimarães, 1981).

A canatoxina (CNTX), uma proteína tóxica isolada dessa leguminosa por Carlini & Guimarães, em 1981, corresponde a ~0,5% do peso seco da semente. A CNTX é tóxica para ratos e camundongos quando administrada via intraperitonial (LD_{50} = 2 mg/Kg), mas é inativa por via oral (Carlini & Guimarães, 1991). *In vitro*, a toxina induz exocitose em diversos tipos celulares, incluindo plaquetas, sinaptosomas, ilhotas pancreáticas, macrófagos, neutrófilos e mastócitos, mediada por ativação do metabolismo do ácido araquidônico via lipoxigenases (Carlini et al., 1985, Barja-Fidalgo et al., 1991a, 1991b, Benjamin et al., 1992) e por alterações dos níveis intracelulares de Ca^{2+} (Ghazaleh et al., 1997).

A principal forma da CNTX apresenta um peso molecular de 184 kDa quando analisada em gel-filtração em pH 7,5. Em SDS-PAGE, em meio redutor ou não, seu peso molecular é de 95 kDa, sugerindo que a forma nativa da proteína é provavelmente um dímero mantido por ligações não covalentes. A determinação parcial da sequência de aminoácidos da canatoxina indicou um alto grau de homologia com a urease (EC 3.5.1.5) majoritária da mesma semente (Follmer et al., 2001). A CNTX apresenta ~30-40% da atividade enzimática da urease, e um sítio ativo híbrido, contendo 1 mol Ni^{2+} e 1 mol de Zn^{2+} por monômero de 95 kDa (Follmer et al., 2002). Em contraste, a urease majoritária é um homohexâmero de uma cadeia de 90 kDa, e contém dois átomos de níquel por subunidade (Sirko & Brodzik, 2000).

Para uma comparação mais abrangente das atividades biológicas das duas isoformas de urease do feijão de porco, bem como estudar as variações no conteúdo de metais, tornou-se necessária a purificação das duas isoformas a partir da mesma partida de sementes. Através de cromatografia de afinidade por metal imobilizado (IMAC) é possível separar as isoformas. O protocolo inicialmente desenvolvido por Follmer et al., 2001, utilizava uma resina experimental dipipecolilamina~Sepharose (Porath 1992). Posteriormente, essa metodologia foi adaptada para uso com resinas disponíveis comercialmente, como Sepharose acoplada com ácido iminodiacético (Follmer et al., 2004b)(ver anexo). Visando evitar qualquer contaminação da amostra com Zn^{2+} na etapa de IMAC como descrita por Follmer e colaboradores em 2001, utilizamos Co^{2+} imobilizado na IDA-Sepharose, condição em que apenas a canatoxina fica retida na coluna (Follmer et al., 2004b).

Verificamos ainda que a urease majoritária da *C. ensiformis* apresenta as propriedades biológicas conhecidas para a CNTX, como indução de agregação plaquetária e interação com gangliosídeos, mas não sua toxicidade em camundongos (Carlini & Grossi-de-Sá, 2002). Esses efeitos farmacológicos não dependem da atividade ureolítica das proteínas, já que a CNTX tratada com 200 μM de p-hidroximercuribenzoato (um agente oxidante de grupos tióis) perde totalmente a atividade ureásica, mas mantém inalterada a sua atividade tóxica em camundongos, ainda induz agregação plaquetária e liga-se a gangliosídeos. As mesmas observações foram feitas para a urease tratada com p-hidroximercuribenzoato (Follmer et al., 2001).

Em 1997, Carlini e colaboradores descreveram a atividade inseticida da CNTX (Carlini et al., 1997, Ferreira-DaSilva et al., 2000). Também nesse caso, o efeito inseticida não está associado à atividade ureolítica da toxina, pois depende da

formação de peptídeos entomotóxicos a partir da hidrólise das proteínas por enzimas digestivas dos insetos. Um dos insetos que é extremamente sensível às urease(s) de *C.ensiformis* é o percevejo *Dysdercus peruvianus*, uma praga do algodão (Carlini & Grossi-de-Sá, 2002, Staniscuaski et al., 2005).

A tabela 1 apresenta uma comparação dos dados biológicos e físico-químicos obtidos nos estudos da urease com a canatoxina (Carlini & Grossi-de-Sá, 2002).

Tabela 1. Comparação das propriedades biológicas e físico-químicas obtidos nos estudos da urease e da canatoxina.

	Canatoxin	Urease
Physicochemical properties		
Molecular mass		
SDS–PAGE	95–100 kDa	90–97 kDa
Native form	Dimer	Hexamer
Urease activity		
K_m (pH 6.5–8.5)	2–5 mM	3–8 mM
V_{max} (pH 6.5–8.5)	4–10 U/mg	15–45 U/mg
Inhibitors, IC50		
p-Hydroxi-mercuribenzoate	5 μM	0.5 μM
Acetohydroxamic acid	3 μM	3 μM
Metal content (per 95 kDa)	Nickel-1 mol/mol Zinc-1 mol/mol	Nickel-2 mol/mol Zinc-not detected
Biological properties		
Toxicity		
Mouse, i.p.	LD_{50}—2 mg/kg (*p*-OHMB treated, 100% active)	Not toxic with 10 LD50
C. maculatus, ingested	100% death—0.25% (w/w)	Not determined
R. prolixus, ingested	LD_{50}—0.4–0.8 mg/kg	LD_{50}—0.4–0.8 mg/kg
Indirect hemagglutination		
End-point (10^6 rabbit cells)	1–2 μg	1–2 μg
Inhibitors	Gangliosides-fetuin (*p*-OHMB treated-100% active)	Gangliosides-fetuin (*p*-OHMB treated-100% active)
Platelet aggregation (rabbit)	EC_{50}—2–3 μg (*p*-OHMB treated-100% active)	EC_{50}—2–3 μg (*p*-OHMB treated-100% active)

(Retirado de Carlini & Grossi-de-Sá 2002)

Nossos resultados sugerem a existência, nestas proteínas, de domínios protéicos distintos, responsáveis por atividades biológicas diferentes: um domínio

com atividade hidrolítica sobre uréia, susceptível de inibição por agentes quelantes e oxidantes; e um segundo domínio, níquel e tiol-independente, que é responsável pela toxicidade intraperitoneal da canatoxina, bem como pelo efeito pró-agregante e capacidade de interação com glicoconjugados tanto da canatoxina como da urease.

Em outro estudo (Follmer et al., 2004b)(ver anexo), comparamos ureases de diferentes organismos para saber se estas possuíam propriedades biológicas independentes da atividade ureolítica, como demonstrado para as isoenzimas de *C. ensiformis*. Para tal escolhemos a urease embrião específica de soja (SBU) e a urease de *B. pasteurii* (BPU), esta com estrutura 3D já caracterizada, composta por três tipos de cadeias unidas formando hexâmeros $(\alpha\beta\gamma)_3$ (Benini et al., 2000). Para comparar as atividades biológicas entre ureases, utilizamos ensaios de agregação de plaquetas de coelho (Carlini et al., 1985), de atividade inseticida em *Dysdercus peruvianus* (Carlini & Grossi-de-Sá, 2002) e avaliação de parâmetros cinéticos enzimáticos, e efeito de inibidores.

Observamos nesse estudo que algumas atividades biológicas parecem ser comuns às ureases de plantas e à enzima da bactéria *B. pasteurii*, como a indução de agregação plaquetária. Por outro lado, a toxicidade em *D. peruvianus*, uma praga da cultura do algodão, aparentemente é exclusiva das isoenzimas vegetais de *C. ensiformis* e *G. max* (Follmer et al., 2004b). Mesmo apresentando maior atividade na clivagem de uréia, a urease de *B. pasteurii* é menos ativa sobre plaquetas, cerca de 20 vezes menos em termos de massa, o que demonstra que essas propriedades biológicas são independentes e corrobora os resultados de inibição química da atividade ureásica com manutenção de efeitos biológicos. Concluímos assim que, apesar dessas enzimas apresentarem um alto grau de similaridade de seqüência primária, ocorrem variações importantes em suas atividades biológicas.

1.4 Eicosanóides

Os eicosanóides são autacóides derivados do ácido araquidônico por rotas metabólicas distintas, entre as quais a via das cicloxigenases e a via das lipoxigenases (figura 2). O ácido araquidônico, atuando diretamente ou na forma de seus metabólitos, eicosanóides, regula uma série de funções celulares. Os eicosanóides são hoje reconhecidos como segundo mensageiros envolvidos na transdução de sinais de uma vasta gama de fenômenos fisiológicos (Metz, 1988, Naor, 1991, Piomelli & Greengard, 1990, Goetzl et al., 1995). Vários indícios sugerem que produtos de lipoxigenases, como os leucotrienos, estariam envolvidos nos processos secretórios de diferentes tipos celulares (Sasakawa et al., 1984, Naor et al., 1985, Metz 1986, Piomelli & Greengard, 1990). Além disso, modificações no metabolismo do ácido araquidônico estão envolvidas em muitas alterações patológicas (Metz, 1988, Piomelli & Greengard, 1990, Naor, 1991, Goetzl et al., 1995, Lotzer et al., 2005, Phillis et al., 2006, Rossi et al., 2007).

Os leucotrienos são ainda considerados mediadores de distúrbios anafiláticos e inflamatórios (Hedi & Norbert, 2004, Kuhn & O'Donnell, 2006). Os produtos de lipoxigenases e cicloxigenase estão envolvidos na patogênese de várias doenças inflamatórias (Linton & Fazio, 2004, Zhao & Funk, 2004, Takahashi et al., 2005, Hatmi et al., 2006), bem como de problemas vasculares decorrentes da diabetes (Natarajan & Nadler, 2003).

Estudos anteriores do nosso grupo mostraram que eicosanóides, em particular derivados da rota da 12-lipoxigenase, modulam ou são mediadores de vários efeitos farmacológicos da canatoxina, como a agregação de plaquetas de coelho (Carlini et

al., 1995; Barja-Fidalgo et al., 1991a), secreção de neuro-transmissores por sinaptosoma, de serotonina por plaquetas e de insulina por células β do pâncreas (Barja-Fidalgo, 1991a,1991b). A participação de eicosanóides nos efeitos observados *in vivo* em ratos tratados com canatoxina, tais como hiperglicemia, hipóxia, convulsões (Ribeiro-DaSilva et al., 1989), e a atividade pró-inflamatória são também bloqueados por pretratamento com inibidores de lipoxigenase (Benjamin et al., 1992).

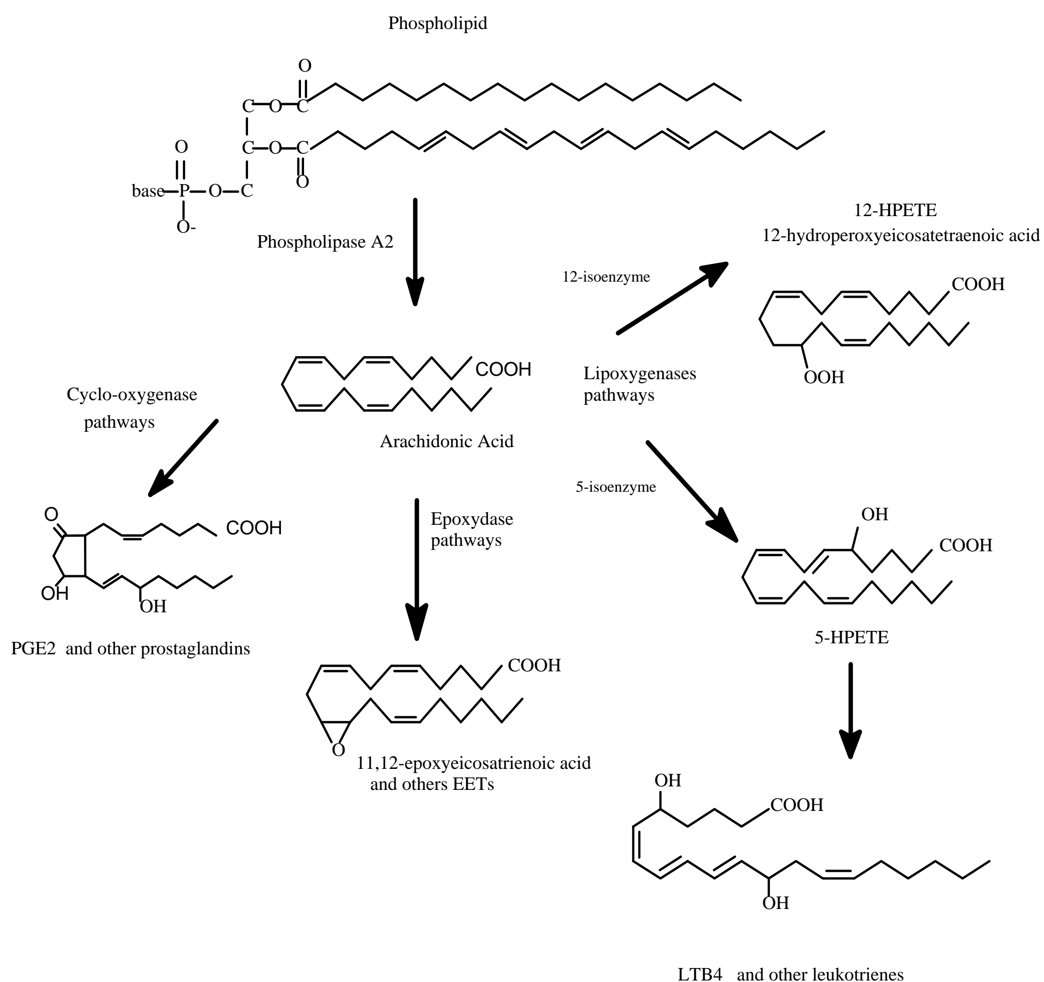

Figura 2: Principais vias do metabolismo dos eicosanóides. A liberação de ácido araquidônico de fosfolipídios de membranas é catalisada por fosfolipases tipo A_2. o ácido araquidônico liberado será substrato para diferentes rotas metabólicas, característica de cada tipo celular. Através da via da cicloxigenase formam-se prostaglandinas e tromboxanas, enquanto que a ação das diferentes lipoxigenases levará á formação dos hidroperóxidos correspondentes. Adaptado de (Stanley-Samuelson & Ogg 1994).

2. Objetivos

Este trabalho teve como objetivo caracterizar propriedades biológicas de ureases independentes de seu potencial hidrolítico sobre a uréia, para diferentes isoenzimas de plantas e bactérias.

Em especial, visamos estudar o mecanismo farmacológico da interação de ureases de bactérias com plaquetas, e correlacionar os efeitos biológicos observados com um possível papel da urease de *H. pylori* na patogênese de doenças gástricas produzidas por essa bactéria.

3. RESULTADOS

A seção de resultados dessa tese é composta por três capítulos, que correspondem a artigos publicados ou a manuscritos prontos para serem submetidos à publicação. Cada capítulo inicia-se com um texto introdutório em português com uma breve apresentação do contexto do artigo e resumo dos resultados obtidos, seguido de cópia do artigo impresso, ou do manuscrito em inglês.

O capítulo I corresponde ao artigo de revisão "*Ureases display biological effects independent of enzymatic activity. Is there a connection to diseases caused by urease-producing bacteria?*" publicado em 2006, no *Brazilian Journal of Medical and Biological Research*.

O capitulo II corresponde ao artigo "*Bacillus pasteurii urease shares with plant ureases the ability to induce aggregation of blood platelets*" publicado em 2006, no *Archives of Biochemistry and Biophysics*.

O capítulo III corresponde ao manuscrito "*Helicobacter pylori Urease Shares with Plant Ureases the Ability to Activate Blood Platelets and Edematogenic Activity*".

As metodologias utilizadas são descritas nos artigos. No final da tese, apresentamos uma discussão geral dos resultados e conclusões, seguidas das referências bibliográficas citadas na Introdução e na Discussão.

Constam como anexos, além do *Curriculum vitae*, dois artigos já publicados e um manuscrito submetido à publicação no periódico *Toxicon*.

Capítulo I

Olivera-Severo, D[#]. **Wassermann, G.E.**[#], Carlini, C.R.

Ureases display biological effects independent of enzymatic activity. Is there a connection to diseases caused by urease-producing bacteria?

Brazilian Journal of Medicinal and Biological Research 39, 851-86, 2006.

[#] Esses autores contribuíram igualmente para o artigo.

Ureases de bactérias têm papel fundamental na patogênese de diversas condições clínicas. Esse papel tem sido associado à atividade ureolítica dessas proteínas, mas em alguns casos, a atividade de hidrólise de uréia não justifica um papel tão importante dessas enzimas. As homologias entre ureases bacterianas e vegetais não se restringem à estrutura primária ou à atividade enzimática. Propriedades biológicas independentes da atividade ureolítica foram descritas para ureases das sementes de *C. ensiformis* (Follmer et al., 2001), e de soja, bem como para a urease da bactéria não patogênica *B. pasteurii* (Follmer et al. 2004b, anexo).

Neste trabalho revisamos dados da literatura que sugerem que a urease de *H. pylori* apresenta muitas das atividades biológicas observadas em ureases de *C. ensiformis*, em particular um efeito secretagogo e atividade pró-inflamatória modulada por eicosanóides derivados de lipoxigenases. Apresentamos também dados preliminares mostrando que uma urease recombinante de *H. pylori* é capaz de agregar plaquetas. Estes achados podem ser importantes para elucidar a real contribuição da urease na patogênese das doenças gastroduodenais causadas por *H. pylori*.

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Ureases display biological effects independent of enzymatic activity. Is there a connection to diseases caused by urease-producing bacteria?

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Abstract

Ureases are enzymes from plants, fungi and bacteria that catalyze the hydrolysis of urea to form ammonia and carbon dioxide. While fungal and plant ureases are homo-oligomers of 90-kDa subunits, bacterial ureases are multimers of two or three subunit complexes. We showed that some isoforms of jack bean urease, canatoxin and the classical urease, bind to glycoconjugates and induce platelet aggregation. Canatoxin also promotes release of histamine from mast cells, insulin from pancreatic cells and neurotransmitters from brain synaptosomes. *In vivo* it induces rat paw edema and neutrophil chemotaxis. These effects are independent of ureolytic activity and require activation of eicosanoid metabolism and calcium channels. *Helicobacter pylori*, a Gram-negative bacterium that colonizes the human stomach mucosa, causes gastric ulcers and cancer by a mechanism that is not understood. *H. pylori* produces factors that damage gastric epithelial cells, such as the vacuolating cytotoxin VacA, the cytotoxin-associated protein CagA, and a urease (up to 10% of bacterial protein) that neutralizes the acidic medium permitting its survival in the stomach. *H. pylori* whole cells or extracts of its water-soluble proteins promote inflammation, activate neutrophils and induce the release of cytokines. In this paper we review data from the literature suggesting that *H. pylori* urease displays many of the biological activities observed for jack bean ureases and show that bacterial ureases have a secretagogue effect modulated by eicosanoid metabolites through lipoxygenase pathways. These findings could be relevant to the elucidation of the role of urease in the pathogenesis of the gastrointestinal disease caused by *H. pylori*.

Key words
- Urease
- Canatoxin
- *Helicobacter pylori*
- Inflammation
- Neutrophils
- Eicosanoids

Introduction

Ureases (urea amidohydrolase; EC 3.5. 1.5) are nickel-dependent enzymes (1) that catalyze the hydrolysis of urea to form ammonia and carbon dioxide. They have been isolated from a wide variety of organisms including plants, fungi and bacteria. While fungal and plant (e.g., jack bean and soybean) ureases are homo-oligomeric proteins of ca. 90 kDa subunits, bacterial ureases are multimers of two or three subunit complexes (Figure 1) (2,3). Amino-terminal residues of the monomers of plant and fungal enzymes

are similar in sequence to the small subunits of bacterial enzymes (e.g., UreA and UreB of *Klebsiella aerogenes*). The large subunits of bacterial ureases (e.g., UreC of *K. aerogenes*) resemble the carboxy-terminal portions of plant and fungal subunits. So far only bacterial ureases have had their 3-D crystallographic structure successfully resolved, e.g., *K. aerogenes* (1FWJ), *Bacillus pasteurii* (4UBP), and *Helicobacter pylori* (1E9Z) (2, 3). However, the high sequence similarity of all ureases indicates they are variants of the same ancestral protein and are likely to possess similar tertiary structures and catalytic mechanisms (2,3). Despite their highly conserved structures and enzymatic action, little is known about the physiological role of ureases in the source organisms. Urease activity enables bacteria to use urea as a sole nitrogen source. Some bacterial ureases play an important role in the pathogenesis of human and animal diseases such as those from *Proteus mirabilis* and *H. pylori* (2).

The wide distribution of ureases in leguminous seeds as well as the accumulation pattern of the protein during seed maturation suggest an important physiological role. Soybean mutants lacking the embryo-specific highly active isoform of urease do not exhibit any of the abnormalities associated with loss of the less active ubiquitous isoform, suggesting that this enzyme probably does not have an essential physiological role (4). *In vitro* cultures of developing soybean cotyledons have indicated that ureases do not play an important role in embryo nutrition since urea is an extremely poor nitrogen source (4). The obvious question from this observation is why would the developing soybean embryo invest in producing a very active ureolytic protein when it usually does not “encounter” urea. Polacco and Holland (4) have proposed that plant ureases may have a role in plant defense against predators due to the high toxicity of the ammonia released.

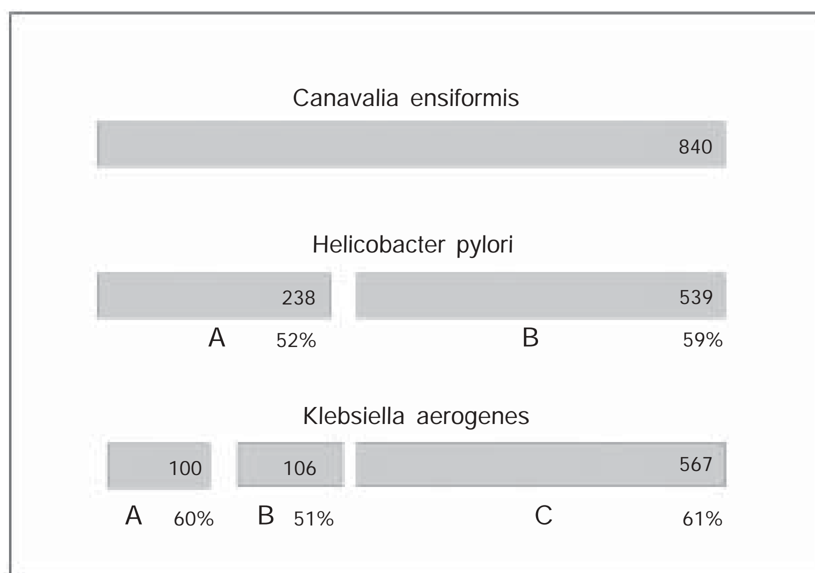

Figure 1. Schematic structure of ureases. Plant ureases such as the jack bean (Canavalia ensiformis) enzyme have a single subunit while bacterial ureases have two (Helicobacter pylori) or three (Klebsiella aerogenes) subunits. The number of amino acid residues of each subunit is indicated. Note that the length of each box is not drawn to scale of total amino acids. The percentage of identity to the corresponding fragment of the jack bean urease is shown under the bacterial proteins.

Plant-derived ureases: canatoxin and jack bean urease

The jack bean *Canavalia ensiformis* is the source of interesting proteins which have contributed significantly to modern Biochemistry. One of these is no doubt urease, the first protein ever crystallized (5), and the first nickel-containing enzyme described (1). In 1981, we isolated a toxic protein, named canatoxin, which accounts for 0.5% of seed dry weight of jack beans (6). Canatoxin is lethal to rats and mice by intraperitoneal injection, but it is inactive when given orally (7). Canatoxin, which consists of a noncovalently linked dimer of 95-kDa acidic polypeptide chains, was characterized recently as a variant form of the classical more abundant jack bean urease (8). RT-PCR applied to mRNA isolated from *C. ensiformis* tissues and Southern blots confirmed the presence of a family of urease-related genes with at least two members sharing 86% similarity (9). Jack bean ureases presented differential behavior in immobilized metal affinity chro-

matography enabling separation of the isoenzymes (8,10). A particle-induced X-ray emission technique applied to determine the metal content of the isoforms showed that canatoxin displays ca. 1 atom of nickel and 1 of zinc per monomer, contrasting with 2 atoms of nickel and absence of zinc in the monomer of the major form of urease in *C. ensiformis* (8,11).

Insecticidal properties of plant ureases

The insecticidal properties of plant ureases were first described for canatoxin (12) and later for *C. ensiformis* major urease and soybean embryo-specific urease (13). The kissing bug *Rhodnius prolixus*, and three economically important crop pests, the cowpea weevil *Callosobruchus maculatus*, the green stinkbug *Nezara viridula* and the cotton stainer bug *Dysdercus peruvianus*, are susceptible to the lethal effect of these proteins when they are added to their diets at 0.02 to 0.1% (w/w) levels (14,15). Susceptible insects have cathepsins of type B and D as their main digestive enzymes. Canatoxin and urease are hydrolyzed by these enzymes to release an internal entomotoxic peptide of 10 kDa (16). No effects of intact canatoxin/urease were seen in insects relying on trypsin-like digestive enzymes, which apparently degrade the proteins more extensively (12). A recombinant peptide, equivalent to that produced by hydrolysis of canatoxin with insect cathepsins, was obtained by heterologous expression in *Escherichia coli.* This peptide presented potent insecticidal effects (17) and did not affect mice or neonate rats upon oral or intraperitoneal administration. In contrast, the urease from the soil bacterium *B. pasteurii* is devoid of insecticidal properties, as expected from its three-chain structure, since part of the sequence of the entomotoxic peptide is absent in microbial ureases. In plant ureases this corresponds to a fragment located between the UreB and UreC chains of *B. pasteurii* urease (13).

Taken together, our results indicate that plant ureases are probably involved in defense mechanisms of plants against insect predation and that their insecticidal properties are independent of their enzymatic activity, being associated with an internal peptide of these proteins.

Biological properties of canatoxin and similarities with other ureases

Canatoxin administered intraperitoneally to rats or mice (LD_{50} 0.4-0.6 and 2-3 mg/kg, respectively) induces respiratory distress, convulsion, and death (6,18). At subconvulsant doses canatoxin promotes increased gonadotropin (19) and plasma insulin levels (20) and pro-inflammatory effects in rats (21). *In vitro*, canatoxin displays potent secretagogue activity at nanomolar doses in several isolated cellular systems, inducing platelet secretion and aggregation (22,23), secretion of labeled dopamine and serotonin from rat brain synaptosomes (23), histamine release from mast cells (24), and secretion of insulin from isolated pancreatic islets (23,25). Thus, canatoxin induces dose-dependent aggregation of platelets from different species at concentrations as low as 20 nM (22). Rat isolated pancreatic islets secrete insulin when exposed to canatoxin (1 µM), making this protein about 20,000-fold more potent than glucose in provoking insulin release (25). Most of these effects, either *in vivo* or *in vitro*, apparently involve activation of arachidonic acid metabolism mainly through the lipoxygenase pathway, since they are blocked by lipoxygenase inhibitors such as nordihydroguaiaretic acid and esculetin, but not by cyclooxygenase inhibitors (Table 1) (21-23,25,26). Pretreatment of animals with lipoxygenase inhibitors protected them also against the lethal effect of canatoxin (26).

Canatoxin was shown to disrupt Ca^{2+} transport by the Ca^{2+},Mg^{2+}-ATPase of sarcoplasmic reticulum membrane vesicles (27)

and to alter Ca^{2+} flux across the plasma membrane of platelets through a verapamil-inhibitable Ca^{2+} channel (28). Canatoxin does not activate phospholipase C, and the intracellular calcium mobilization mediated by inositol 1,4,5-triphosphate does not play a role in platelet activation by this toxin. Preincubation of platelets with 8-bromo-guanosine 3',5'-cyclic monophosphate inhibited the canatoxin-evoked calcium influx, arachidonate release, ATP secretion, and cell aggregation, showing that the calcium influx is an early step in the mechanism of platelet activation by canatoxin, being modulated by cGMP (28).

Canatoxin displays pro-inflammatory activity (21). Thus, intraplantar injection of 50-300 µg canatoxin induced a dose-dependent rat hind-paw edema characterized after 3 h by an intense cellular infiltration at the site of administration. Pharmacological studies suggested that canatoxin-induced edema is a phenomenon mediated by several components. Initially histamine, serotonin, platelet aggregating factor, and prostaglandins play a role as agonists while lipoxygenase metabolites, probably leukotrienes, may account for the development of an intense cellular infiltration at the inflammatory site. Canatoxin also induced neutrophil migration into rat peritoneal and pleural cavities and into air pouches (29). This effect was dependent on the resident macrophage population and was inhibited by glucocorticoids but not by non-steroidal anti-inflammatory drugs. It has also been shown that rat macrophage monolayers treated with canatoxin release a neutrophil chemotactic factor (29). Mouse peri-

Table 1. Modulation of canatoxin-induced effects by inhibitors of the lipoxygenase pathway.

Model/Effect	Canatoxin EC_{50}	Inhibitor	Dose	% inhibition	Ref.
Rabbit platelets					
Aggregation	300 nM	NDGA	0.52 mM	50	22
		ETYA	0.02 mM	50	22
		BW755C	0.05 mM	50	22
5-HT secretion	300 nM	NDGA	0.5 mM	75	23
		Esculetin	0.1 mM	85	23
Rat brain synaptosomes					
5-HT secretion	500 nM	NDGA	0.2 mM	90	23
		Esculetin	0.1 mM	90	23
Dopamine secretion	2 µM	NDGA	0.5 mM	42	23
Rat pancreatic islets					
Insulin secretion	200 nM	NDGA	0.2 mM	76	25
		Esculetin	0.1 mM	36	25
Rat mast cells					
Histamine secretion	0.5 mM	Not tested	-	-	24
Mouse macrophages					
Release of lysosomal enzymes	0.3 mM	NDGA	0.15 mM	No inhibition	#
Rat - in vivo					
Hypoglycemia	0.4 mg/kg	NDGA	125 mg/kg	100	26
		Esculetin	125 mg/kg	100	26
Hyperinsulinemia	0.4 mg/kg	NDGA	125 mg/kg	100	20
Hypoxia	0.4 mg/kg	NDGA	125 mg/kg	72	26
		Esculetin	125 mg/kg	50	26
Paw edema	0.3 mg/paw	NDGA	100 mg/kg	66	21
		Esculetin	50 mg/kg	No inhibition	21
Convulsions	0.4 mg/kg	NDGA	125 mg/kg	75	26

5-HT = 5-hydroxytryptamine; NDGA = nordihydroguaiaretic acid; ETYA = eicosatetraynoic acid; BW755C = 3-amino-l-[m-(trifluoromethyl)-phenyl]-2-pyrazoline. [#]Ghazaleh FA, unpublished results.

toneal macrophages release lysosomal enzymes when exposed to canatoxin through a pathway involving nitric oxide (NO) signaling and guanyl cyclase activation (Ghazaleh FA, unpublished data).

In addition to lethality to insects, jack bean urease unexpectedly also displays other relevant biological properties observed for canatoxin, such as activation of blood platelets and monovalent lectin activity, but no toxicity when administered intraperitoneally to mice, probably due to its larger size (540 kDa as opposed to 185 kDa) (8). Canatoxin as well as urease interact with polysialogangliosides (GD1b and GT1b) and sialoproteins (mucin, tireoglobulin, fetuin) on the surface of erythrocytes and in ELISA microplates (7,8). This property of binding carbohydrates probably "directs" the proteins to cell surfaces enriched with this type of glycoconjugates and may provide an explanation for their selective tissue specificity. Pretreatment of the proteins with the thiol oxidant *p*-hydroxymercuribenzoate (pHMB) irreversibly abolished the ureolytic activity of urease (IC_{50} 0.5 mM) and of canatoxin (IC_{50} 5 mM) (8). In contrast, pHMB-treated canatoxin or urease was still fully active to promote platelet aggregation and binding to glycoconjugates. Moreover, the intraperitoneal toxicity of canatoxin was also not affected by pHMB treatment, indicating that these biological effects are not related to the enzymatic activity (8,10,13).

In order to determine if ureases from other sources share with jack bean ureases the property of inducing biological effects independent of their ureolytic activity, soybean embryo-specific urease and *B. pasteurii* urease (30) were tested in rabbit platelets. Both ureases induced platelet aggregation even after being treated with pHMB (13). Furthermore, purified recombinant *H. pylori* urease also displays platelet aggregating activity (Figure 2) (Wassermann GE, unpublished data).

The pattern of platelet response to all the ureases tested so far was very similar, with a collagen-type shape-change reaction. As is the case for canatoxin (22,23), platelet aggregation induced by *B. pasteurii* or *H. pylori* ureases also depends on lipoxygenase-derived metabolites. Thus, treatment of platelets with indomethacin, a cyclooxygenase inhibitor, potentiates urease-induced aggregation while the lipoxygenase inhibitor esculetin blocks platelet responses to the microbial enzymes (Figure 3).

Taken together, our data indicate that plant and microbial ureases form a group of multifunctional proteins with at least two distinct domains: 1) a thiol-dependent domain containing the ureolytic active site, and 2) thiol-independent domain(s) involved in toxic effects on insects (and mice, only for canatoxin), binding to glycoconjugates and in the activation of blood platelets.

In addition to the platelet aggregating activity found for *B. pasteurii* and *H. pylori*, these and other bacterial ureases may share other biological activities and pro-inflammatory properties with canatoxin, including the ability to activate lipoxygenases and the metabolism of eicosanoids. If true, these considerations could change our present understanding of the pathogenesis of some diseases caused by urease-producing bacteria, such as urolithiasis due to *Proteus mirabilis* or gastric ulcers consequent to *H. pylori* infection.

***Helicobacter pylori*, its urease and its implications in gastric diseases**

H. pylori is a micro-aerophilic spiral-shaped Gram-negative bacterium with polar flagella that colonizes the human stomach mucosa (31,32). The presence of this bacterium has now been established as the main risk factor in the development of diseases such as duodenal and stomach ulcers, gastric carcinomas and lymphomas (33,34). Gastric cancer is the first or second most common cancer in many developing countries, affect-

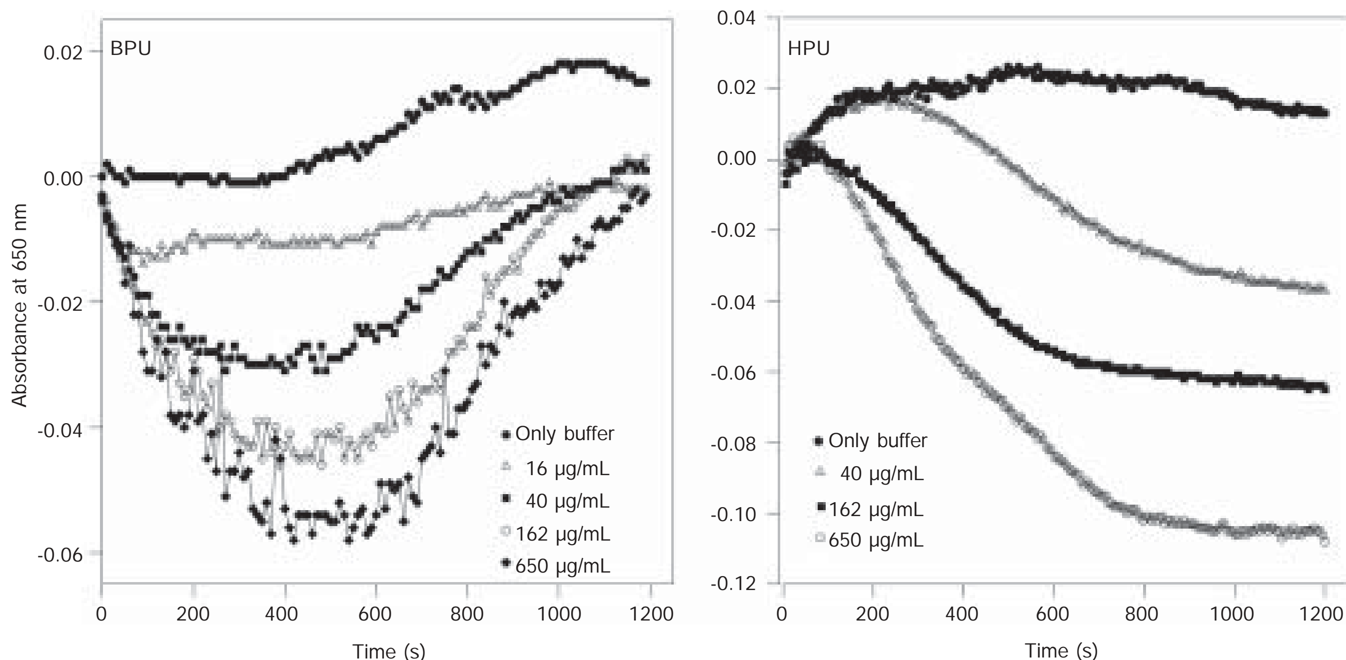

Figure 2. Dose-response curve of platelet aggregation induced by purified Bacillus pasteurii (BPU, left panel) and Helicobacter pylori ureases (HPU, right panel). Rabbit platelet-rich plasma suspensions were challenged with different concentrations of purified B. pasteurii (Sigma, St. Louis, MO, USA) or recombinant H. pylori (Ref. 44) ureases. The decrease in absorbance at 650 nm indicating platelet aggregation was monitored every 11 s for 20 min with a plate reader (13). Typical experiments are shown.

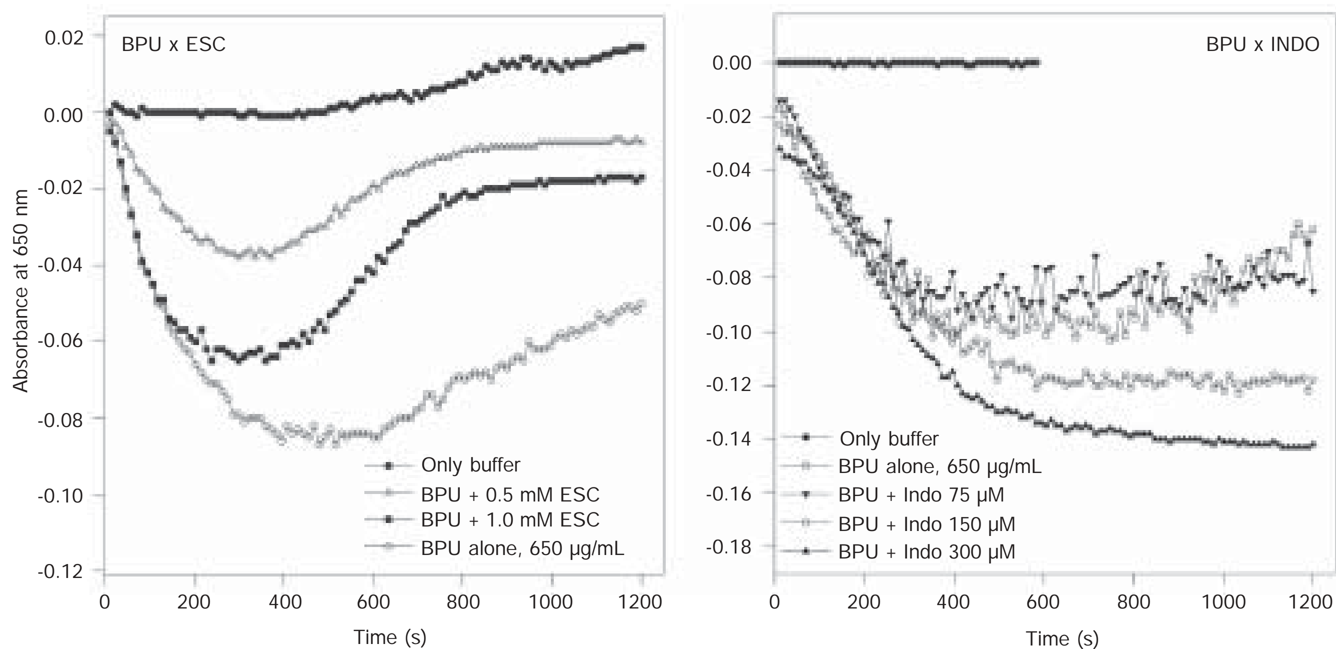

Figure 3. Effect of inhibitors of arachidonate metabolism on platelet aggregation induced by Bacillus pasteurii urease. Rabbit platelet-rich plasma suspensions were pretreated for 2 min with the indicated final concentrations of esculetin (ESC, left panel) and indomethacin (INDO, right panel) and then exposed to 0.7 mg/mL B. pasteurii urease (BPU; Sigma). The decrease in absorbance readings at 650 nm indicating platelet aggregation was monitored every 11 s for 20 min with a plate reader (13). Typical experiments are shown.

ing 40 to 60 persons per 100,000 yearly. Population-based intervention studies have shown the beneficial effect of early treatment of *H. pylori* in the prevention of gastric cancer. Notably, the most common lymphoma of the stomach, mucosa-associated lymphoid tissue (MALT) lymphoma, is strongly associated with *H. pylori* infection and *H. pylori* eradication therapy is today the widely accepted initial treatment of stage I gastric MALT lymphoma, leading to remission rates of as much as 70% (34).

H. pylori is estimated to infect half of the world's population, with peaks of 90% of the population infected in countries with poor sanitary conditions and of low socioeconomic level (33,34). The prevalence of the organism in the Brazilian population ranges from 34% among urban children in the richer Southeast region to over 82% among adults in the poorer Northeast region (35,36). For the discovery of *H. pylori* and demonstration of its association with gastric disease, the Australian gastroenterologists Warren and Marshall (31) received the 2005 Nobel Prize in Medicine.

H. pylori-induced gastroduodenal disease depends on the inflammatory response of the host and on the production of main virulence factors, such as the vacuolating cytotoxin VacA and the cytotoxin-associated protein CagA, and of urease, all of which cause damage to gastric epithelial cells (37-39). *H. pylori* resides within the mucus and on the apical surface of epithelial cells, where it attaches firmly via adhesin molecules. All *H. pylori* isolates, as well as each of the gastric *Helicobacter* species identified to date, produce large quantities of the enzyme urease, which may account for 10-15% of the bacterial protein. Urease and urea influx through UreI, a pH-gated urea channel, have been shown to be essential for gastric colonization and for acid survival *in vivo*. Intrabacterial urease generation of ammonia and a membrane-anchored periplasmic carbonic anhydrase regulate inner membrane potential and periplasmic pH to approximately 6.1 under acidic conditions, allowing adequate bioenergetics for survival and growth (40). Upon bacterial autolysis urease is released and adsorbed onto the extracellular surface of viable bacteria where it represents about 30% of the total cell urease content (38-39).

The native urease of *H. pylori* has a molecular mass of approximately 540 kDa and is a nickel-containing hexameric molecule consisting of two subunits, UreA (26.5 kDa) and UreB (60.3 kDa), in a 1:1 molar ratio (41,42). The residues involved in the coordination of the two active site Ni^{2+} ions are completely conserved between *H. pylori* and *K. aerogenes* ureases (42). The circular genome of *H. pylori* encodes about 1500 genes, depending on the strain (43). The biosynthesis of urease is coordinated by a gene cluster composed of two structural genes encoding the UreA and UreB subunits and five accessory proteins which are responsible for Ni^{2+} uptake and insertion into the active site of the apoenzyme (44). Active recombinant *H. pylori* urease was produced in *E. coli* transformed with pHP8080, a plasmid encoding the whole operon with the two subunit structural genes and the NixA nickel transporter (44).

Pathogenesis of gastroduodenal diseases and *Helicobacter pylori*

The exact mechanisms by which *H. pylori* contributes to the development of gastroduodenal injury is unclear. Studies have shown that *H. pylori* infection has no direct effect on basal or peak acid secretion, thus raising doubts as to the importance of hypergastrinemia in mucosal injury pathogenesis (38,39). The main role of urease is thought to be the neutralization of the acidic microenvironment by producing ammonia. A stable urease-negative ($ureB^-$) mutant strain was reported to be unable to colonize the stomach of nude mice (45). The co-inoculation of a urease-negative (ure^-) strain with a urease-positive strain in the

stomach of gnotobiotic piglets resulted in preferential colonization of the urease-positive bacteria (46), suggesting that neutralization of the gastric acidity is not the sole role of urease for colonization.

Several reports have demonstrated *H. pylori*-induced apoptosis in gastric epithelial cells *in vivo* and *in vitro* (47). Neutrophil apoptosis and subsequent clearance by phagocytes are critical to the resolution of acute inflammation (48), a process modulated by the NF-kappaB pathway and inflammatory regulators such as interleukin-8 (IL-8), lipopolysaccharide, or leukotriene B4 (48,49).

Although this organism is known to be noninvasive, *H. pylori* infection elicits gastric mucosal infiltration of inflammatory cells, especially neutrophils (38,50). Histological observation in humans has indicated that the degree of *H. pylori* infection and the severity of mucosal injury are directly correlated with the extent of neutrophil infiltration into the mucosa. Superfusion of the exposed mesentery with aqueous extracts of *H. pylori*, which are rich in urease and devoid of significant contamination by lipopolysaccharide from the cell wall, resulted in a three-fold increase in adherent leukocytes within the venules and a four-fold increase in those that had emigrated into the interstitium resulting in gastric mucosal injury (50). Kim and co-workers (51) reported that supernatants of *H. pylori* cultures enhance neutrophil degranulation and adhesion capacity and up-regulate IL-8 expression by activated neutrophils. Purified *H. pylori* urease was shown to directly activate primary human blood monocytes and to stimulate dose-dependent production of inflammatory cytokines (IL-1b, IL-6, IL-8, and tumor necrosis factor-alpha) (52). Recently, Enarsson et al. (53) reported that *H. pylori* induced significant T-cell migration in a model system using human umbilical vein endothelial cells. $CD4^+$ and $CD8^+$ T cells migrated to the same extent in response to *H. pylori*. Although the presence of a functional *cag* pathogenicity island contributed to the transendothelial migration, purified *H. pylori* urease alone induced a migration effect similar to that of whole live bacteria. On the other hand, mutant *H. pylori* negative for urease A subunit still promoted significant cell migration (53), suggesting that the ability of the bacterial urease to induce this effect may rely only on its B chain.

Inducible NO-synthesizing enzyme (iNOS) is expressed after activation by pro-inflammatory cytokines in macrophages, endothelial cells and other cells. NO acts as a messenger in various inflammatory pathways and contributes to defense mechanisms against microorganisms but can also have cytotoxic effects. iNOS levels are up-regulated in a subgroup of patients with chronic active gastritis (54). Recombinant *H. pylori* urease was shown to stimulate directly macrophage iNOS expression (55).

Ischemic lesions due to vascular insufficiency may lead to the development of ulcers within the gastric mucosa. Studies using fluorescent *in vivo* microscopy have shown that *H. pylori* infection alters blood flow, the endothelial lining of the vessels, and leukocyte activity and often induces the formation of circulating or adherent platelet aggregates, consistent with epidemiological studies that suggest a possible association between *H. pylori* infection and the incidence of cardiovascular diseases, as reviewed by Kalia and Bardhan in 2003 (56). So far, there are no published studies on the effects of purified *H. pylori* urease on platelets. On the other hand, it is well known that platelets participate in the inflammatory response by modulating the activity of other inflammatory cells and as a storage site of vasoactive substances and inflammatory mediators such as histamine, serotonin, platelet aggregating factor, thromboxane A2 and other eicosanoids, as well as by generating cytotoxic superoxide and hydroxyl radicals which may induce microcirculatory disturbances (56).

Histamine not only contributes to gastric

secretion but is also a major vasoactive mediator in microcirculatory physiology. A major source of histamine within the gastrointestinal tract is the mast cell and it was demonstrated that *H. pylori*, and in particular its cell wall materials, could potentiate secretogogue-induced histamine release from isolated mast cells (57).

H. pylori adhesion to the gastric mucosa represents the initial contact between the bacterium and its host. Numerous adhesive properties of *H. pylori* have been described, including hemagglutination, attachment to epithelial cells, and binding to oligosaccharides or proteins of the extracellular matrix. Several research groups have reported that *H. pylori* cells contain proteins that bind Neu5Ac (39,58).

Adhesins are bacterial proteins, glycoconjugates, or lipids involved in the initial stages of colonization mediating the interaction between the bacterium and the host cell surface. It is predicted by genome sequencing that *H. pylori* possesses a supergene family of 32 genes encoding putative outer membrane proteins. Among these, the bacterial adhesin BabA2 has been identified to bind human blood group antigen Lewis b in the gastrointestinal mucosa. Two other members of this family, Alp A and B, are necessary for *H. pylori* to attach to human gastric tissue. A sialic acid-binding adhesin, SabA, was identified using a sialyl-Lex saccharide as a probe. The adhesion of *H. pylori* to fibronectin and lactoferrin is not dependent on BabA or SabA activities because the *babA/sabA* double mutant still binds to these proteins. Thus, the presence of an additional binding activity of *H. pylori* has been suggested (58,59).

In general, adhesin receptors are carbohydrate moieties on glycoproteins or glycosphingolipids. Extracellular matrix proteins such as laminin and collagen type IV have been proposed as receptors for *H. pylori.* For cellular receptors, phosphatidylethanolamine, laminin, and sialic acid-containing molecules are regarded as potential receptors other than Lewis b. *H. pylori*-binding gangliosides and sialylated glycoproteins are present in relatively high amounts in human neutrophils (59). Bacterial binding to normal gastric cells may be through nonsialylated receptors, like Lewis b antigenic structures, or lactotetraose. However, the level of sialylated structures increases accompanying inflammation. Sialyllactose has been reported to inhibit binding of *H. pylori* to cultured gastrointestinal epithelial cells and chronic atrophic gastritis in mice has been shown to be associated with increased synthesis of Neu5Aca3Gal structures (58,59).

It has become increasingly clear that urease has other functions in the physiology of *H. pylori* besides alkalization of the medium. Icatlo's group (60) has shown that purified *H. pylori* urease binds to gastric mucin and sulfated cell membrane glycolipids in an acidic setting. This property is expressed independently of its ureolytic activity which requires pH above 5.0. The interaction of urease with sulfated glycoproteins, heparin and heparinoids at pH 4.0 was shown to be dose- and time-dependent, and affected by the pH and salt concentration of the medium.

Reports of antibiotic-resistant *H. pylori* clinical isolates are increasing. Therefore, specific drugs targeting factors important for bacterial colonization, such as urease and chemotaxis, may be useful to minimize generation of drug-resistant bacteria. Carbohydrates and their chemical analogs are relevant candidates for anti-adhesion therapy (58-60).

Perspectives

In view of our data on the biological activities of jack bean ureases, particularly the secretagogue, platelet-activating and pro-inflammatory effects described for the canatoxin isoform, our present research is based on the hypothesis that bacterial ureases display the same properties. There are several

lines of evidence that point in this direction for *H. pylori* urease. These include a) pro-inflammatory activity accompanied by mononuclear phagocyte activation and neutrophil chemotaxis; b) platelet aggregating activity; c) histamine release from mast cells; d) lectin-like activity towards sialic-acid-containing glycoconjugates. Most studies reported so far were carried out with whole *H. pylori* cells or non-fractionated aqueous extracts and therefore the conclusion of involvement of urease in these phenomena is merely circumstantial.

As reviewed here, similar findings have been reported for the plant urease canatoxin. Thus, it is possible that *H. pylori* urease may also have other biological activities presented by canatoxin, particularly the secretagogue effect modulated by eicosanoid metabolites through lipoxygenase pathways, dependent on verapamil inhibitable calcium channels, and involving cGMP and NO signaling. Another important aspect to be investigated is whether or not the biological activities displayed by *H. pylori* urease depend on its ureolytic activity. If proven to be true, these findings could be extremely relevant to the elucidation of mechanisms leading to gastrointestinal disease caused by this bacterium and should be taken into consideration in the development of more efficient therapeutic approaches.

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Capítulo II

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Bacillus pasteurii urease shares with plant ureases the ability to induce aggregation of blood platelets.

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Na quase totalidade dos modelos farmacológicos estudados, os efeitos biológicos da canatoxina parecem estar relacionados com a capacidade da proteína em ativar os sistemas secretórios de diversos tipos celulares (revisto em Olivera-Severo et al. 2006, capítulo I). Tal efeito secretagogo da canatoxina envolve mediação por metabólitos do ácido araquidônico via lipoxigenases.

Neste trabalho caracterizamos a propriedade da urease de *B. pasteurii* (BPU) em induzir agregação plaquetária, sendo menos ativa que a canatoxina (ED_{50} 0,4 e 0,015 mg/mL, respectivamente), mas com as mesmas características cinéticas e farmacológicas. O efeito agregante da BPU foi bloqueado em plaquetas pré-tratadas com dexametasona e esculetina, inibidores de fosfolipase A_2 e da 12-lipoxigenase, respectivamente. Por outro lado, plaquetas tratadas com indometacina, um inibidor de cicloxigenase, apresentaram um grande incremento na resposta de agregação por BPU. Plaquetas em presença de metoxi-verapamil (bloqueador de canais de cálcio) ou AMP (antagonista de ADP) tiveram uma resposta reduzida de agregação induzida por BPU, enquanto WEB 2170, um antagonista de "PAF-acether", não teve nenhum efeito. Estes dados nos permitem concluir que a agregação plaquetária induzida por BPU é mediada por eicosanóides derivados de lipoxigenase, e pela secreção de ADP pelas plaquetas ativadas, através de um mecanismo cálcio dependente. A potencial implicação desses resultados para as interações bactéria-planta foram discutidas, bem como relevância para a patogênese de infecções causadas por bactérias produtoras de urease.

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Bacillus pasteurii urease shares with plant ureases the ability to induce aggregation of blood platelets

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Abstract

Ureases (EC 3.5.1.5) are highly homologous enzymes found in plants, bacteria and fungi. Canatoxin, an isoform *Canavalia ensiformis* urease, has several biological properties unrelated to its ureolytic activity, like platelet-aggregating and pro-inflammatory effects. Here, we describe that *Bacillus pasteurii* urease (BPU) also induces aggregation of rabbit platelets, similar to the canatoxin-induced effect (ED_{50} 0.4 and 0.015 mg/mL, respectively). BPU induced-aggregation was blocked in platelets pretreated with dexamethasone and esculetin, a phospholipase A_2 and a lipoxygenase inhibitor, respectively, while platelets treated with indomethacin, a cyclooxygenase inhibitor, showed increased response to BPU. Methoxyverapamil (Ca^{2+} channel blocker) and AMP (ADP antagonist) abrogated urease-induced aggregation, whereas the PAF-acether antagonist Web2170 had no effect. We concluded that platelet aggregation induced by BPU is mediated by lipoxygenase-derived eicosanoids and secretion of ADP from the platelets through a calcium-dependent mechanism. Potential relevance of these findings for bacterium–plant interactions and pathogenesis of bacterial infections are discussed.
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Keywords: *Bacillus pasteurii*; Urease; Platelet aggregation; Canatoxin; *Canavalia ensiformis*; Lipoxygenase

Ureases (EC 3.5.1.5) are nickel-dependent enzymes found in plants, bacteria and fungi, that hydrolyze urea into ammonia and carbon dioxide [25,45]. Ureases from plants and fungi are homotrimers or hexamers of a ~90 kDa subunit, while bacterial ureases are multimers of two or three subunits complexes [46,55]. The N-terminal halves of plant or fungal urease monomers are similar to the small subunits of bacterial enzymes (e.g. β and γ chains of *Bacillus pasteurii* urease). The C-terminal portions of plant and fungal chains resemble the large subunits of bacterial ureases (e.g. α chain of *B. pasteurii* urease). The high sequence similarity of all ureases indicate they are variants of the same ancestral protein and are likely to possess similar tertiary structures and catalytic mechanisms [46]. Only bacterial ureases had their 3D crystallographic structure successfully resolved so far, e.g. *Klebsiella aerogenes* (1FWJ), *B. pasteurii* (4UBP), and *Helicobacter pylori* (1E9Z).

Some bacterial ureases play an important role in the pathogenesis of human and animal diseases such as those from *Proteus mirabilis* and *H. pylori* [46]. Urease activity enable bacteria to use urea as a sole nitrogen source [46]. *B. pasteurii* is a widespread soil bacterium remarkable for its large urease production [7]. *B. pasteurii* urease (BPU)[2] was purified [42] and crystallized [9] being a heteropolymer $(\alpha\beta\gamma)_3$ of three chains (α, 61.4 kDa; β, 14.0 kDa; γ, 11.1 kDa) featuring an active site containing two nickel ions [10]. *B. pasteurii* urease (2BPU) and jackbean (*Canavalia ensifor-*

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[2] *Abbreviations used:* BPU, *Bacillus pasteurii* urease; CNTX, canatoxin; AMP, adenosine monophosphate; ADP, adenosine diphosphate; D-600, methoxyverapamil; COX, cyclooxygenase; 12-LOX, 12-lipoxygenase; 12-HETE, 12-hydroxyeicosatetraenoic acid; TXA_2, thromboxane A_2; PAF platelet-aggregating factor.

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mis) major urease (P07374), the first protein ever crystallized [59], share 55% identity and 73% similarity despite the difference in their quaternary structures. The high ureolytic activity of living *B. pasteurii* cells and the presence of its urease free in the soil [24,51] enable the use of urea as a fertilizer. However, this widespread urease activity may also cause plant damage by ammonia toxicity and soil pH increase [16].

Despite the ubiquity of urease in virtually all plants, little has been revealed about its physiological roles [50,55]. We have shown that canatoxin (CNTX) [19], an isoform of jackbean (*C. ensiformis*) urease [28], displays several biological properties independent of its ureolytic activity, as activation of blood platelets, pro-inflammatory effect and interaction with glycoconjugates [5,6,11,18–20,35] and insecticidal activity, suggestive of a role in plant defense [18,21,27,30,31]. The insecticidal activity is due to a ~10 kDa internal peptide released from plant ureases upon digestion by insect cathepsins [27,49,58].

Canatoxin induces exocytosis in platelets, synaptosomes, pancreatic islets, macrophages, neutrophils, and mast cells. The protein disrupts Ca^{2+}-transport across membranes [1,35] and lipoxygenase metabolites modulate most its pharmacological effects [5,6,11,20]. Soybean and *B. pasteurii* ureases also induced aggregation of platelets in nanomolar concentrations independent of urease activity [30].

Platelet release reaction with secretion of ADP promotes aggregation of CNTX-stimulated platelets [20]. Blood platelets are anucleated secretory cells derived from megakaryocytes. In the event of vascular injury or exposition to agonists such as ADP, collagen or thrombin, the disc-shaped non-stimulated platelets become spherical (shape change) and adherent to each other and to surrounding tissues [26,52]. Platelets secrete two types of vesicles, the α-granules and the dense granules, whose contents contribute to hemostasis [3,37]. Primary reversible platelet aggregation induced by direct agonists such as ADP, PAF-acether or thromboxane A_2 does not require the release reaction. When platelets undergo the release reaction, the released ADP amplifies the secondary aggregation response [34].

Platelet agonist-coupled receptors activate membrane-bound phospholipase A_2 which hydrolyzes membrane phospholipids yielding free arachidonic acid, which is the precursor of eicosanoids either resulting from the cyclooxygenase pathway, such as thromboxane A_2, or from the lipoxygenase pathway, such as 12-hydroperoxy-eicosatetranoic acid, which mediate platelet's response to the agonist [2,38,47]. Platelets also synthesize from arachidonic acid the phospholipid 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine or platelet-aggregating factor (PAF-acether) which interacts as a direct agonist with its own receptors on the platelets [12,15].

Platelet ADP-receptors are P2-class G protein-coupled purinergic receptors which regulate Ca^{2+}-dependent events and the "shape-change" reaction [33,34,48]. ADP induces phospholipase C activation seconds after addition to platelets, through a receptor-mediated mechanism [33]. Elevated intracellular levels of Ca^{2+} are necessary for platelet aggregation and release reaction (exocytosis) resulting from influx of external Ca^{2+} through voltage-dependent channels, inhibition of Ca^{2+} ATPases and/or by the action of phosphatidylinositol-triphosphate upon intracellular Ca^{2+} pools [8,13,40].

In the present work, we studied the mechanism of action of platelet aggregation induced by *B. pasteurii* urease and compared it with canatoxin-induced platelet responses.

Materials and methods

The following drugs were obtained from Sigma Chemical Co., St. Louis, USA: adenosine monophosphate (AMP), adenosine diphosphate (ADP), esculetin, dexamethasone, indomethacin, and methoxyverapamil (D-600). PAF-acether (platelet-aggregating factor: 1-*O*-alkyl-2-acetyl-sn-glycero-phosphocholine) and Web 2170 (Bepafant; 5-(2-chloro-phenyl)-3,4-dihydro-10-methyl-3-[(4-morphol- inyl) carbonyl]-2H, 7H-cyclopenta (4,5) thieno [3,2-*f*] [1,2,4 triazolo-[4,3-*a*] [1,4] diazepine]) were a kind gift from Dr. João Baptista Calixto, Dept. Pharmacology, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil. Stock solutions were prepared as follows: dexamethasone and esculetin were dissolved in absolute ethanol and diluted in saline to give final concentrations of ethanol in the platelet assay of no more than 0.2% v/v; indomethacin was first dissolved in 0.1 M Na_2CO_3 then diluted with saline and adjusted to pH 6.0; ADP and AMP were diluted in Tris buffer, pH 8.2 and 6.0, respectively; PAF-acether was dissolved in a 0.1 w/v % bovine serum albumin solution and used in the same day; Web 2170 was dissolved in saline.

Bacillus pasteurii urease

Bacillus pasteurii urease (BPU) (194 U/mg of dry weight, U-7127, Sigma Chem. Co.) was used in all experiments. The freeze-dried protein was solubilized in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 5 mM 2-mercaptoethanol to give 0.5 mg protein/mL solutions.

Purification of jackbean ureases

The jackbean enzymes were purified from jack bean meal according to [31]. The final step of the purification protocol consists of an affinity chromatography on a Co^{2+} loaded iminodiacetic acid-Sepharose column. The major isoform of jackbean urease (JBU, ca. 85% total urease protein) is recovered in the non-retained fraction while the isoform corresponding to canatoxin (CNTX, ca.15% total urease protein) binds to the resin, being eluted with ammonium chloride [31]. CNTX and JBU solutions were dialyzed against 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 2 mM 2-mercaptoethanol to give 0.5 mg protein/mL solutions.

Protein determination

The protein content of samples was determined by their absorbance at 280 nm. Alternatively, the method of Spector [57] was used.

Urease activity

The ammonia released was measured colorimetrically by the alkaline nitroprussiate method [62]. One unit of urease releases 1 μmol of ammonia per minute, at 37 °C, pH 7.5.

Platelet aggregation

Platelet-rich plasma was prepared from rabbit blood collected from the ear central artery in the presence of sodium citrate to a final concentration of 0.313% (v/v). Blood samples were then centrifuged at 200*g* for 20 min at room temperature, to give a platelet-rich plasma suspension [20]. Platelet aggregation and shape change were monitored turbidimetrically by the method of Born and Cross [14], using a Lumi-aggregometer (Chrono-Log Co. Havertown, Pa.) and light transmission across the rabbit platelet-rich plasma suspension was registered on a chart recorder for 3 min. Platelet aggregation assays were also performed on a SpectraMax microplate reader (Molecular Devices, USA) as described [32]. Briefly, urease samples with or without potential inhibitors in 96-well flat-bottomed plates were completed to a final volume of 50 μL with saline. Aggregation was triggered by the addition of 100 μL of platelet suspension. The plate was incubated for 2 min at 37 °C before beginning of agitation and readings were performed at 650 nm every 11 s, during 20 min. When testing potential inhibitors, platelets and the compounds were pre-incubated for 2 min at 37 °C under stirring and aggregation was triggered by addition of BPU or control inducer (ADP or PAF-acether). Change in turbidity was measured in absorbance units, and results are expressed as area under the aggregation curves.

Statistical analysis

Data were analyzed by ANOVA followed by the Tukey–Kramer test using the Instat Graph Pad software and values of $p < 0.05$ (∗), $p < 0.01$ (∗∗) or $p < 0.001$ (∗∗∗) were considered statistically significant.

Results and discussion

Fig. 1 illustrates the pattern of aggregation of platelet-rich rabbit platelet suspensions triggered by a commercial preparation of *B. pasteurii* urease (BPU). Attempts at further purifying this commercial preparation showed no other fractions active upon platelets besides those containing urease activity (data not shown). However, contrasting to the commercial material, the highly purified enzyme was unstable making it very difficult to work with. We thus decided to conduct the experiments with the commercial enzyme without further purification. BPU induced aggregation of rabbit platelets with an ED_{50} of ca. 0.4 mg/mL, with a time course and collagen-type shape change reaction very similar those induced by canatoxin (ED_{50} 15.8 μg/mL) [20,28,30]. Table 1 shows that the ureolytic specific activity

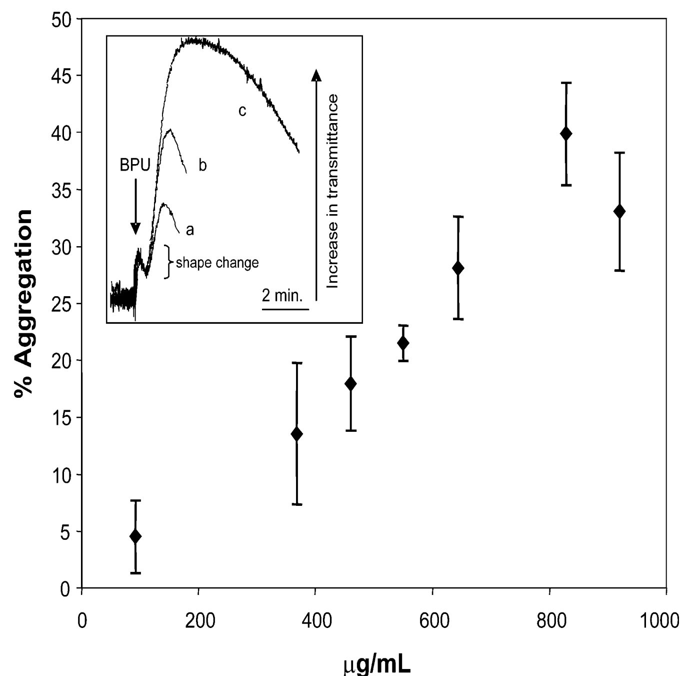

Fig. 1. Dose–effect curve of aggregation of rabbit platelet-rich plasma suspensions induced by *B. pasteurii* urease. Rabbit platelet-rich plasma suspension in microwell plates were exposed to increasing concentrations of BPU or 5 μM ADP (100% aggregation). Aggregation of platelets was monitored ever 11 s during 20 min in a SpectraMax plate reader. Results (means ± SD) are expressed as percentage of maximal aggregation for four replicates. The inset shows the aggregation reaction induced by BPU (arrow indicates addition of BPU) at 200 (a), 400 (b), and 800 (c) μg/mL concentrations as registered on a Lumi-aggregometer apparatus. Note the shape change reaction (decrease in transmittance) of platelets before the aggregation takes place.

Table 1
Comparison of ureolytic and platelet-aggregation inducing activities of three plant-derived ureases and *B. pasteurii* urease

Source/urease	Urease activity[a] (U/mg)	Platelet aggregation[b]	
		EC_{50} (μg/mL)	EC_{50} (mU/mL)
Canavalia ensiformis			
JBU	22.2 ± 0.7	15.8	0.350
CNTX	11.6 ± 3.2	15.8	0.183
Glycine max (soybean) SBU	14.2 ± 0.6	22.2	0.315
Bacillus pasteurii BPU	194.0	400	77.6

Data for JBU and SBU were taken from [28,30,31].
[a] One unit of urease activity was defined as the amount of enzyme releasing 1 μmol of ammonia per minute at 37 °C and pH 7.5.
[b] Aggregation of rabbit platelet-rich plasma suspension was measured turbidimetrically and the effective dose 50% (EC_{50}) of the proteins producing half-maximal aggregation in the conditions described was determined.

of BPU is about 8- to 15-fold greater than that of CNTX or jackbean urease [30]. Expressing the 50% effective dose in terms of urease activity gives an ED_{50} of 77 U/mL for BPU and 0.18 U/mL for CNTX, indicating that the ureolytic activity of the proteins is not related to their platelet-aggregating property. We have previously shown that treating jackbean ureases [28] or the soybean embryo-specific urease [30] with the irreversible inhibitor *p*-hydroxy-mercuribenzoate abolished their ureolytic activity but did not affect their ability to induce platelet aggregation, clearly demonstrating that these two biological activities are not related.

To elucidate the pathway(s) recruited by BPU to induce platelet aggregation, pretreatments of platelets with antagonists such as adenosine monophosphate (AMP), which blocks the receptor for adenosine diphosphate (ADP) [48], or Web 2170, an antagonist of platelet-activating factor (PAF-acether), were carried out. Fig. 2 shows that 1 mM AMP was able to inhibit 70% the extent of BPU-induced aggregation while Web2170 [22] had no significant inhibitory effect. These results indicate that BPU-induced aggregation does not require synthesis and release of PAF-acether. In contrast, the inhibitory effect of 1 mM AMP indicated that at least part of the aggregation response to BPU is mediated by ADP released from the stimulated platelets. ADP-induced platelet aggregation is known to involve activation of P2 purinergic receptors which are

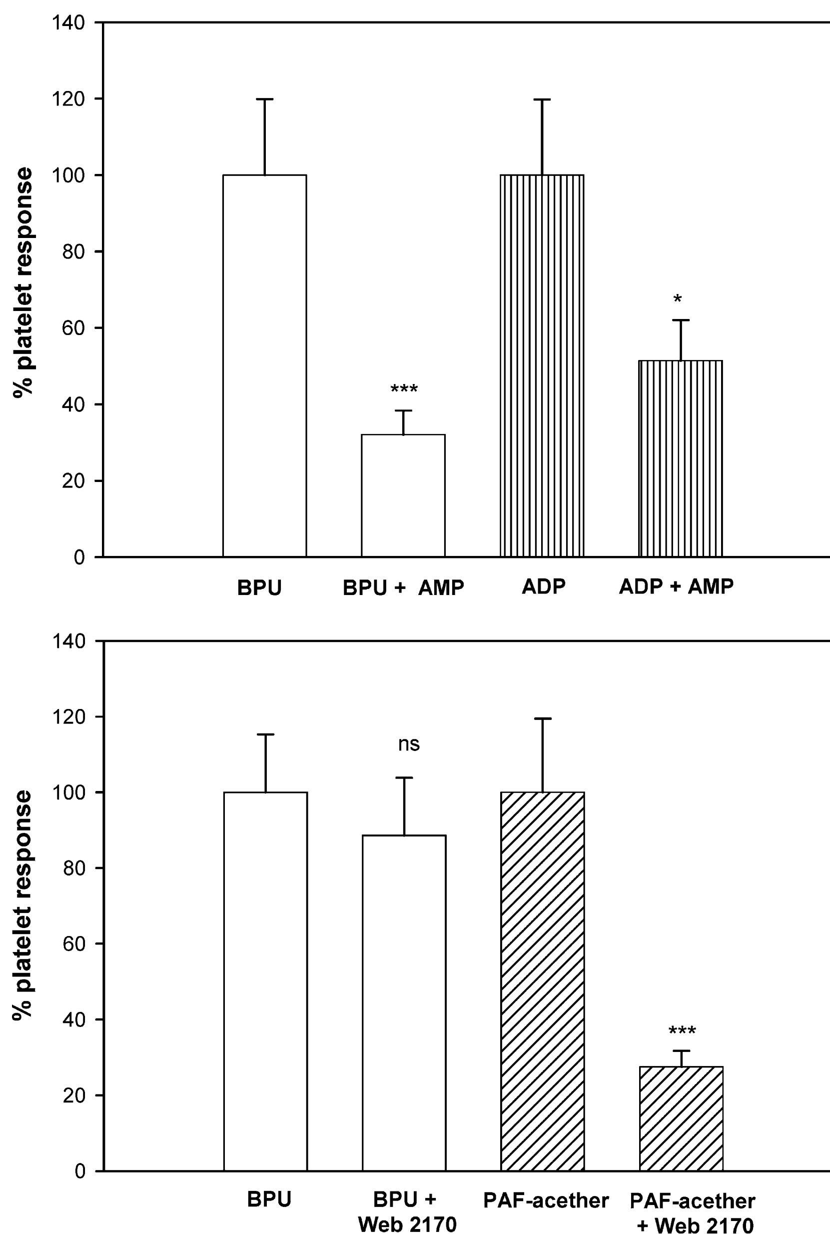

Fig. 2. Effect of adenosine monophosphate (AMP) (upper panel) or Web 2170 (a PAF antagonist) (lower panel) on the BPU-induced platelet aggregation. Rabbit platelet-rich plasma suspension in microwell plates were exposed to BPU (0.7 mg/mL), 5 μM ADP or 50 nM PAF (100% aggregation), in the presence or absence of the indicated concentrations of AMP or Web 2170. Aggregation of platelets was monitored ever 11 s during 20 min in a SpectraMax plate reader. Results (means ± SD) are expressed as percentage of maximal aggregation for four replicates. Values of $*p < 0.05$ or $***p < 0.001$ were considered statistically significant and ns is not significant.

competitively blocked by AMP [34,48]. In a previous study, we have demonstrated that CNTX-induced platelet aggregation also does not require PAF-acether and depends on the ADP secreted by stimulated platelets being inhibited by AMP and by the creatine phosphokinase/creatine phosphate scavenger system. Moreover thrombin-degranulated platelets were shown to be irresponsive to CNTX [20].

The involvement of arachidonic acid metabolites in BPU-induced platelet aggregation was investigated in platelets pretreated with dexamethasone (a phospholipase A_2 inhibitor), or indomethacin (a cyclooxygenase inhibitor) or esculetin (a 12-lipoxygenase inhibitor). Fig. 3 shows that dexamethasone blocked aggregation induced by BPU, indicating a requirement of free arachidonic acid. In indomethacin-treated platelets, BPU-induced aggregation was augmented up to 175%, excluding the participation of thromboxane A_2, an indirect product of cyclooxygenase activity in the aggregation response. In esculetin-pretreated platelets, BPU-induced aggregation was reduced up to 70% indicating that product(s) of the 12-lipoxygenase, which is specifically inhibited by this compound [54], mediated platelet's response to the protein. This agrees with the potentiation of BPU-induced aggregation of indomethacin-treated platelets as more arachidonic acid would be available for the 12-lipoxygenase. Thus, similar to what we described previously for CNTX [6,20], platelet aggregation induced by *B. pasteurii* urease is also mediated by lipoxygenase-derived eicosanoids.

In platelets treated with methoxyverapamil (D-600), a blocker of voltage-dependent Ca^{2+}-gated channels, the aggregating effect of BPU was greatly diminished, as shown in Fig. 4. In CNTX-stimulated platelets we also observed inhibition (IC_{50} 25 μM) of aggregation and release reaction by D-600, indicating that these responses depend on the influx of external Ca^{2+} into the platelets [35]. We also showed that CNTX-induced aggregation was independent of activation of phospholipase C and the phosphatidyl-inositol pathway [35].

Fig. 5 summarizes our present knowledge on the mechanism of platelet aggregation induced by *B. pasteurii* urease. Except for its lower potency compared to CNTX, as indicated by their respective ED_{50}, both proteins activate rabbit platelets through a Ca^{2+}-dependent mechanism involving arachidonic acid metabolite(s) of the 12-lypoxygenase enzyme leading to exocytosis of platelet's dense granule and culminating with the secreted ADP promoting aggregation.

Thus, in spite of the tri-chain structure as compared to plant ureases consisting of single chain oligomers, the platelet-aggregating activity (a model for exocytosis) is a common feature of bacterial and plant ureases. Preliminary data of our group indicate that other bacterial ureases, such as *H. pylori* urease, also display platelet-aggregating properties. This fact contrast with the lack of insecticidal activity reported for BPU [30]. We previously demonstrated that this biological property relies on an internal peptide of plant ureases which is partially absent in the microbial enzymes, corresponding to a fragment located between UreB and UreC chains of *B. pasteurii* urease [27,30]. The fact that bacterial and plant ureases evolutionarily conserved the property of inducing exocytosis in some cell types independent of ureolytic activity may shed new lights into the so far poorly understood biological functions of these proteins.

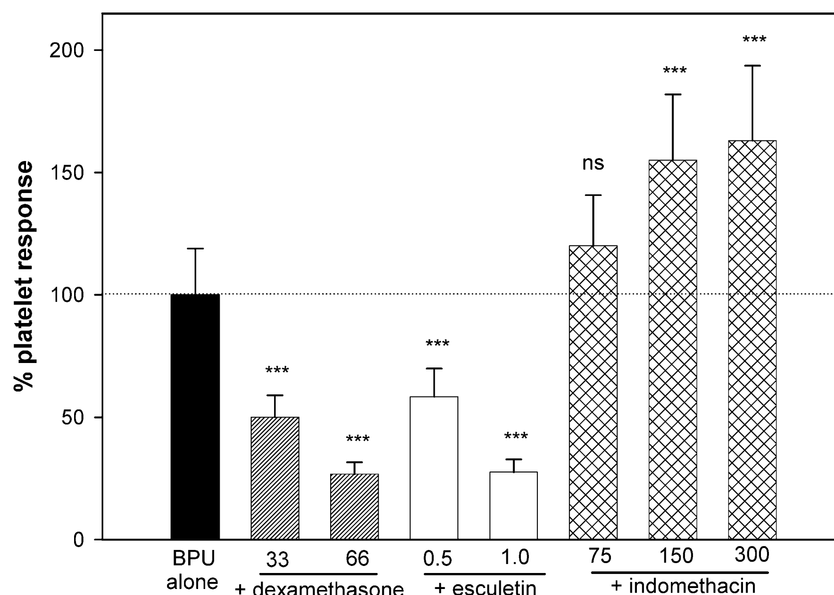

Fig. 3. Effect of inhibitors of platelet phospholipase A_2 or arachidonic acid metabolism on the BPU-induced platelet aggregation. Rabbit platelet-rich plasma suspensions in microwell plates were incubated for 2 min at r.t. in the presence or absence of the indicated concentrations of the drugs and aggregation was triggered by addition of BPU (0.7 mg/mL). Aggregation of platelets was monitored ever 11 s during 20 min in a SpectraMax plate reader. Results (means ± SD) are expressed as percentage of maximal aggregation for four replicates. Values of $***p < 0.001$ were considered statistically significant and ns is not significant.

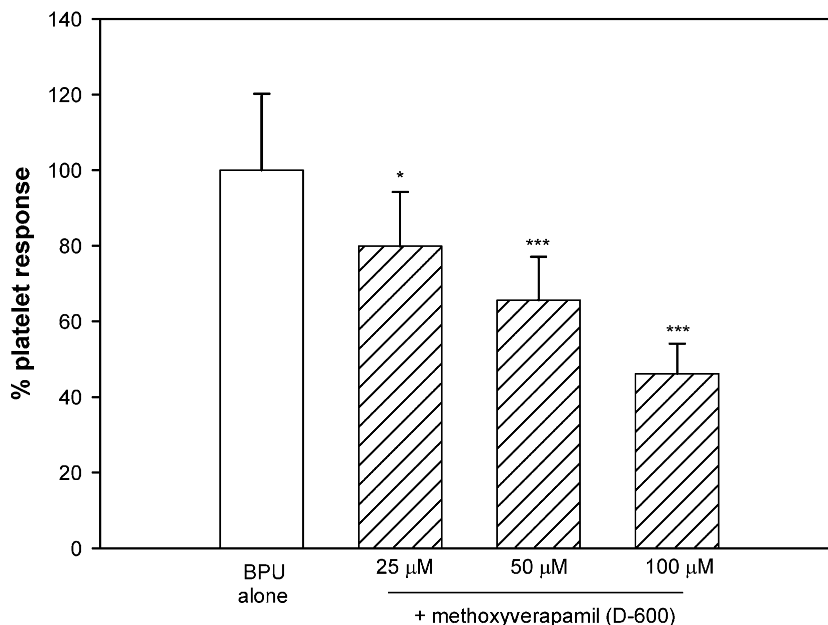

Fig. 4. Effect of methoxyverapamil (D-600), a blocker of voltage-gated Ca^{2+} channels, on the BPU-induced platelet aggregation. Rabbit platelet-rich plasma suspensions in microwell plates were incubated for 2 min at r.t. in the presence or absence of D-600 at the indicated concentrations and aggregation was triggered by addition of BPU (0.7 mg/mL). Aggregation of platelets was monitored ever 11 s during 20 min in a SpectraMax plate reader. Results (means ± SD) are expressed as percentage of maximal aggregation for four replicates. Values of $*p < 0.05$ or $***p < 0.001$ were considered statistically significant.

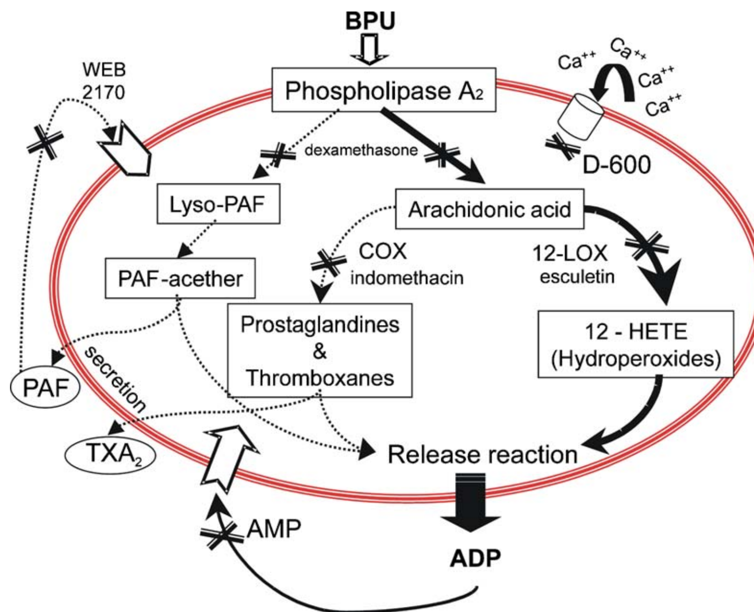

Fig. 5. Proposed mechanism of platelet aggregation induced by *Bacillus pasteurii* urease. The biochemical pathways that underlie the platelet-aggregating activity of *Bacillus pasteurii* urease (BPU) are indicated as continuous lines. BPU activates platelets through a phospholipase A_2 and calcium-dependent pathway that makes arachidonic acid available for the 12-lipoxygenase enzyme and leads to secretion of platelet's dense granules. The ADP contained in these granules is released into the medium and triggers aggregation of platelets. Dotted lines indicate other pathways tested in this work that are not relevant to BPU-induced platelet aggregation. Inhibition sites of pathways are marked by (**X**).

The ability to secrete a vast array of compounds is a remarkable metabolic feature of plant roots, with as much as 20% of the photosynthetically fixed carbon being transferred to the rhizosphere through root exudates [43,61]. Roots actively secrete high-Mr compounds forming a mucilage, which consist of 95% polysaccharides and 5% protein [36,44,53]. The mucilage is secreted largely from the root cap, but the root epidermis, including root hairs, also release mucilage [36,60,61]. It has been shown that urease activity is significantly protected from thermal denaturation and proteolysis by immobilization on Ca^{2+}-polygalacturonate gels such as those produced by roots [4,23]. Thus it is conceivable that extracellular BPU present in the vicinity of roots is active upon nearby cells, e.g.

root hairs, inducing or potentiating the secretion of mucilage that would ultimately be consumed by living *B. pasteurii* cells. Studies have shown that 64–86% of the carbon released by roots into the rhizosphere are respired by microorganisms [17,39,41,61]. On the other hand, beside its role in the mobilization of urea N for the plant, BPU may also affect mineral availability for to the plant by increasing the soil pH and inducing $CaCO_3$ precipitation [4,23,56]. Moreover, ureases are nickel-containing proteins [25] and we have shown that the jackbean urease, and probably BPU, specifically binds copper [29]. Thus, the presence of BPU in the soil may result beneficial to both, bacterium and plant. Studies are under way in our laboratory to investigate the effects of BPU upon plant protoplasts.

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Capítulo III

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Helicobacter pylori Urease Shares with Plant Ureases the Ability to Activate Blood Platelets and Edematogenic Activity

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A urease de *H. pylori* é considerada um fator de virulência, sendo a sua atividade enzimática um marcador utilizado amplamente para diagnóstico. Supõe-se que a principal função dessa enzima está relacionada com a formação, no lúmen gástrico, de um microclima neutro favorável à sobrevivência do microrganismo.

A infecção pelo *H. pylori* causa uma resposta inflamatória persistente da mucosa gástrica (Kalia & Bardan, 2003) que pode progredir para o adenocarcinoma distal, linfoma MALT ou úlcera páptica, bem como ao comprometimento de outros sistemas fisiológicos. O *H. pylori* tem sido associado, ainda, a doenças extra-gástricas, como por exemplo a púrpura trombocitopênica imunológica, uma doença que leva à problemas de coagulação causados por deficiência de plaquetas, sendo que a erradicação da bactéria acompanha a regressão do quadro hemorrágico em parte dos pacientes (40-50%) (Emilia et al., 2001, 2002). Outros estudos mostram ainda que alterações na microcirculação da mucosa gástrica podem levar a danos na mucosa, tanto por ativação de espécies reativas de oxigênio, quanto por infartos causados por agregados de plaquetas e linfócitos (Kalia & Bardan, 2003). Considerando a atividade pró-agregante de plaquetas de ureases vegetais e de uma bactéria não patogênica, descritas nessa tese (capítulos I e II), nosso objetivo foi explorar a hipótese da urease de *H. pylori* também apresentava tais propriedades.

Neste trabalho caracterizamos a atividade pró-agregante em plaquetas e pró-inflamatória mediada pela urease recombinante de *H. pylori* e também mostramos que esses processos, como descrito anteriormente para a canatoxina, estão relacionados com eicosanóides da via das lipoxigenases e com alterações em canais de cálcio.

Nature Cell Biology

Helicobacter pylori urease shares with plant ureases blood platelet aggregating and edematogenic activities.

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http://www.ufrgs.br/laprotox

Running title: Platelet activation and edematogenic effect of *Helicobacter pylori* urease.

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Abstract (max 150 words)

The bacterium *Helicobacter pylori* causes peptic ulcers and gastric adenocarcinoma in humans by mechanisms not yet fully understood. *H. pylori* produces urease which neutralizes the acidic medium permitting its survival in the stomach. Ureases (EC 3.5.1.5) are enzymes that hydrolyze urea into ammonia and CO_2. We have shown ureases from jackbean, soybean or *Bacillus pasteurii* induce blood platelet aggregation independently of their enzyme activity by a pathway requiring platelet secretion, activation of eicosanoid metabolism and of calcium channels. *H. pylori* urease (HPU) also induces ATP secretion and aggregation of rabbit platelets (ED_{50} 0.15 mg/mL). HPU-induced platelet aggregation was blocked by lipoxygenase inhibitors, but not by a cyclooxygenase inhibitor, which resulted in increased response. We also show that HPU induces mouse paw edema in an eicosanoid-dependent manner. These newly described pharmacological properties of HPU could play an important role in the pathogenesis of the gastroduodenal disease caused by *H. pylori*.

Keywords: *Helicobacter pylori* – urease – platelet aggregation – canatoxin – paw edema– lipoxygenase

Introduction

Ureases (EC 3.5.1.5) are highly homologous nickel-dependent enzymes widespread among plants, bacteria and fungi, that hydrolyze urea into ammonia and carbon dioxide (Dixon et al. 1975; Mobley and Hausinger 1989). Plant and fungal ureases are homotrimers or hexamers of a ~90 kDa subunit, while bacterial ureases are multimers of two or three subunits complexes (Mobley, Island, and Hausinger 1995; Sirko and Brodzik 2000). The N-terminal halves of plant or fungal urease single chain align with the primary sequence of the small subunits of most bacterial enzymes (e.g. β and γ chains of *Bacillus pasteurii* urease or the A subunit of *Helicobacter pylori* urease). The C-terminal portions of plant and fungal chains resemble the large subunits of bacterial ureases (e.g. α chain of *B. pasteurii* urease or the B subunit of *H. pylori* enzyme). Considering the similarity in their sequences, all ureases are likely to possess similar tertiary structures and catalytic mechanisms indicating they are variants of the same ancestral protein (Mobley, Island, and Hausinger 1995). The 3D crystallographic structures of three bacterial ureases were successfully resolved: *Klebsiella aerogenes* (1FWJ), *B. pasteurii* (4UBP), and *H. pylori* (1E9Z).

Urease activity enables bacteria to use urea as a sole nitrogen source (Mobley, Island, and Hausinger 1995). Some bacterial ureases play an important role in the pathogenesis of human and animal diseases such as those from *H. pylori* or *Proteus mirabilis* (Mobley, Island, and Hausinger 1995). *H. pylori*, a Gram-negative bacterium that colonizes the human gastric mucosa, causes peptic ulcer and gastric adenocarcinoma by mechanisms that not completely understood yet (Fischbach, Chan, and Wong 2005). This bacterium

produces factors that damage gastric epithelial cells, such as the vacuolating cytotoxin VacA, the cytotoxin-associated protein CagA, and a urease (up to 10% of bacterial protein) that neutralizes the acidic medium permitting its survival in the stomach. *H. pylori* urease (1E9Z) and jackbean (*Canavalia ensiformis*) major urease (P07374), the first protein ever crystallized (Sumner, 1926), share about 50 % identity despite the difference in their quaternary structures. The gastro-duodenal illness induced by *H. pylori* depends on the host inflammatory response elicited by the several virulence factors produced by the microorganism. There are reports showing that *H. pylori* whole cells or extracts of its water-soluble proteins promote inflammation, activate neutrophils and release cytokines. Recent reviews on *H. pylori* and its involvement in stomach illness were published (Olivera-Severo, Wasserman, and Carlini 2006b; Kabir, 2007)

The physiological role of urease in plants is still largely unknown despite its ubiquity in virtually all plants (Polacco and Holland 1993; Sirko and Brodzik 2000). Jackbean and soybean ureases display fungicidal and insecticidal activity, suggestive of a role in plant defense (Carlini et al. 1997; Ferreira-DaSilva et al. 2000; Carlini and Grossi-de-Sá 2002; Follmer et al. 2004a, b). The insecticidal activity is due to a ~10 kDa internal peptide released from plant ureases upon digestion by insect cathepsins (Ferreira-DaSilva et al. 2000; Mulinari et al. 2004; Staniscuaski et al. 2005).

We have previously reported that canatoxin (Carlini and Guimarães 1981), an isoform of jackbean (*C. ensiformis*) urease (Follmer et al. 2001), presents biological properties that are independent of its enzyme activity, as binding to sialylated glycoconjugates, activation of blood platelets and pro-

inflammatory effect (Carlini and Guimaraes 1981; Carlini et al. 1985; Barja-Fidalgo et al. 1991b; Benjamin et al. 1992; Ghazaleh et al. 1997; Follmer et al. 2001). Submicromolar concentrations of canatoxin induced exocytosis in a number of cell system *in vitro* including platelets, synaptosomes, pancreatic islets, macrophages, neutrophils and mast cells. Canatoxin also induced hypothermia, bradycardia, hypoxia, hypoglycemia, hyperinsulinemia and paw edema in rats and mice, preceding convulsions and death of the animals. Canatoxin disrupted Ca^{2+}-transport across membranes (Alves et al. 1992; Ghazaleh et al. 1997) and lipoxygenase metabolites were shown to modulate most of its pharmacological effects (Carlini et al. 1985; Barja-Fidalgo, et al. 1991a; 1991b; Benjamin et al. 1992; Olivera-Severo et al. 2006b), either in vivo or in vitro. More recently we reported that jackbean, soybean and *B. pasteurii* ureases also induced aggregation of platelets in nanomolar concentrations independently of enzyme activity (Follmer et al. 2004; Olivera-Severo et al. 2006a).

Blood platelets are anucleated disc-shaped cells derived from megakaryocytes. Upon vascular injury or exposition to agonists such as ADP, collagen or thrombin, non-stimulated platelets become spherical (shape change) and adherent to each other and to surrounding tissues (Erusalimsky and Martin 1996; Ruggeri 1997). Stimulated platelets may undergo release reaction, with exocytosis of α-granules and dense granules, whose contents contribute to hemostasis (Hawiger 1989; Andrews et al. 2004). Primary reversible platelet aggregation induced by direct agonists such as ADP, PAF-acether or thromboxane A_2 does not require the release reaction. When platelets secrete ADP it amplifies the aggregation response (Gachet et al.

1997). Elevated intracellular levels of Ca^{2+} are necessary for platelet aggregation and secretion resulting from external Ca^{2+} influx through voltage-dependent channels, inhibition of Ca^{2+} ATPases and/or release of intracellular Ca^{2+} pools by the action of phosphatidylinositol-triphosphate (Belville et al. 1979; Blache et al. 1987; Jackson et al. 2003).

Platelet membrane-bound phospholipase A_2 activated by agonist-coupled receptors hydrolyzes phospholipids into free arachidonic acid, which serves as substrate for the synthesis of eicosanoids either resulting from the cyclooxygenase pathway, such as thromboxane A_2, or the lipoxygenase pathway, such as 12-hydroperoxy-eicosatetranoic acid, which in turn mediates platelet's response to the agonist (Moncada and Vane 1979; Hawiger 1995; Andrews and Berndt 2004). Platelets also synthesize platelet-aggregating factor (PAF-acether, 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine) from arachidonic acid which interacts with its own receptors on the platelets (Benveniste et al. 1982; Braquet, Bourgain, and Mencia-Huerta 1989).

Paw edema is a well accepted model of the inflammatory process (Bertelli and Soldani, 1979). We have previously shown (Benjamin et al. 1992) that intraplantar injection of canatoxin induced a dose-dependent rat hind-paw edema which was distinguished by two phases. In the initial 2nd hour after canatoxin injection, the increase in paw volume apparently did involve inflammatory phagocytic cells. The second phase after the 3rd hour was characterized by an intense cellular infiltration and a further increase in paw swelling. CNTX-induced edema is a multi-mediated phenomenon with histamine, serotonin, PAF and prostaglandins are likely involved in the first phase, while lipoxigenase metabolites, probably leukotrienes, may account for

the development of cellular infiltration in the inflammatory site during the second phase.

In the present work we studied the mechanism of action of platelet activation and mouse paw edema induced by *H. pylori* urease and compared these pharmacological activities with those previously reported for the plant urease, canatoxin.

Results

Figure 1 illustrates the pattern of aggregation of platelet-rich rabbit platelet suspensions triggered by *H. pylori* urease (HPU) and two physiological agonists, ADP and collagen. HPU induced aggregation of rabbit platelets with an ED_{50} of ca. 150 μg/mL (0.28 μM), with a time course and collagen-type shape change reaction very similar to those induced by canatoxin or the major jackbean urease (ED_{50} 15.8 μg/mL) (Follmer et al. 2001; Carlini et al. 1985; Follmer et al. 2004a). We have previously shown that treating jackbean ureases (Follmer et al. 2001) or the soybean embryo-specific urease (Follmer et al. 2004a) with the irreversible inhibitor *p*-hydroxy-mercuribenzoate abolished their ureolytic activity but did not affect their ability to induce platelet aggregation, clearly demonstrating that these two biological activities are not related. Platelet aggregation induced by *B. pasteurii* urease also does not correlate with the protein's enzyme activity (Olivera-Severo et al. 2006a), as it is also the case for HPU (Table 1).

Figure 2a shows that platelets stimulated by HPU undergo release reaction and secrete ATP from dense granules. Platelets stimulated by 0.3 μM HPU secreted about 60 % of the ATP secreted on a collagen-induced release

reaction with a slower kinetics and longer lag phase and peaking after 3 min. Scanning electron microscopy of platelets exposed to HPU showed a morphology typical of activated platelets, the cells clumped together with numerous pseudopods and no signs of cell lysis (Figure 2b).

To elucidate the pathway(s) recruited by HPU to induce platelet aggregation we investigated the involvement of arachidonic acid metabolites in platelets pretreated with dexamethasone (a phospholipase A_2 inhibitor), or indomethacin (a cyclooxygenase inhibitor) or esculetin (a 12-lipoxygenase inhibitor). Figure 3 shows that dexamethasone reduced aggregation induced by HPU, indicating a requirement of free arachidonic acid. In indomethacin-treated platelets, HPU-induced aggregation was augmented up to 3 fold, excluding the participation of thromboxane A_2, an indirect product of cyclooxygenase activity in the aggregation response. HPU-induced aggregation was reduced in esculetin-pretreated platelets, indicating that product(s) of the 12-lipoxygenase, which is specifically inhibited by this compound (Sekiya et al. 1982, Matsunaga et al. 1998) mediated platelet's response to the protein. The potentiation of HPU-induced aggregation in indomethacin-treated platelets was expected as more arachidonic acid would be available as substrate for the 12-lipoxygenase. Thus, similar to what we described previously for CNTX (Carlini et al. 1985; Barja-Fidalgo et al. 1991b), and *Bacillus pasteurii* urease (Olivera-Severo et al. 2006a), platelet aggregation induced by *H. pylori* urease is also mediated by lipoxygenase-derived eicosanoids.

To investigate whether HPU possesses pro-inflammatory activity the model of mouse paw edema was used. Figure 4 shows the time course and dose-dependency curves of paw edema induced by recombinant HPU. As low

as 0.5 μg of protein injected into the mouse hind paw produced a significant edema after 2 hours with maximal edema at 4-6 hours. For higher doses the edema peaked at 4-6 hours and lasted more than 24 hours. In mice pre-treated with dexamethosone or esculetin (Figure 5), there was a significant reduction in the paw edema indicating that eicosanoids, particularly lipoxygenase metabolites, mediate the pro-inflammatory activity of HPU.

Discussion

Recently we reported on the platelet aggregating activity of the trichain urease from *Bacillus pasteurii* (Olivera-Severo et al. 2006a). Here we reported the same property for the two-chain HPU. Thus, independent of their quartenary structures, the property of activating blood platelets inducing aggregation and release reaction (a model for exocytosis) is a common feature of bacterial and plant ureases. There is no correlation between the enzyme activity and the ability of ureases to stimulate platelets. Platelet aggregation induced by all three ureases is consequent to the release of ADP from dense granules, stored together serotonin (BarjaFidalgo et al. 1991b; Carlini et al. 1985) and ATP (this work) which could be detected in the medium. The responses of platelets to all three proteins were inhibited by different inhibitors of the endogenous phospholipase A_2 and of the platelet 12-lipoxygenase. In agreement with a modulation by lipoxygenase metabolites, indomethacin pre-treated platelets showed significantly enhanced reactivity to ureases (BarjaFidalgo et al. 1991b; Carlini et al. 1985, this work). Platelet aggregation induced by canatoxin and *B.*

pasteurii does not involve PAF-acether receptors (BarjaFidalgo et al. 1991; Carlini et al. 1985) (Olivera-Severo et al. 2006a) and requires activation of voltage-gated Ca^{2+} channels inhibitable by verapamil (Ghazaleh et al. 1997) (Olivera-Severo et al. 2006a). Thus the same behavior can be expected from HPU-stimulated platelets. As reviewed by Kalia and Bardhan 2003, fluorescent *in vivo* microscopy studies have shown that *H. pylori* infection alters blood flow, the endothelial lining of the vessels, and leukocyte activity and often induces the formation of circulating or adherent platelet aggregates, consistent with epidemiological studies that suggest a possible association between *H. pylori* infection and the incidence of cardiovascular diseases. On the other hand, platelets participate in the inflammatory response by modulating the activity of other inflammatory cells and ischemic lesions due to vascular insufficiency may contribute to ulcers within the gastric mucosa (Kalia and Bardhan 2003).

Pro-inflammatory activity is also a common property shared by canatoxin, a plant urease, and the bacterial urease HPU. The time-course of HPU-induced mouse paw edema is very similar to the rat paw edema induced by intraplantar injections of canatoxin (Benjamin et al. 1992). HPU is about 10-fold more potent in inducing paw edema, although differences in inflammatory reactions of animal models have also to be considered. We have shown that canatoxin-induced rat paw edema is a multi-mediated phenomenon with two phases (Benjamin et al. 1992). During the first two hours, histamine and serotonin are major contributions to the inflammatory process induced by canatoxin. After that, the paw edema is accompanied by an intense cellular infiltration. Polymorphonuclear phagocytes predominate in the tissues up to 6 hours after canatoxin injections, while mononuclear cells were seen at 24 and 48 h (Barja-

Fidalgo et al. 1992; Benjamin et al. 1992). As described for canatoxin, eicosanoids derived from lipoxygenase(s) pathways play an important role in HPU-induced inflammation, as evidenced by the reduction in paw edema in mice pre-treated with esculetin.

Purified *H. pylori* urease was shown to directly activate primary human blood monocytes and to stimulate dose-dependent production of inflammatory cytokines (Harris et al. 1996). Enarsson et al. 2005 reported that *H. pylori* induced significant T-cell migration in a model system using human umbilical vein endothelial cells. Purified *H. pylori* urease alone induced a migration effect similar to that of whole bacteria. On the other hand, mutant *H. pylori* negative for urease A subunit still promoted significant cell migration which the authors imparted to a contribution of the functional *cag* pathogenicity island to the transendothelial migration (Enarsson et al. 2005). Another interpretation of the data would be that the ability of the bacterial urease to induce this effect relies only on its B chain. Interestingly, polyclonal anti-canatoxin antibodies recognize only the B chain of HPU (result not shown), suggesting these parts of the jackbean and *H. pylori* urease molecules have common antigenic epitopes and conserved a higher degree of homology than their corresponding N-terminal moieties.

The fact that bacterial and plant ureases evolutionarily conserved the property of inducing exocytosis in some cell types independent of ureolytic activitiy may shed new lights into the so far poorly understood biological functions of these proteins. Another important aspect to be investigated is whether or not other biological activities displayed by *H. pylori* urease depend on its ureolytic activity. This finding and the modulation of its pro-inflammatory

activity by lipoxygenase-derived eicosanoids could be particularly relevant to the elucidation of mechanisms leading to gastroduodenal disease caused by this bacterium and should be taken into consideration in the development of more efficient therapeutic approaches.

Materials and Methods

The following drugs were obtained from Sigma Chemical Co., St. Louis, USA: reagents for electrophoresis, adenosine diphosphate (ADP), esculetin, dexamethasone, indomethacin, bovine acid soluble collagen, luciferin, luciferase. Stock solutions were prepared as follows: dexamethasone and esculetin were dissolved in absolute ethanol and diluted in saline to give final concentrations of ethanol in the platelet assay of no more than 0.2% v/v; indomethacin was first dissolved in 0.1 M Na_2CO_3 then diluted with saline and adjusted to pH 6.0; ADP was diluted in Tris buffer pH 8.2.

Helicobacter pylori recombinant urease

Helicobacter pylori recombinant urease (HPU) expressed in *Escherichia coli* SE5000 transformed with plasmid pHP8080 (McGee et al. 1999), a kind gift from Dr. Harry T Mobley, University of Michigan Medical School, was used in all experiments. HPU was purified from bacterial extracts by a modification of the method of (Hu et al. 1992) as follows: after cultivation, cells were harvested by centrifugation, suspended in 20 mM sodium phosphate, pH 7.5 containing 1 mM EDTA, 5 mM 2-mercaptoethanol (extracting buffer, EB) and lysed using a Ultrasonic Homogenizer 4710, 10 pulses of 30 sec in an ice bath. After

centrifugation (20 min, 20.000 *g*, 4 ℃), the supernatant was fractionated by ammonium sulfate precipitation. The precipitate formed between 0.3-0.7 saturation was dissolved in EB and dialysed to remove the excess of salt. This material was then submitted to anion exchange chromatography in Q-Sepharose (GE Healthcare) at a ratio of 10 mg protein per 1 mL resin equilibrated in EB pH 7.8. After removing the unbound proteins, the column was eluted stepwise with the equilibration buffer containing 100 mM NaCl and the urease-enriched fraction was eluted with 200 mM NaCl in EB pH 7.8. After dialysis to remove excess of salt and concentration on Centriprep (Millipore) cartridges this material was applied into a size exclusion Superose 6 HR column equilibrated in EB pH 7.8, mounted on a FPLC apparatus at a flux of 0.3 mL per min. After gel filtration the fractions with urease activity were pooled and freeze-dried. SDS-PAGE analysis of the purified HPU protein showed two major bands of 60 and 30 kDa (not shown). For the experiments, the freeze-dried protein was solubilized to 0.5 mg protein/mL in 20 mM sodium phosphate, pH 7.5, containing 1 mM EDTA and 5 mM 2-mercaptoethanol.

Protein determination

The protein content of samples was determined by their absorbance at 280 nm or alternatively, by the method of Spector, 1978 using bovine serum albumin as standard.

Urease activity

The ammonia released was measured colorimetrically by the alkaline nitroprussiate method (Weatherburn 1967). One unit of urease releases one

μmol of ammonia per min, at 37°C, pH 7.5.

Platelet aggregation

Platelet-rich plasma (PRP) was prepared from rabbit blood collected from the ear central artery in the presence of sodium citrate to a final concentration of 0.313 % (v/v). Blood samples were then centrifuged at 200 x g for 20 min at room temperature, to give a platelet-rich plasma suspension. Platelet aggregation and shape change were monitored turbidimetrically by the method of Born and Cross 1963, using a Lumi-Aggregometer (Chrono-Log Corporation, Havertown, PA) and light transmission across the rabbit platelet-rich plasma suspension was registered on a chart recorder for 3 min. Platelet aggregation assays were also performed on a SpectraMax microplate reader (Molecular Devices, USA) as described by Francischetti et al. 2000. Briefly, urease samples with or without potential inhibitors in 96-well flat-bottomed plates were completed to a final volume of 50 μL with saline. Aggregation was triggered by the addition of 100 μL of platelet suspension. The plate was incubated for 2 min at 37°C before beginning of agitation and readings were performed at 650 nm every 11 sec, during 20 min. When testing potential inhibitors, platelets and the compounds were pre-incubated for 2 min at 37°C unde r stirring and aggregation was triggered by addition of HPU or control inducer (ADP). Change in turbidity was measured in absorbance units, and results are expressed as area under the aggregation curves.

Platelet Secretion

ATP release from platelet-rich plasma suspension was measured as a change in bioluminescence in the presence of the Chronolume® reagent containing a

luciferin-luciferase mixture (Chrono-Log Corporation, Havertown, PA) according to manufacturer instructions.

Scanning electron microscopy of HPU-stimulated platelets

The procedure for sample preparation for SEM was modified from Gear, 1984. Briefly, PRP samples were pre-warmed at 37 ℃ and e xposed to ADP, saline or HPU under low stirring for 2 min. Platelets were then fixed by adding glutaraldehyde in 0.1 M sodium cacodylate pH 7.2 to a final 2.5% concentration and incubated overnight at 4°C. The samples were washed twice for 30 minutes in 0.1 M sodium cacodylate and filtered on 0.4 µm polycarbonate membranes (Millipore, USA). The fixed platelets were sequentially dehydrated in 30, 50, 70 and 90% (v/v) acetone for 5 minutes each and finally twice in 100% acetone for 10 minutes. Critical-point drying and gold coating treatments were performed at our University's Center of Electron Microscopy (CEM-UFRGS, Brazil). Specimens were visualized in a JEOL-JSM 6060 scanning electron microscope with automated image digitalization and archiving.

Paw Edema

Male Swiss mice (20 - 22 g), housed at 22 ± 3 C with a 12/12 h light/dark cycle (lights on at 06:00 a.m.) were used for the experiments. On the day of the experiments, the mice received, under alight ether anesthesia, a 30 µL intraplantar injection of different doses of HPU into the right hind paw. The left hind paw was used as control receiving an injection 0.03 mL of PBS. In some experiments the animals were pre-treated with anti-inflammatory drugs given

subcutaneously 1 hour (esculetin, 50 mg/Kg) or 4 hours (dexamethasone, 0,5 mg/kg) before HPU administration.

Increased paw thickness due to edema was measured with a micrometer (Mitutoyo, 0 to 25 mm at 0.002 mm increments) at the indicated time intervals after the injections. Paw edema is expressed as the difference between the thickness in mm of right and left paws of the same animal. Thus the results represent the net edema (mm) induced by HPU at the mean of 9 animals per group.

Animal experimentation

All procedures involving animals were conducted in strict accordance to Brazilian national legislation (Law no. 6.638/1979) and are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised in 1985).

Statistical analysis

Data were analyzed by ANOVA followed by the Tukey-Kramer test using the Instat Graph Pad software and values of $p < 0.05$ were considered statistically significant.

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Table 1. Comparative data on physicochemical and biological properties of the isoform of jackbean urease canatoxin (CNTX), *B. pasteurii* urease (BPU) and a recombinant *H. pylori* urease (HPU).

Physicochemical properties	CNTX [1]	BPU [2]	rHPU [3]
Molecular mass:			
SDS-PAGE	95 kDa	11-13 - 61 kDa (chains A, B and C, respectively)	30-62 Kda (chains A and B, respectively)
native form	dimer	trimer	hexamer
Urease activity			
U/ mg protein	11.6	194.0	252.0
Biological properties			
Toxicity to:			
mouse, i.p.	toxic	not toxic	ND
Treated with pHMB	100% active		
D. peruvianus, LD50	DL50<0.01 % (w/w)	not toxic	ND
Treated with pHMB	100% active		
Platelet aggregation, EC_{50} (rabbit)	22.2 μg/mL	400 μg/mL	150 μg/mL
Treated with pHMB	100% active	ND	ND
Platelet secretion	yes (serotonine)	ND	yes (ATP)
Lipoxigenase inhibitors	inhibition	inhibition	inhibition
Cyclooxygenase inhibitors	potentiation	potentiation	potentiation
PAF-acether inhibitors	inhibition	inhibition	ND
Ca^{2+} channel blocker	inhibition	inhibition	ND
Paw Edema	yes (rats)	ND	yes (mice)
Lipoxigenase inhibitors	inhibition	ND	inhibition

ND: not determined

[1] Carlini et al., 1985; Benjamin et al., 1992; Follmer et al., 2001; Follmer et al., 2004b.

[2] Follmer et al., 2004a; Olivera-Severo et al., 2006[a]

[3] Hu et al., 1992; this paper.

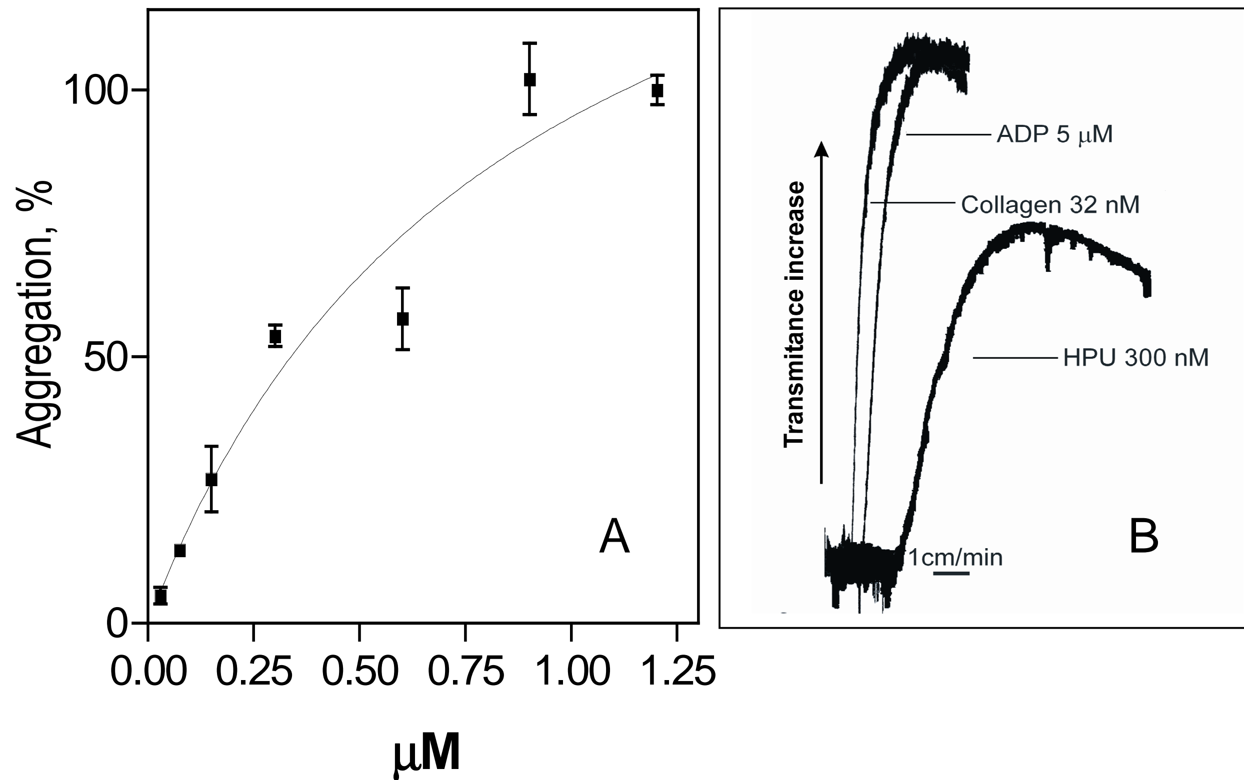

Figure 1. Aggregation of rabbit platelets induced by recombinant *Helicobacter pylori* urease (HPU).
Panel A. Rabbit platetet-rich plasma suspension in microwell plates were exposed to increasing concentrations of HPU or 5 mM ADP (100% aggregation). Aggregation of platelets was monitored ever 11 sec during 20 minutes in a SpectraMax plate reader. Results (means ± sd) are expressed as percentage of maximal aggregation for 4 replicates. Panel B. Comparison of aggregation tracings in the Lummi-aggregometer of platelets stimulated with HPU (0.3 mM), ADP (5 mM) or collagen (0.03 mM)

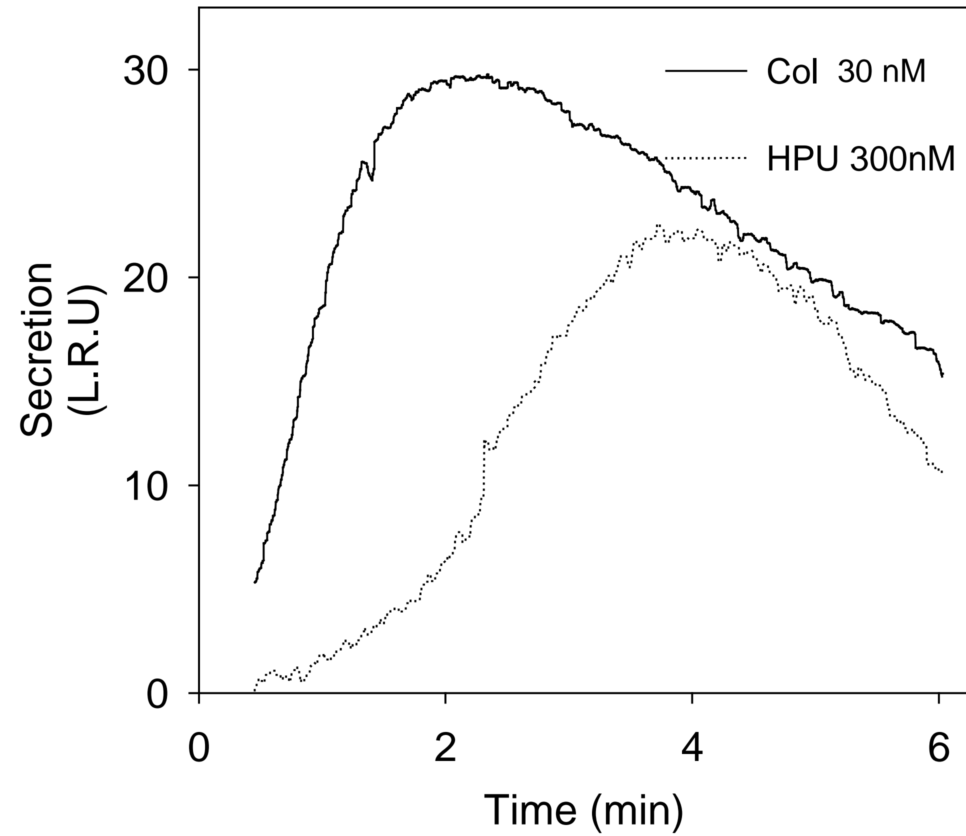

Figure 2. Release reaction and ATP secretion from rabbit platelets stimulated by HPU or collagen.
ATP secretion by activated platelets is detected as light emitted in the presence of a luciferin-lucipherase mixture.
L.R.U. – light relative units. A typical result out of 3 replicates is shown.

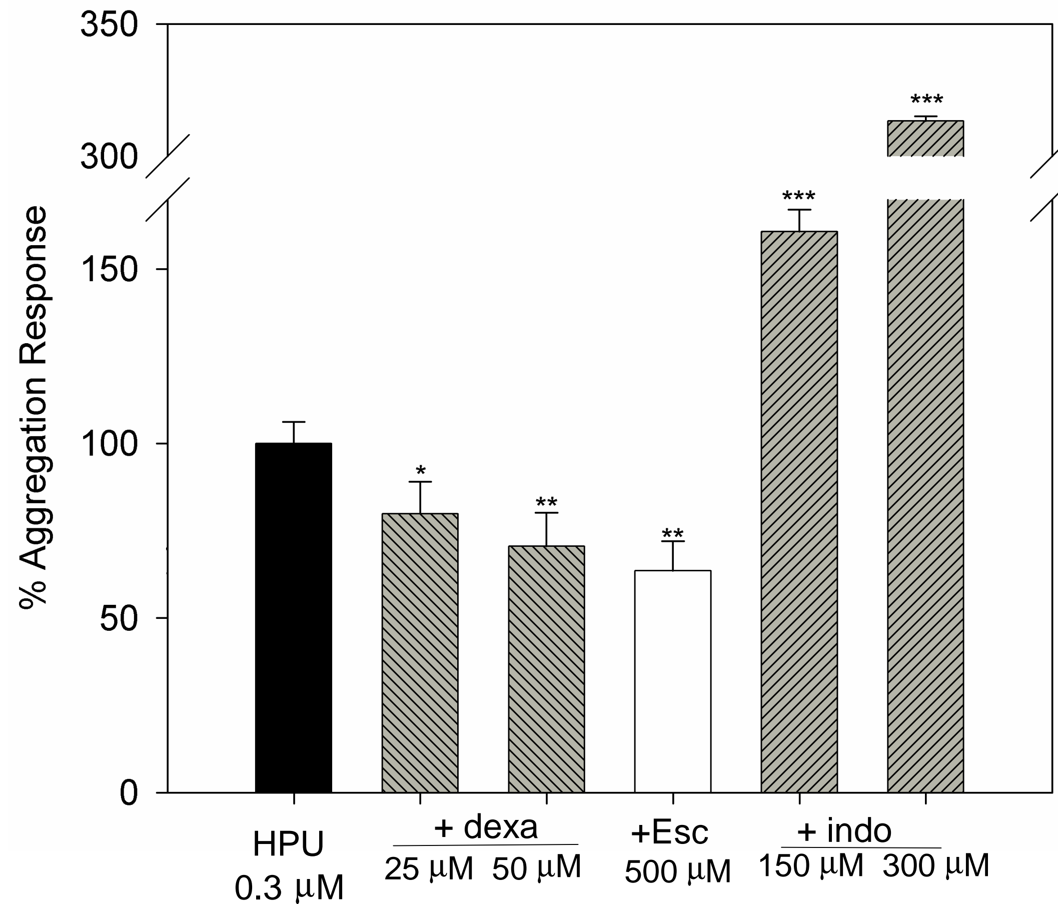

Figure 3. Involvement of phospholipase A_2 and eicosanoids in HPU-induced platelet aggregation.
Rabbit platetet-rich plasma suspensions in microwell plates were incubated for 2 min at room temperature in the absence or in the presence of the indicated concentrations of the drugs and aggregation was triggered by addition of HPU (0.3 μM). Aggregation of platelets was monitored ever 11 seconds during 20 minutes in a SpectraMax plate reader. Results are means ± sd of 4 replicates of each condition. Values of p <0.05* , p<0.01** or p <0.001*** were considered statistically significant.

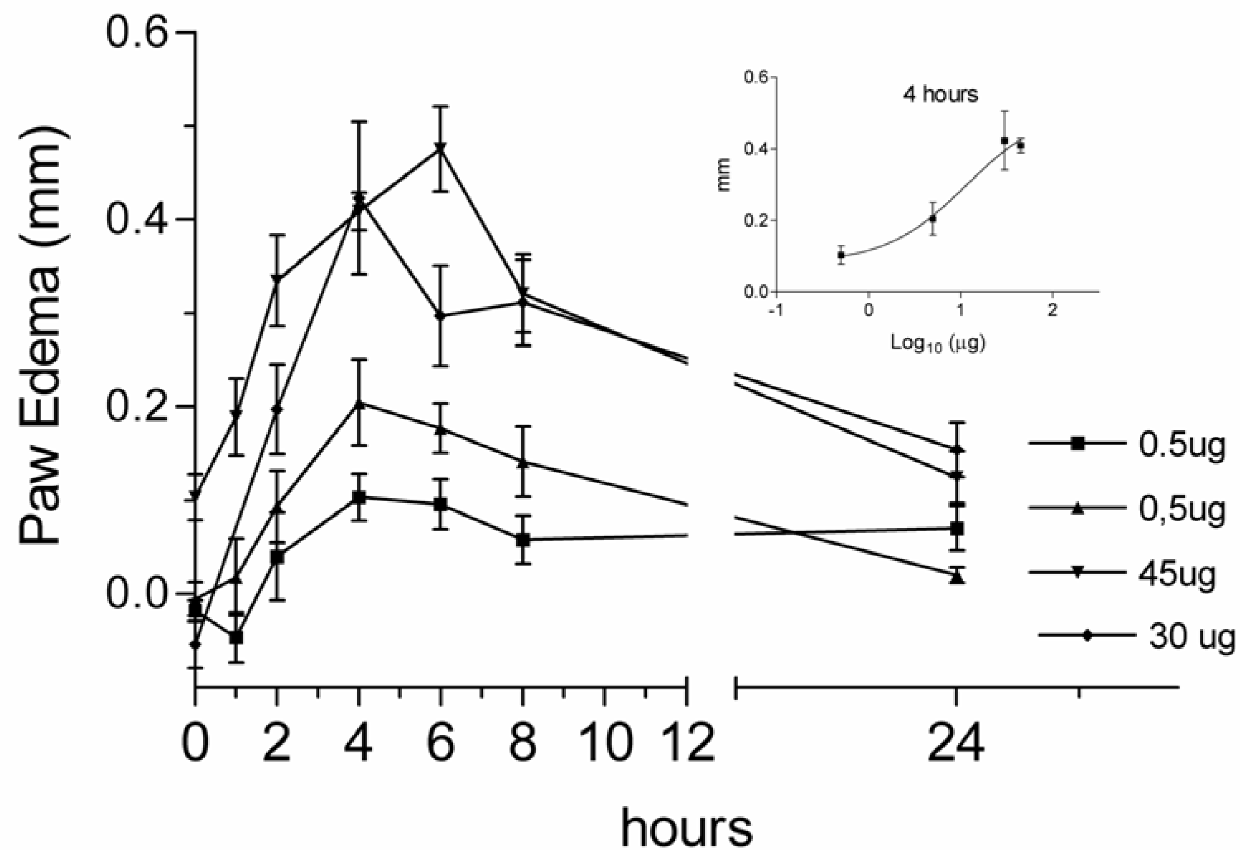

Figure 4. Time course and dose-response curve (Inset) of HPU-induced mice paw edema.
HPU was injected into the right paw of mice in a final volume of 30 µL and the left paw of the same animal received vehicle (PBS). Results are expressed as net increase in thickness (mm) of the right paw as compared to the left. Each point represents mean ± sd from 9 animals. Values of p <0.05* , p<0.01** or p <0.001*** were considered statistically significant.

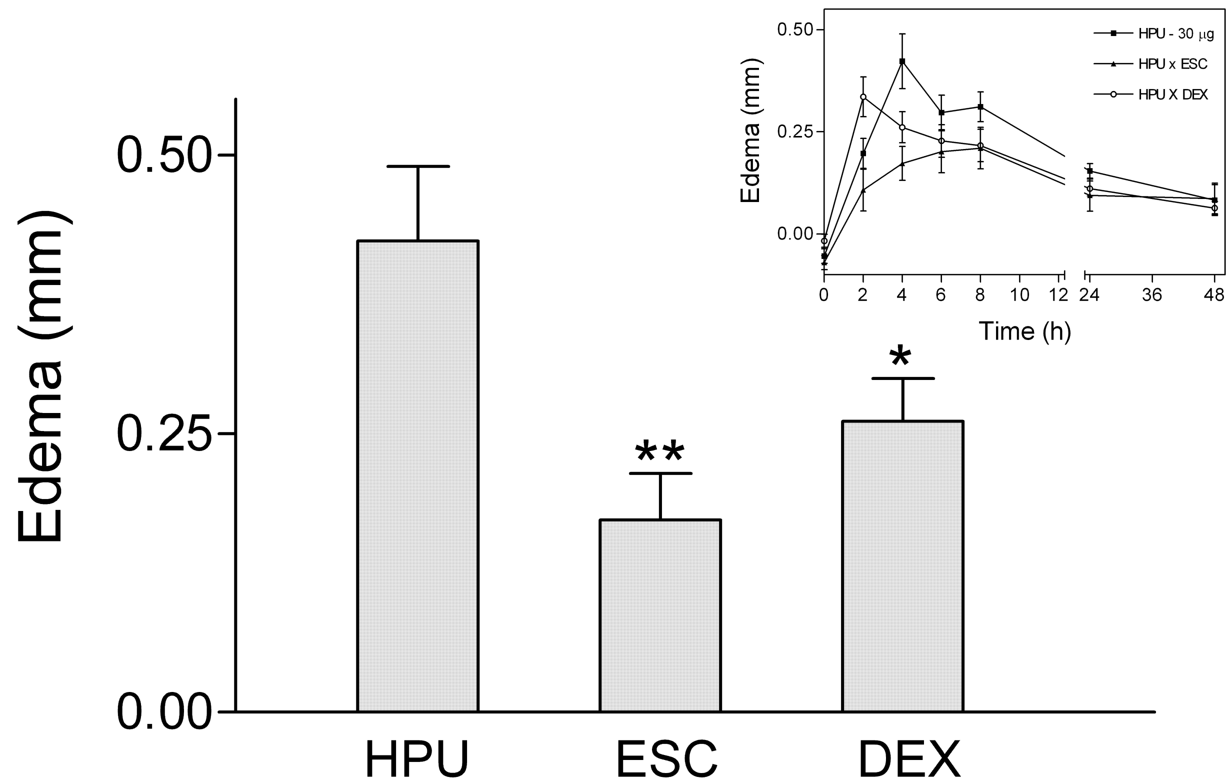

Figure 5. Involvement of phospholipase A_2 and lipoxygenase-derived eicosanoids (the time course results inset) in HPU-induced mice paw edema. Mice were received subcutaneously esculetin (50 mg/Kg) or dexamethasone (0.5 mg/kg), 1 hour or 4 hours before HPU administration. HPU (30 µg) was injected into the right paw of mice in a final volume of 30 µL and the left paw of the same animal received vehicle (PBS). Results are expressed as net increase in thickness (mm) of the right paw as compared to the left. Each point represents mean ± sd from 9 animals. Values of $p < 0.05$* , $p < 0.01$** or $p < 0.001$*** were considered statistically significant.

4. DISCUSSÃO

As principais informações contidas nesta tese podem ser sumarizadas na Tabela 2, comparando propriedades físico-químicas e os efeitos biológicos estudados para as diferentes isoenzimas de urease analisadas.

Tabela 2. Comparação de propriedades físico-químicas e biológicas de ureases de plantas e de bactérias.

Propriedades Físico-químicas	CNTX	JBU	SBU	BPU	HPU
Subunidades (kDa)	95	90	90	11-13-61	30 - 62
Forma nativa	Dímero	Hexâmero	Hexâmero	$(\alpha\beta\gamma)_3$	$(\alpha\beta)_6$
Conteúdo de metais	Zn^{2+} e Ni^{2+}	2 Ni^{2+}	2 Ni^{2+}	2 Ni^{2+}	2 Ni^{2+}

Modelo/Efeito	CNTX	JBU	SBU	BPU	HPU
Toxicidade *ip* [1](mg/kg) Camundongos	DL_{50}=2,0	[2] NT, até 20	NT, até 20	[3] ND	ND
Toxicidade em Insetos (% em peso/peso) *D. peruvianus*	[4]DL_{50}<0,01	DL_{50}=0,017	DL_{50}=0,052	NT, até 0,1	ND
Plaquetas de coelho Agregação (µg/mL)	[5]DE_{50}=15	DE_{50}=15,8	DE_{50}=22,2	DE_{50}=400	DE_{50}=150
Inibidores de lipoxigenase [6]	Inibição	Inibição	ND	Inibição	Inibição
Inibidor de Ciclooxigenase [7]	Potenciação	ND	ND	Potenciação	Potenciação
Bloqueador Canal de Ca^{2+} [8]	Inibição	ND	ND	Inibição	ND
Inflamação Edema de pata	SIM	ND	ND	ND	SIM
Inibidores de Lipoxigenase [6]	Inibição	ND	ND	ND	Inibição

CNTX= canatoxina, JBU= urease de *C. ensiformis*, SBU= urease de soja *G. max*, BPU= urease de *B. pasteurii*, HPU= urease de *H. pylori*,

[1] *ip*.= intraperitonial,
[2] NT= não tóxico
[3] ND= não determinado
[4] DL_{50}= dose letal para 50%,
[5] DE_{50}=dose para 50% do efeito
[6] ácido nordihidroguaiarético e/ou esculetin
[7] indometacina
[8] metoxy-verapamil

A canatoxina (CNTX) possui um sítio catalítico híbrido contendo Zn^{2+} e Ni^{2+}, diferente da isoenzima da mesma semente (JBU), que possui dois átomos de Ni^{2+}, assim como todas as outras ureases já estudadas. As diferenças estruturais se refletem na atividade ureolítica, que é 30-40% menor para a CNTX, e na susceptibilidade à inibidores da atividade ureolítica, como ácido acetohidroxâmico, um quelante de metais divalentes. As diferenças estruturais das duas moléculas também são evidenciadas pela avidez a metais, visto que a CNTX tem afinidade por Co^{2+} e Zn^{2+}, enquanto que a JBU só interage com o Zn^{2+} (Follmer et al., 2004b).

As diferenças estruturais entre as ureases têm repercussão em suas atividades biológicas, como a toxicidade por via intraperitonial em camundongos, só observada até o momento para a canatoxina. A atividade inseticida para o percevejo *D. peruvianus* é conservada entre as ureases de plantas, CNTX, JBU e SBU, mas não foi detectada para a urease de *B. pasteurii*, uma bactéria presente no solo. Por outro lado, o efeito pró-agregante em plaquetas foi identificado em todas as isoenzimas, sendo as ureases de bactéria em torno de 10-20 vezes menos potentes.

Além dos efeitos decorrentes da atividade hidrolítica sobre a uréia, como formação de microclima na mucosa gástrica para *H. pylori* (Montecucco et al., 1999) ou precipitação de sais de “struvite” em infecções por *P. mirabilis* (Burall et al., 2004) as ureases podem ter uma participação importante no desenvolvimento das patologias. No caso da infecção por *H. pylori*, uma diferença nas respostas do paciente à essas agressões pode ser a diferença da simples presença da bacatéria na mucosa para o quadro patológico, visto que 50% da população mundial está infectada e as estimativas são de que menos de 20% irá desenvolver enfermidades (Queiroz & Luzza, 2006).

Assim, a interação da urease de *H. pylori* com plaquetas levando à formação de microagregados na circulação gástrica (Kalia & Bardan, 2003) e sua atividade pró-inflamatória, ambas envolvendo a rota dos eicosanóides, são de particular importância. Soma-se a isso o fato de que fármacos antiinflamatórios não esteroidais, inibidores da síntese de prostaglandinas que aumentam a disponibilidade de ácido araquidônico para outras vias, são um importante fator de risco para disfunções gástricas, mesmo na ausência da bactéria. O mecanismo pelo qual a infecção de *H. pylori* evolui para uma condição clínica mais severa está relacionada com a inflamação e a resposta do hospedeiro à infecção (Cinque et al., 2006). Além dos fatores de virulência já caracterizados, como Vac A e Cag A, a urease do *H. pylori*, por possuir efeitos modulatórios sobre a inflamação e agregação plaquetária pode contribuir efetivamente na patogênese de lesões gástricas.

Os efeitos biológicos da CNTX parecem ser, quase na totalidade, mediados por eicosanóides via lipoxigenase. Plaquetas possuem a isoenzima 12-lipoxigenase e essa via de formação de segundos mensageiros não está completamente caracterizada, visto que muito poucas moléculas estimulam plaquetas à agregação por essa rota. As ureases de *B. pasteurii* e de *H. pylori* ativam aparentemente a mesma rota de sinalização disparada pela CNTX para indução de secreção e agregação plaquetária, sendo bloqueadas por inibidores da via da 12-lipoxigenase, como a esculetina. Recentemente, foi reconhecida a importância da 12-lipoxigenase para ativação plaquetária em resposta ao agonista fisiológico colágeno, envolvendo interação com a glicoproteína VI, que é o seu receptor específico, (Coffey et al., 2004a, Coffey et al., 2004b, Poeckel et al., 2006). Assim, esperamos que o conhecimento da capacidade das ureases em ativar essa rota de sinalização não só em plaquetas, mas como os resultados previamente obtidos com a CNTX deixam

antever, provavelmente também em outros tipos celulares importantes para a resposta inflamatória, ou ainda em células secretoras de uma maneira geral, possa contribuir para reavaliar a importância dessas enzimas como fator de virulência, bem como para a adequação de procedimentos terapêuticos em doenças causadas por bactérias produtoras de ureases.

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6. ANEXOS

Anexo 6.1

Follmer C., **Wassermann G.E.**, Carlini C.R.

Separation of jack bean (*Canavalia ensiformis*) urease isoforms by immobilized metal affinity chromatography and characterization of insecticidal properties unrelated to ureolytic activity

Plant Science 167, 241-246, 2004.

Nesse artigo apresentamos um novo método de separação das ureases de *C. ensiformis*, confirmamos o conteúdo diferencial de metais no sítio ativo das isoformas, e observamos que, como a canatoxina, a urease majoritária também apresenta atividade inseticida no inseto modelo *Dysdercus peruvianus*. O efeito inseticida de ambas as ureases não foi afetado por pré-tratamento com p-OHMB, um inibidor irreversível da atividade ureásica, indicando que o mecanismo de entomotoxicidade não depende do sitio da atividade ureásica.

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Separation of jack bean (*Canavalia ensiformis*) urease isoforms by immobilized metal affinity chromatography and characterization of insecticidal properties unrelated to ureolytic activity

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Abstract

In this work we described the separation of two isoforms of urease from jack bean seeds, the "classical" jack bean urease (JBU) and canatoxin (CNTX), using immobilized metal affinity chromatography (IMAC). Jack bean urease isoforms presented differential behavior on a cobalt-loaded iminodiacetic acid (IDA)–Sepharose column and IMAC is proposed as an efficient method to isolate the isoenzymes. The metal content of the urease isoforms was determined by particle induced X-ray emission (PIXE). CNTX displays ca. 1 eq. of nickel per monomer, contrasting with 2 eq. of nickel found per monomer for JBU. Beside nickel, CNTX contains 1 eq. of zinc per monomer, while no zinc is found in JBU. The insecticidal property of these ureases was investigated in feeding trials with the cotton sucker bug, *Dysdercus peruvianus* (Hemiptera) as an insect model. Both ureases were lethal to the insects, being CNTX more potent than JBU. This property was not affected by treatment with *p*-hydroxymercurybenzoate (pHMB), an irreversible inhibitor of ureolytic activity. Altogether the data show that IMAC is a suitable method for separating jack bean urease isoforms and that the isoenzymes display entomotoxic effects, independent of their ureolytic activity, suggestive of a role in plant defense against insect predators.
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Keywords: Urease; Canatoxin; *Canavalia ensiformis*; IMAC; Insecticide; Nickel; Zinc

1. Introduction

Urease (urea amidohydrolase; EC 3.5.1.5) is a nickel dependent enzyme [1] that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. Ureases have since been isolated from a wide variety of organisms including high plants, fungi and bacteria [2]. In 1926, jack bean urease (JBU) was crystallized by Sumner [3] and these were the first crystals of a characterized enzyme.

In despite of the abundance of urease in some tissues, namely seeds of some members of the families Fabaceae (Leguminosae) and Curcubitaceae, and its ubiquity in virtually all plants [4], little has been revealed about its role. Soybean contains two distinct urease isoenzymes: the ubiquitous urease is synthesized in all tissues examined, whereas synthesis of the embryo-specific urease is confined to the developing embryo and is retained in the mature seed where its specific activity is roughly 1000-fold greater than that of ubiquitous urease in many tissue [5–8]. It has been suggested that the embryo-specific urease plays no urea assimilatory role and that ureases may be involved in seed chemical defense [9].

Recently our group has shown that canatoxin (CNTX), an isoform of jack bean urease [10,11], displays several biological properties independent of its ureolytic activity, such as activation of blood platelets [12,13] and interaction with glycoconjugates [14]. Moreover, canatoxin is lethal to rats and mice when injected intraperitoneally (LD_{50} 2.0 mg/kg body weight for mice) [10,11] and displays insecticidal activity when fed to some groups of insects, reinforcing the suggestion that ureases may be involved in plant defense [15,16]. The kissing bug *Rhodnius prolixus*, and three eco-

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nomically important crop pests, the cowpea weevil *Callosobruchus maculatus*, the green stinkbug *Nezara viridula* and the cotton stainer bug *Dysdercus peruvianus* are susceptible to canatoxin [17].

In a previous work [11], we found that jack bean urease isoenzymes could be separated by immobilized metal affinity chromatography (IMAC) using a zinc-loaded column. The resin used for that purpose, dipipecolylamine–Sepharose, is not commercialized. In the present work we aimed to develop a method to separate the isoenzymes using a readly available resin, iminodiacetic acid (IDA)–Sepharose, loaded with a metal other than zinc, to avoid any interference in the determination of the content of this metal in the proteins. We have successfully separeted two isoforms of urease from jack bean seeds on a cobalt-loaded resin, measured the metal content of the purified isoforms and found that both of them displayed insecticidal properties unrelated to their ureolytic activity.

2. Material and methods

2.1. Purification of urease isoforms

Jack bean urease isoenzymes were purified from seed meal based on a modification of the method of Blakeley [18,19]. Briefly, dry seeds (Casa Agrodora, São Paulo, Brazil) were powdered and 50 g of deffated meal were extracted with buffer A (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol) for 1 h at 4 °C. The meal was removed by centrifugation (30,000 × *g*, 20 min, 4 °C), and 28% (v/v, final concentration) ice-cold acetone was added to the supernatant. The suspension was kept at 4 °C overnight and the precipitated proteins were removed by centrifugation (30,000 × *g*, 20 min, 4 °C). The concentration of acetone in the supernatant was then increased to 31.6% (v/v) and, after stirring at room temperature for 10 min, the precipitate was removed by centrifugation (30,000 × *g*, 20 min, 4 °C). The supernatant was dialysed against buffer B (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol) and then mixed with 25 mL of Q-Sepharose resin (Amersham-Biotech Pharmacia) equilibrated in buffer B. After stirring in a beacker for 30 min in ice bath, the mixture was filtered and the resin was washed with 100 mM NaCl in buffer B to remove the non-retained proteins. Elution of urease-enriched fractions was achieved adding 300 mM NaCl to buffer B. Active fractions were pooled and concentrated by using a CentriPrep cartridge (10,000 cut-off, Millipore). The urease-enriched material was then applied into a Superose 6 HR 10/30 gel filtration column (Amersham-Biotech Pharmacia) equilibrated in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, mounted in a FPLC system. The protein peak containing urease activity was collected and concentrated. The material resultant from this step is equivalent to the highly purified urease previously used for crystallography studies [18,19]. The urease fraction obtained after gel-filtration was then submitted to an affinity chromatography in an iminodiacetic acid–Sepharose (Chelating Sepharose Fast Flow, Amersham Pharmacia Biotech) column loaded with Co^{2+} ions [20], and equilibrated in 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.5. The flow-through fraction (JBU) was recovered by washing the resin with equilibration buffer and the retained fraction (canatoxin) was eluted with 2 M ammonium chloride. The purity of the protein fractions after the Co^{2+}–IDA–Sepharose column was analyzed by SDS-PAGE and capillary electrophoresis (CE).

2.2. Protein content determination

The protein content of samples was determined by their absorbance at 280 nm, or by the Coomassie Blue G (Sigma) method of Spector [21], with bovine serum albumin (Sigma) as standard.

2.3. Urease activity

Urease activity was estimated colorimetrically measuring the ammonia released by the phenylnitroprussiate-hypochlorite method [22]. One unit of urease activity was defined as the mass of enzyme that releases 1 μmol of ammonia/min, at 37 °C, pH 7.5. For inhibition studies, proteins (0.1–0.2 mg/mL in 20 mM phosphate pH 7.5, 1 mM EDTA) were incubated 18 h at 4 °C with *p*-hydroxymercurybenzoate (pHMB) (5 or 50 μM final concentrtion), and then tested for urease or insecticidal activity.

2.4. SDS-polyacrylamide electrophoresis

Electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate [23] were run at 20 mA for 2–3 h. The gels were stainned with Coomassie Blue R-250.

2.5. Toxic activity in *Dysdercus peruvianus*

The insecticidal activity of ureases was evaluated in feeding trials with the cotton stainer bug *D. peruvianus* (Hemiptera), which is an economically important pest of cotton crop. Groups of second instar insects (from a colony kept in our laboratory) were fed on cotton seed meal mixed with freeze-dried urease in a final protein concentration of 0.05% (w/w). Control groups fed on cotton seed meal containing equivalent volumes of the protein solution diluents. The insects were kept at 26 °C, 70–80% air humidity, 12 h dark: 12 h light cycle, and were examined every 2 days during 20 days for lethality, body weight and developmental stage (the insect goes through five instar stages before becoming adult). The results are expressed as survival rate (mean and S.E.M.) of triplicated points.

2.6. Determination of metal content

Determination of metal content of the proteins was carried out by the particle-induced X-ray emission (PIXE) technique. For this, protein samples (0.2 mg/mL) were previously dialyzed extensively against several changes of 20 mM sodium phosphate buffer, 10 mM EDTA, and then ice-cold acetone was added for a final 80% (v/v) proportion. The precipitated proteins were collected after 10 min by filtration onto regenerated cellulose acetate filters (Sartorius, 0.45 μm pore, 40 mm diameter). The resulting mass of the samples corresponded to a filter density in the range of 0.1–0.7 nmol of protein per square centimeter. A blank filter, which was also washed with ice-cold acetone, was used for background counts.

The PIXE [24,25] analysis were carried out at a 3 MV Tandetron accelerator (at the Instituto de Física, Universidade Federal do Rio Grande do Sul) using a 2 MeV proton beam with an average current of 5 nA and 1 h of acquisition time per sample. X-rays induced by the proton beam were detected by an HPGe detector (EG&G; GLP series), with an energy resolution of 172 eV at 5.9 keV, and the data were analysed by the GUPIX code [26].

2.7. Capillary eletrophoresis

Capillary electrophoresis [27] was performed on a HP3DCE model apparatus (Agilent, Waldborn, Germany) equipped with a diode array for UV-Vis detection, at the Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil. Urease samples in 20 mM sodium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA, at 37 °C, were injected under a pressure of 50 mbar, −15 kV, during 6 s. The capillar (PVA) dimensions were 60 cm (51.7 cm to detector) long and 50 μm internal diameter. The proteins were monitored by the absorbance at 200, 214 and 254 nm.

3. Results

3.1. Isolation of jakbean urease isoforms by IMAC

Fig. 1A shows the capillary electrophoresis analysis of jack bean urease preparation, which has been purified according to previously described procedures [18,19]. This material clearly contained two distinct proteins that we successfully separated using immobilized metal affinity chromatography in a Co^{2+} chelated form of iminodiacetic acid–Sepharose (Co^{2+}–IDA) (Fig. 1B). Both, the non-retained and the eluted protein fractions produced a single protein peak in the CE (not shown) and a single band in the SDS-PAGE analysis (Fig. 1C), showing similar molecular mass for the monomers. Almost 80–90% of the total protein obtained by the previously described procedures [18,19] did not bind to the immobilized cobalt, resulting in only a small fraction eluted with 2 M ammonium chloride, with recoveries near to 90–95% protein (Table 1). Kinetic data of ureolytic activity and amino acid sequence revealed that the non-retained fraction is the “classical” jackbean urease [28], refered here as JBU, with a specific activity of 22.2 U/mg, while the retained fraction is the isoform designated as canatoxin, characteristically with a lower specific activity of 11.6 U/mg (Table 1) [10,11].

3.2. Determination of metal content of JBU isoforms

Table 1 shows the metal content as determined by PIXE of the jack bean urease isoforms separated on the

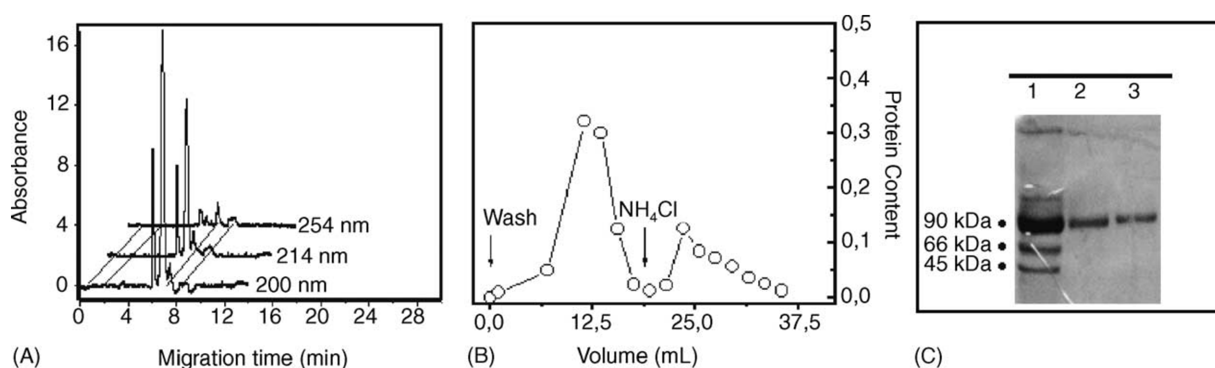

Fig. 1. Separation of jackbean urease isoforms by capillary electrophoresis and IMAC. (A) Capillary electrophoresis (CE) of the urease fraction purified according to [18,19] (after the gel-filtration step) shows the existence of two major protein peaks. CE was performed on a HP3DCE model apparatus (Agilent, Waldborn, Germany) equipped with a diode array for UV-Vis detection. Urease samples in 20 mM sodium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA, at 37 °C, were injected under a pressure of 50 mbar, −15 kV, during 6 s, in a PVA capillar 60 cm long with 50 μm internal diameter. The protein peaks were monitored by the absorbance at 200, 214 and 254 nm. (B) The urease fraction from the gel filtration step (same as in A), in 20 mM sodium phosphate pH 7.0, 0.5 M sodium chloride, was applied on to a Co^{2+}–IDA–Sepharose column (∼2 mL resin/mg protein), equilibrated in the same buffer. After washing out the non-retained fraction, the retained protein was eluted with 2 M ammonium chloride, pH 5.5. (C) SDS-PAGE bands pattern of jack bean ureases: lane 1: molecular mass standards are shown (in kDa) on the left; lane 2: the non-retained fraction (JBU) from the Co^{2+}–IDA–Sepharose column in panel (B); lane 3: the fraction eluted with ammonium chloride (canatoxin) from the Co^{2+}–IDA–Sepharose column in panel (B).

Table 1
Comparative data on JBU and CNTX after the IMAC procedure

	JBU	CNTX
Protein (% total)[a]	87.5 ± 2.5	12.5 ± 2.5
Urease activity (U/mg)	22.2 ± 0.7	11.6 ± 3.2
Insecticidal activity in *D. peruvianus*		
Survival rate (%)	36.5 ± 1.4	15.7 ± 2.4
Treatment with pHMB	100% active	100% active
Metal content[b]		
Nickel	1.75± 0.13	1.20 ± 0.11
Zinc	0.17 ± 0.06	1.01 ± 0.18

[a] Represents percentage of total protein recovered of each isoform after the IMAC procedure.

[b] Determined by particle induced X-ray emission (PIXE). Metal content is expressed as moles of metal per mole of protein monomer (90 kDa).

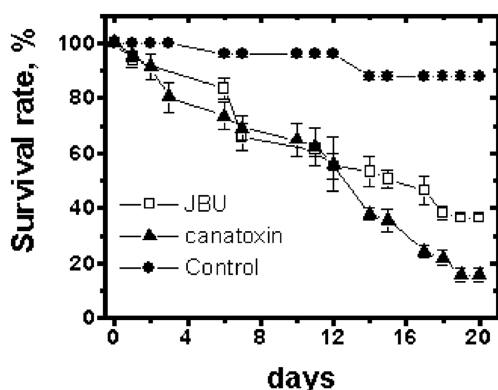

Fig. 2. Insecticidal properties of the jackbean urease isoforms. The ureases, JBU (□) and canatoxin (▲), in 20 mM sodium phosphate pH 7.0, 1 mM EDTA, were mixed with cotton seed meal to give a final concentration of 0.05% (w/w), the mixtures were freeze-dried and offered as feed to groups of second instar *D. peruvianus*. The control group (●) fed on cotton meal containing the same volume of freeze-dired buffer alone. The lethality was observed every 2 days and is expressed as the percentage of survival. Data are means ± S.E.M. of six experiments ($P < 0.001$).

Co^{2+}–IDA–Sepharose column. The data confirmed our previous results with atomic absorption spectroscopy indicating that CNTX contains equimolar amounts of nickel and zinc [11], while the “classical” urease (JBU) contains 2 eq. of nickel per monomer and no zinc.

3.3. Insecticidal properties of jack bean isoforms

In order to evaluate the insecticidal properties of the jackbean urease isoforms we have monitored their toxicity for the cotton stainer bug *D. peruvianus* in feeding trials. Both proteins JBU and CNTX displayed entomotoxic properties, decreasing the survival rate of the tested insects to 35 and 20% after 20 days, respectively, under similar experimental conditions (Table 1 and Fig. 2).

To investigate if the enzymatic activity of the proteins is related to their insecticidal properties, the urease isoforms were treated with the irreversible urease inhibitor, *p*-hydroxymercurybenzoate and then bioassayed in the *D. peruvianus* model. While both pHMB-treated proteins fully maintained their entomotoxic effect, their enzymatic activity was totally abolished (Fig. 3). As we reported previously [11], the isoenzymes differ in their susceptibility to *p*-hydroxymercurybenzoate, being JBU more susceptible to *p*-hydroxymercurybenzoate than is canatoxin.

4. Discussion

Capillary electrophoresis data (Fig. 1A) confirmed that preparations of jack bean urease considered to be highly purified and suitable for growing good quality crystals [18,19] consisted of a mixture of two different proteins. In other studies we have shown the existence of a family

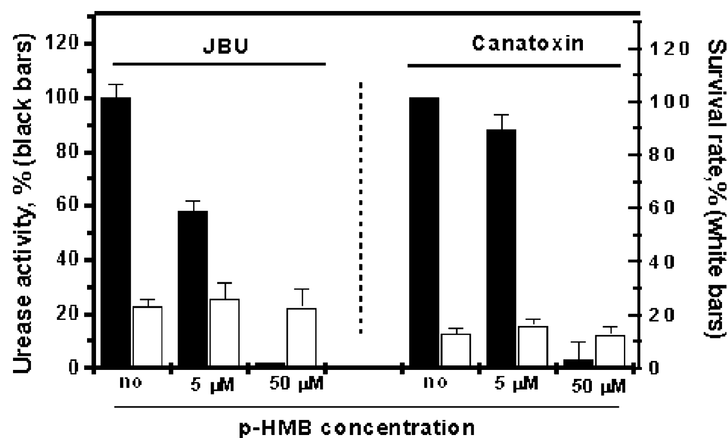

Fig. 3. Effect of *p*-hydroxymercurybenzoate (pHMB) on ureolytic activity and insecticidal properties of the jackbean urease isoforms. JBU and canatoxin were pre-treated with pHMB at final concentrations of 5 and 50 μM pHMB and then tested for ureolytic activity (black bars, percentage of residual activity) and toxicity to *D. peruvianus* (open bars). For this, the proteins (concentration 0.05%, w/w) or buffer containing pHMB were added to cotton seed meal, the mixtures were freeze-dried and offered to the insects in feeding trials. The lethality recorded after 20 days was expressed as percentage of survival. Control groups fed on cotton seed containing only buffer or 50 μM pHMB showed no lethality after the same period (not shown). Data are means ± S.E.M. of six experiments.

of urease-related genes in *Canavalia ensiformis* [29]. The existence of genetically determined jack bean urease isoenzymes might explain most of the complex behavior reported for this enzyme and the presence of isoenzymes may be the reason of the difficulties encountered in obtaining crystals of JBU diffracting at low angles [19,30].

We have previously characterized canatoxin as an isoform of jack bean urease and explored the differential affinity of the isoenzymes for zinc (immobilized in dipipecolylamine–Sepharose) to eliminate residual traces of the "classical" urease isoform from highly purified canatoxin [11]. Both proteins are able to bind zinc although with different strenght, since JBU could be eluted with 100 mM imidazol while canatoxin was eluted only in presence of EDTA. Part of our goal in this paper was to develop a new IMAC procedure to separate jack bean urease isoforms using a commercially available resin. For an accurate determination of the zinc content of the isoforms, adsorption to another metal ion in the IMAC step was also investigated. This was fully accomplished with a cobalt-loaded Chelating Sepharose column (Fig. 1B). Under the conditions tested (20 mM sodium phosphate–500 mM sodium chloride, pH 7.0) only canatoxin binds to cobalt while JBU showed no interaction with this metal, thus making IMAC the method of choice to achieve good separation of the isoforms. A differential exposition of histidine residues at the surface of the proteins is expected from their behavior in the immobilized metal affinity chromatography [20].

Furthermore, our data suggest that the urease preparation used for crystallization studies probably contained about 10% of canatoxin, resulting in low quality crystals [19].

The results shown here confirm our previous observation that the jack bean urease isoforms differ in their metal content. The stoichiometric data (Table 1) obtained by particle induced X-ray emission analysis, a sensitive and multi-elemental determination method, indicated that JBU contains two mols of nickel per 90 kDa monomer, confirming the results available in the literature for Sumner's JBU [1]. Furthermore, the concentration of zinc in JBU is close to zero. In contrast, canatoxin contains about one mol of zinc and one of nickel per monomer (90 kDa). The protein had no contact with zinc during the purification that could account for a contamination and the metal is tighly bound to canatoxin as it could not be removed by extensive dialysis against EDTA. The results presented here also confirmed the previously observed [11] difference in specific ureolytic activity of the isoenzymes, being the "classical" urease about 1.5–2-fold more active than canatoxin, the less abundant isoform. The participation of a hybrid nickel–zinc metallocenter in the catalytic activity of canatoxin may explain its lower specific activity [11].

The wide distribution of ureases in leguminous seeds as well as the accumulation pattern of the protein during seed maturation are suggestive of an important physiological role. Canatoxin, a JBU isoform, first isolated as a highly toxic protein [10], displays insecticidal effects when fed to insects relying on cathepsin-like digestive enzymes such as hemipteran bugs *R. prolixus* [15,16], *N. viridula* and *D. peruvianus* [17] or bruchid weevils, for example, *C. maculatus* (Coleoptera) [15]. A second goal in the present study was to investigate if the more abundant isoform of jack bean urease, the "classical" urease (JBU), displays insecticidal properties similar to canatoxin. For that purpose, JBU and canatoxin were given in feeding trials to the cotton sucker bug, *D. peruvianus*. Both ureases isoforms induced entomotoxic effects (Table 1 and Fig. 2), being canatoxin more potent than JBU. There is no correlation between insecticidal and enzymatic activities as the protein displaying lower urease activity (canatoxin) showed more potent insecticidal effect. Both ureases kept their insecticidal properties after being treated with *p*-hydroxymercurybenzoate, clearly indicating this feature is independent of ureolytic activity (Fig. 3). Beside the lethal effect, the urease isoforms produced significant delay in development of the surviving insects which, after 20 days feeding on a urease-containing diet, showed decreased body weight (∼40% of controls) and were still nymphs while controls have reached the adult stage (data not shown).

Altogether, our data show that jack bean urease isoforms can be separated by immobilized metal affinity chromatography and that insecticidal activity is not unique to canatoxin, as the more abundant JBU also displays entomotoxic properties unrelated to its ureolytic activity. Additional studies are under way in our laboratory to study the mode of action of these entomotoxic proteins in order to establish their biotechnological potential against phytophagous insects. Further elucidation of the three dimensional structures of these molecules should provide new insights for correlating the structural features to their multiple biological effects.

Acknowledgements

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Anexo 6.2

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Jackbean, soybean and *Bacillus pasteurii* ureases: Biological effects unrelated to ureolytic activity.

European Journal of Biochemistry 271,1357-1363, 2004.

Nesse artigo comparamos as propriedades da urease majoritária de C. ensiformis, a urease embrião-específica da soja (*Glycine max*) e a urease da bactéria *Bacillus pasteurii* quanto à capacidade de induzir agragação plaquetária e o efeito inseticida em *Dysdercus peruvianus.* Os resultados mostraram que as três ureases induzem agregação de plaquetas de coelho, sendo que a urease bacteriana é cerca de 10-20 de grandeza menos potente, em termos de massa, do que as enzimas vegetais. Por outro lado, a propriedade inseticida só foi observado para as ureases vegetais, sendo independente da atividade ureolítica, pois foi não afetada pelo tratamento com o inibidor irreversível, p-hidroximercuri-benzoato.

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Jackbean, soybean and *Bacillus pasteurii* ureases

Biological effects unrelated to ureolytic activity

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In this work we compared two plant ureases, jackbean urease (JBU) and embryo-specific soybean urease (SBU) and a bacterial (*Bacillus pasteurii*) urease, for kinetic parameters and other biological properties described recently for ureases that are independent of the ureolytic activity. The insecticidal effect of ureases was investigated in feeding trials with the cotton sucker bug, *Dysdercus peruvianus* (Hemiptera) as an insect model. Contrasting with *B. pasteurii* urease (PBU), both plant ureases presented potent insecticidal activity, with LD_{50} values of 0.017% (w/w) and 0.052% (w/w) for JBU and SBU, respectively. The insecticidal property of JBU or SBU was not affected by treatment with *p*-hydroxymercuribenzoate, an irreversible inhibitor of ureolytic activity of both proteins. Also, contrasting with canatoxin – a urease isoform from jackbean seeds that displays a toxic effect in mice ($LD_{50} = 2$ mg·kg^{-1}) – no lethality was seen in mice injected intraperitoneally with JBU or SBU (20 mg·kg^{-1}). Similarly to canatoxin, the three enzymes promoted aggregation of blood platelets ($EC_{50} = 400.0$ µg·mL^{-1}, 22.2 µg·mL^{-1}, 15.8 µg·mL^{-1} for BPU, SBU and JBU, respectively). This platelet activating property was also independent of urease activity. Comparison of the kinetic properties indicated that SBU is fivefold less susceptible than JBU to inhibition by acetohydroxamic acid, a chelator of Ni^{+2} and Zn^{+2} ions. The ureases also showed different susceptibility to agents that modify cysteine residues, such as *p*-hydroxymercuribenzoate and *p*-benzoquinone. Altogether, these data emphasize that biological properties that are independent of ureolytic activity are not restricted to jackbean ureases and that these proteins may have a role in plant defense against insect predators.

Keywords: *Bacillus pasteurii* urease; insecticide; jackbean urease; platelet aggregation; soybean urease.

Ureases (urea amidohydrolase; EC 3.5.1.5) are nickel dependent enzymes [1] that catalyze the hydrolysis of urea to form ammonia and carbon dioxide. Ureases have been isolated from a wide variety of organisms including plants, fungi and bacteria [2]. While fungal and plant (e.g. jackbean and soybean) ureases are homo-oligomeric proteins of ≈ 90 kDa subunits, bacterial ureases are multimers of two or three subunit complexes [3,4]. The UreA, UreB and UreC subunits of *Bacillus pasteurii* and most other bacterial ureases are colinear with the single subunit of fungal and plant ureases, the major difference being two gaps, between UreA and UreB and between UreB and UreC. *Helicobacter pylori* urease has two subunits, one being a fusion of UreA and UreB [2,3]. So far only bacterial ureases have had their 3D crystallographic structure successfully resolved, e.g. *Klebsiella aerogenes* (1FWJ), *Bacillus pasteurii* (4UBP) and *Helicobacter pylori* (1E9Z). However, the high sequence similarity of all ureases indicates they are variants of the same ancestral protein and are likely to possess similar tertiary structures and catalytic mechanisms [3].

Urease activity enables bacteria to use urea as a sole nitrogen source. Some bacterial ureases play an important role in the pathogenesis of human and animal diseases such as those from *Proteus mirabilis* and *Helicobacter pylori* [3].

Despite the abundance of urease in some plant tissues, e.g. seeds of members of the families Fabaceae (Leguminosae) and Curcubitaceae, and its ubiquity in virtually all plants [3,4], little has been revealed about its physiological roles. Soybean contains two distinct urease isoenzymes: an ubiquitous urease that is synthesized in all tissues examined and an embryo-specific urease that is confined to the developing embryo and is retained in the mature seed where its activity is roughly 1000-fold greater than that of the ubiquitous urease in many tissues [5,6]. One role of the ubiquitous urease, in recycling metabolically derived urea, has been demonstrated in a number of experimental conditions [4,7–9]. In spite of the high concentration of the protein in the seeds, it has been suggested that the embryo-specific urease plays no role in nitrogen assimilation from urea [4,7,10]. To our knowledge, no recent work has addressed the question of the physiological relevance of this highly active enzyme.

Recently, our group has shown that canatoxin, an isoform of jackbean urease consisting of a dimer of 95 kDa subunits, displays several biological properties

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Abbreviations: AHA, acetohydroxamic acid; BPU, *Bacillus pasteurii* urease; JBU, jackbean urease; *p*-BQ, *p*-benzoquinone; *p*-HMB, *p*-hydroxymercuribenzoate; SBU, soybean urease.

Enzyme: urea amidohydrolase (EC 3.5.1.5).

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independent of its ureolytic activity, such as activation of blood platelets and interaction with glycoconjugates [11–15]. Moreover, canatoxin is lethal to rats and mice when injected intraperitoneally (LD_{50} 2.0 mg per kg body weight) and presents insecticidal activity when fed to some groups of insects, suggesting that ureases may be involved in plant defense [16–18]. The kissing bug *Rhodnius prolixus*, and three economically important crop pests, the cowpea weevil *Callosobruchus maculatus*, the Southern green soybean stinkbug *Nezara viridula* and the cotton stainer bug *Dysdercus peruvianus* are highly susceptible to the entomotoxic effect of canatoxin [18].

In order to investigate if ureases from other sources share, with jackbean ureases, the property of inducing biological effects not related to their ureolytic activity, we have tested soybean embryo-specific urease (SBU) and *Bacillus pasteurii* urease (BPU) [19] for their lethality in mice and for their insecticidal and platelet aggregating activities. Kinetic parameters and susceptibility of SBU and BPU to different inhibitors were also compared with those of the jackbean urease (JBU).

Material and methods

Protein determination

The protein content of samples was determined by their absorbance at 280 nm or, alternatively, by the method of Spector [20].

Bacillus pasteurii urease

A commercially available preparation of BPU (U-7127, Sigma Chemical Co.) was used in all experiments without further purification. The freeze-dried protein was resuspended in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol to give 0.5 mg protein per mL solutions.

Purification of jackbean urease

The jackbean enzyme was purified from jackbean meal based on the method of Blakeley *et al.* [21] with modifications. Dry seeds (Casa Agrodora, São Paulo, Brazil) were powdered and 50 g of defatted meal were extracted with buffer A (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol) for 1 h at 4 °C. The meal was removed by centrifugation (30 000 ***g***, 20 min, 4 °C), and 28% (v/v) ice-cold acetone (final concentration) was added to the supernatant. The suspension was kept at 4 °C overnight and the precipitated proteins were removed by centrifugation (30 000 ***g***, 20 min, 4 °C). The concentration of acetone in the supernatant was then increased to 31.6% (v/v) and, after stirring at room temperature for 10 min, the precipitate was removed by centrifugation (30 000 ***g***, 20 min, 4 °C). The supernant was dialysed against buffer B (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol) and then mixed with 25 mL of Q-Sepharose resin (Amersham-Biotech Pharmacia) equilibrated in buffer B. After stirring in a beaker for 30 min in an ice bath, the mixture was filtered and the resin was washed with 100 mM NaCl in buffer B to remove the nonretained proteins. Elution of an urease-enriched fraction was achieved by adding 300 mM NaCl to buffer B. The active fraction was concentrated using a CentriPrep cartridge (Millipore). The urease-enriched material was then applied into a Superose 6 HR 10/30 gel filtration column (Amersham-Biotech Pharmacia) equilibrated in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, mounted in a FPLC system. The peak fraction containing urease activity was dialysed against 20 mM sodium phosphate, pH 7.0, 500 mM NaCl (buffer C) and then submitted to affinity chromatography on 10 mL of a Co^{+2} loaded iminodiacetic acid-Sepharose resin equilibrated in buffer C. Highly purified urease was recovered in the nonretained fraction (Fig. 1).

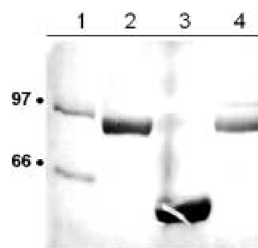

Fig. 1. SDS/PAGE patterns of JBU and SBU. SDS/PAGE analysis was performed using a 10% (w/v) polyacrylamide gel containing 0.1% SDS. After the run, the gel was stained with Coomassie Blue. Lane 1, molecular mass standards; lane 2, JBU (12 µg); lane 3, concanavalin A (12 µg, 27 kDa subunit); lane 4, SBU (10 µg).

Purification of soybean urease

A new method for purifying soybean embryo-specific urease was developed based on the procedure of Blakeley *et al.* [21]. Briefly, dry seeds of soybean (cultivar EM66, Criscium al, RS, Brazil) were powdered and 25 g of defatted meal were extracted with buffer A for one hour at 4 °C. The meal was removed by centrifugation (30 000 ***g***, 20 min, 4 °C), and 20% (v/v) ice-cold acetone (final concentration) was added to the supernatant. The suspension was kept at 4 °C for 2 h and the precipitated proteins were removed by centrifugation (30 000 ***g***, 20 min, 4 °C). The supernatant was dialysed against buffer B and then mixed with 15 mL of Q-Sepharose resin (Amersham-Biotech Pharmacia) equilibrated in buffer B. After stirring in a beaker for 30 min, the mixture was filtered and the resin was washed with 150 mM NaCl in buffer B to remove the nonretained proteins. Elution of an urease-enriched fraction was achieved by adding 300 mM NaCl to buffer B. The gel filtration column and the affinity chromatography in immobilized Co^{+2} were performed as described for JBU. As for JBU, SBU did not bind to immobilized Co^{+2} in the affinity chromatography step. Purified SBU showed a major band in SDS/PAGE analysis (Fig. 1).

SDS-PAGE

Electrophoresis in 10% polyacrylamide minigels containing 0.1% sodium dodecyl sulfate [22] were run at 20 mA for 2–3 h. The gels were stained with Coomassie Blue R-250.

Assay of biological activities of ureases

Toxic activity was expressed as LD_{50} and defined as lethality of mice within 24 h after intraperitoneal injection of single doses (20 mg·kg^{-1}, equivalent to 10 LD_{50} of canatoxin) of the samples [11]. Institutional (IB-UFRGS) protocols designed to minimise suffering and limit the number of animals killed, were followed throughout the experiments.

Platelet-rich plasma was prepared from rabbit blood collected from the ear central artery in the presence of sodium citrate to a final concentration of 0.313% (v/v). Blood samples were then centrifuged at 200 ***g*** for 20 min at room temperature, to give a platelet-rich plasma suspension [12]. Platelet aggregation and shape change were monitored turbidimetrically [23], using a Lumi-Aggregometer apparatus (Chrono-Log Co., Havertown, PA, USA) and light transmission across the rabbit platelet-rich plasma suspension was registered on a chart recorder for 3 min. Platelet aggregation assays were also performed on a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) as described previously [24]. The use of microplate assays has been shown to give results similar to those obtained with Born's aggregometry. Briefly, urease samples (previously dialysed against phosphate buffered saline) in 96-well flat-bottomed plates were prepared to a final volume of 50 µL with saline. Aggregation was triggered by the addition of 100 µL of platelet suspension. Controls were run by adding platelet-poor plasma. The plate was incubated for 2 min at 37 °C before commencing agitation, and readings were taken at 650 nm every 11 s for 20 min. Change in turbidity was measured in absorbance units and results are expressed as the area under the aggregation curves.

The insecticidal activity of ureases was evaluated in feeding trials with the cotton stainer bug *Dysdercus peruvianus* (Hemiptera), which is an economically important crop pest. Groups of 15 second instar insects (from a colony housed in this laboratory) were fed on cotton seed meal mixed with freeze-dried urease in a final protein concentration of 0.02–0.1% (w/w). For this, solutions of ureases were added to cotton seed meal, the mixtures were homogenized, freeze-dried, put inside gelatin capsules and then offered to the insects. Control insects fed on cotton seed meal containing equivalent volumes of freeze dried buffer A alone or containing 20 µM *p*-hydroxymercuribenzoate. For proteins treated with 50 µM *p*-HMB, excess reagent was removed by dialysis against buffer A prior to the bioassays. The insects were kept at 26 °C, 70–80% air humidity, 12-h dark : 12-h light cycle and examined every 2 days during 20 days for lethality, body weight and developmental stage (the insect goes through five instar stages before becoming adult). The results are mean and SEM of triplicates and expressed as survival rate and percentage of body weight of the control insects. LD_{50} values were calculated by linear regression of survival rates after 20 days plotted against five doses of the ureases tested in the feeding trials.

Urease activity

The ammonia released was measured colorimetrically [25]. One unit of urease releases one µmol ammonia per minute, at 37 °C, pH 7.5. Kinetic parameters (K_m and specific activity) were calculated as by Cleland [26]. For inhibitory studies, the proteins were incubated with *p*-hydroxymercuribenzoate (*p*-HMB), acetohydroxamic acid (AHA) and *p*-benzoquinone (*p*-BQ), or the corresponding diluents, for 18–24 h at 4 °C.

Results

Kinetic parameters and inhibitors of urea hydrolysis

Table 1 shows the kinetic parameters for the three ureases, JBU, SBU and BPU. Purified JBU and SBU displayed ureolytic specific activities at pH 7.5 of 22.2 ± 0.7 and 14.2 ± 0.6 U·mg^{-1}, respectively. Susceptibility of the ureolytic activity to different inhibitors was also evaluated (Fig. 2, Table 1). The inhibitors tested were *p*-HMB and *p*-BQ, two cysteine-binding inhibitors, and AHA, a chelator

Table 1. Comparative data on physicochemical and biological properties of soybean embryo-specific urease (SBU), jackbean urease (JBU) and *B. pasteurii* urease (BPU). ND, not determined.

Physicochemical/Biological properties	SBU	JBU	BPU
Physicochemical properties			
Molecular mass, SDS/PAGE	90 kDa	90 kDaA	11,13 and 61 kDa [2,3] (chains A, B and C, respectively)
Native form	hexamer	hexamer	trimer
Urease activity			
K_m	0.2–0.6	2–3.5 [15]	40–130 [2]
Inhibitors, IC_{50}			
p-hydroxymercuribenzoate	38[a]	70	ND
acetohydroxamic acid	216	42	ND
p-benzoquinone	92	54	ND
Biological properties			
Toxicity in mouse, interperitoneal	not toxic	not toxic	ND
Dysdercus peruvianus, LD_{50}	0.052% (w/w)	0.017% (w/w)	not toxic
Treated with *p*-hydroxymercuribenzoate	100% active	100% active	–
Platelet aggregation, EC_{50} (rabbit)	22.2 µg·mL^{-1}	15.8 µg·mL^{-1}	400 µg·mL^{-1}
Treated with *p*-hydroxymercuribenzoate	100% active	100% active	100% active

[a] Values of IC_{50} were taken from Fig. 2 and are expressed as mol of inhibitor per mol of enzyme.

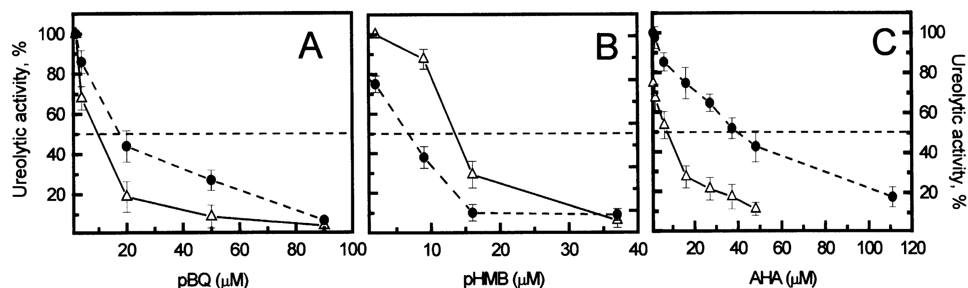

Fig. 2. Inhibition of ureolytic activity of JBU and SBU by *p*-benzoquinone (*p*-BQ), *p*-hydroxymercuribenzoate (*p*-HMB) and acetohydroxamic acid (AHA). Aliquots (0.1 mg·mL^{-1}) of JBU (△) or SBU (●) were incubated for 18–24 h at 4 °C with *p*-BQ (A), *p*-HMB (B) or AHA (C) and then assayed for residual ureolytic activity. Data are means ± SEM of at least four independent experiments.

of Ni^{+2} and Zn^{+2} ions. JBU was fivefold more susceptible to AHA than SBU. Although *p*-HMB and *p*-BQ have the same mechanism of action, different inhibition patterns were seen for JBU and SBU, two highly similar enzymes.

Insecticidal properties of ureases

As described for canatoxin [16,17], JBU and SBU were also highly toxic to the cotton stainer bug *Dysdercus peruvianus* in feeding trials, with calculated LD_{50} values of 0.017% and 0.052% (w/w) of protein added to the cotton meal, respectively (not shown). The time dependency of the entomotoxic effect was similar for both proteins, with a lag-phase of 3–4 days for death of the first insects, and reaching maximal lethality in about two weeks (Fig. 3). Contrasting to the plant ureases, *Bacillus pasteurii* urease was not toxic to the insects in the feeding trials at 0.1% (w/w) concentration (not shown). After treating JBU and SBU with *p*-HMB, an irreversible urease inhibitor, their insecticidal property was re-evaluated. The results showed that *p*-HMB-treated JBU or SBU maintained full toxic activity in the insect (Figs 3B,D and 4), while the enzymatic activity of the proteins was abolished (Fig. 4). Both plant ureases were detrimental for the development of the surviving insects, which showed decreased body weight and delayed progress through the instar stages (Fig. 5).

Platelet aggregation

Similarly to both jackbean ureases, canatoxin [12] and JBU [15], SBU and BPU also induced aggregation of rabbit platelets (Fig. 6). EC_{50} for the platelet aggregation was estimated to be 400.0, 22.2 and 15.8 μg·mL^{-1}, for BPU, SBU and JBU, respectively. As described for canatoxin and JBU [12], SBU was also still fully able to activate platelets after treatment with 50 μM *p*-HMB (Table 1).

Lethality in mice

Canatoxin is lethal to rats and mice (LD_{50} 2 mg·kg^{-1} for mice), while JBU is not [15]. Similarly to JBU, no signs of toxicity were seen after 7 days in animals injected intraperitoneally with 20 mg SBU per kg of body weight. BPU was not tested for intraperitoneal toxicity in mice.

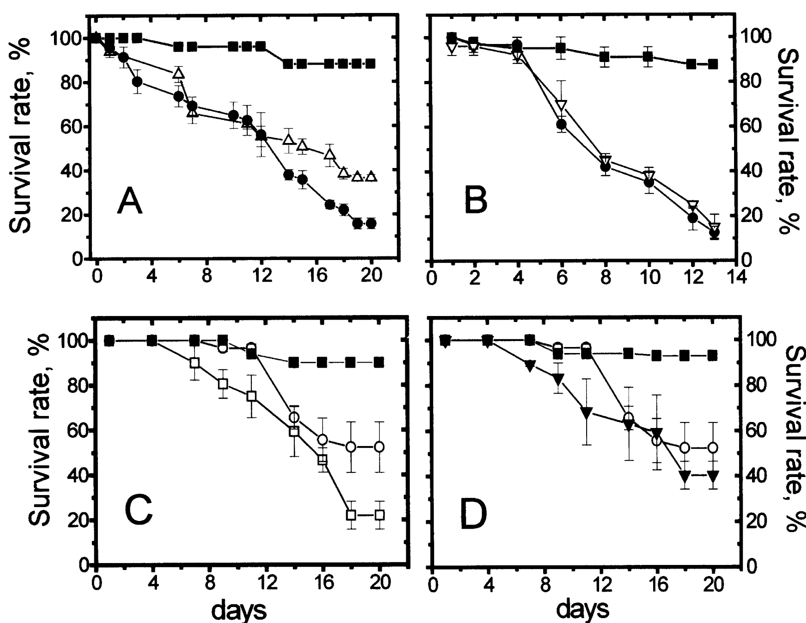

Fig. 3. Insecticidal effect of JBU and SBU in *Dysdercus peruvianus*. The toxic activity of ureases was assayed in feeding trials with *Dysdercus peruvianus* (second instar) using different concentrations of the freeze-dried proteins added to cotton meal. (A) Insecticidal effect of JBU: 0.02% (w/w) (△), 0.05% (w/w) (●); Control: cotton meal alone (■). (B) Effect of *p*-hydroxymercuribenzoate (*p*-HMB)-treatment on the insecticidal activity of JBU. JBU 0.05% (w/w) (●); *p*-HMB-treated JBU (▽); Control: cotton meal containing *p*-HMB (■). (C) Insecticidal effect of SBU: 0.1% (w/w) (□) and 0.05% (w/w) (○); Control: cotton meal alone (■). (D) Effect of *p*-HMB-treatment on the insecticidal activity of SBU. SBU 0.05% (w/w) (○); pHMB-treated SBU (▼); Control: cotton meal containing pHMB (■). Data are mean ± SEM of triplicate points, with 20 insects each.

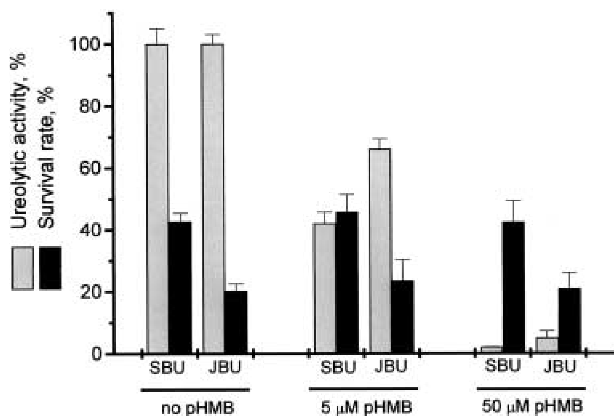

Fig. 4. Effect of *p*-hydroxymercuribenzoate treatment on the insecticidal and ureolytic activities of JBU and SBU. Ureases were incubated for 18–24 h at 4 °C with different concentrations of *p*-HMB (5 μM and 50 μM), dialysed against buffer A and then assayed for ureolytic activity and toxicity for *Dysdercus peruvianus*. Data are means ± SEM of at least four independent experiments.

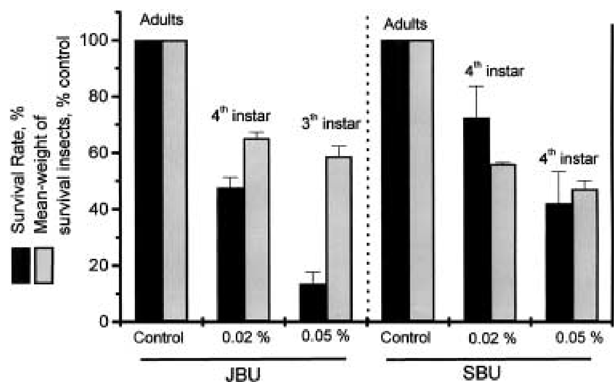

Fig. 5. Detrimental effects of JBU and SBU on the cotton stainer bug, *Dysdercus peruvianus*. The entomotoxic effects of JBU and SBU fed to *Dysdercus peruvianus* were evaluated as survival rate, mean body weight and stage of development of the surviving insects after two weeks. The freeze-dried proteins in concentrations of 0.02% and 0.05% (w/w) were added to the cotton meal, and the insects feeding on them were monitored over 20 days. Data are means ± SEM of at least four independent experiments.

Table 1 summarizes the data on kinetic parameters and biological activities of the three ureases analyzed in this work.

Discussion

Despite their highly conserved structures and similar mechanisms of catalytic action, little is known about the physiological role of ureases in the source organisms.

The wide distribution of ureases in leguminous seeds as well as the accumulation pattern of the protein during seed maturation is suggestive of an important physiological role. As soybean mutants lacking the embryo-specific urease do not exhibit any of the abnormalities associated with loss of the ubiquitous urease, this enzyme probably has no essential physiological function [10]. Studies with developing cotyledons of pea [27] and soybean seedlings [28,29] indicated that urease(s) play little or no role in embryo nutrition. The obvious question from this observation is why the developing soybean embryo would invest in a very active ureolytic protein when it never 'sees' urea.

Canatoxin, first isolated as a highly toxic protein [11] and identified recently as an isoform of jackbean urease [15], displays insecticidal activity against insects of Coleoptera (beetles) and Hemiptera (bugs) orders, such as the cowpea weevil, *Callosobruchus maculatus*, the kissing bug, *Rhodnius prolixus* [16], the cotton stainer bug, *Dysdercus peruvianus* and the green soybean stinkbug, *Nezara viridula* [18]. The entomotoxic property of canatoxin is independent of its enzymatic activity and requires the proteolytic activation of the protein by insect cathepsin-like digestive enzymes in order to produce entomotoxic peptide(s) [17]. The more abundant isoform of urease, here designated JBU, was previously shown to be as lethal as canatoxin in feeding trials with the kissing bug *Rhodnius prolixus* [18].

Here we have analyzed the insecticidal properties of three ureases, JBU, SBU and BPU, using the cotton stainer bug *Dysdercus peruvianus* as the insect model. Only the plant ureases were toxic in the feeding trials. JBU, with a LD_{50} of 0.017% (w/w) was as toxic as canatoxin [16], whereas both jackbean ureases are three-fold more potent than SBU, with a LD_{50} of 0.052% (w/w). Besides lethality, both ureases induced severe detrimental effects in surviving insects, reducing gain in body weight and delaying the developmental stages of nymphs into adults. The insecticidal effect of JBU and SBU was not altered after treating the proteins with *p*-HMB, clearly indicating that this feature is independent of their ureolytic activity (Figs 3 and 4). The lack of insecticidal activity of *Bacillus pasteurii* urease may be explained by its three-chain structure. Part of the region comprising the sequence of the entomotoxic peptide released from canatoxin ([17], patent pending) by insect cathepsins is absent in microbial ureases, corresponding in

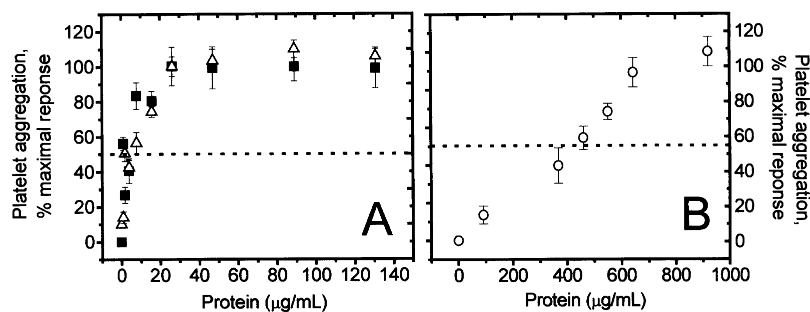

Fig. 6. Platelet aggregation induced by ureases. Platelet suspensions were challenged with (A) JBU (■), SBU (△) or (B) BPU (○), and aggregation of platelets was measured turbidimetrically. Data are means ± SEM of at least four independent experiments ($P < 0.001$).

plant ureases to a fragment located between the UreB and UreC chains of *Bacillus pasteurii* urease. Altogether, our findings suggest that insecticidal activity is a characteristic of plant ureases and provide compelling evidence for a possible defense role of these proteins. Additional studies are under way in our laboratory to characterize and to study the mode of action of entomotoxic ureases in order to establish their biotechnological potential against phytophagous insects.

Contrasting with canatoxin, which is highly toxic in rats and mice [11], both JBU and SBU were not lethal to mice when given intraperitoneally (maximal dose tested 20 $mg \cdot kg^{-1}$). Thus, there is no correlation between the insecticidal activity of ureases and the intraperitoneal toxicity in mice, until now a property displayed only by canatoxin. It is plausible to think that this unique feature of canatoxin may be related to its dimeric form, as compared to the hexameric JBU and the embryo-specific SBU, making it more difficult for the larger proteins to be absorbed from the site of injection into the blood stream.

All three ureases studied here shared with canatoxin the ability of inducing activation of rabbit blood platelets [12–15]. JBU and SBU showed similar potency as inducers of platelet aggregation (Fig. 6), with $EC_{50} = 22.2$ and 15.8 $\mu g \cdot mL^{-1}$ for SBU and JBU, respectively. BPU, on the other hand, showed a 20-fold lower potency, with EC_{50} of 400 $\mu g \cdot mL^{-1}$. The time pattern of platelet response to the ureases was very similar, showing a collagen-type shape change reaction. As already described for canatoxin and JBU [15], this activity was retained in *p*-HMB treated SBU confirming it is independent of the enzymatic activity.

These newly described properties of plant and microbial ureases may shed new light on the physiological roles of these proteins in the source organisms. The involvement of plant ureases in the bioavailability of nitrogen is still controversial. Brodzik *et al.* [30] reported no significant alteration in the growth pattern of tobacco plants expressing *Helicobacter pylori* urease, which caused a two-fold increase in the ureolytic activity and an eight-fold increase in ammonia levels of the transgenic plants as compared to controls. However, these authors did not test the transgenic plants for their resistance to insects or phytopathogens. Polacco and Holland [7] have proposed that plant ureases may have a role in plant defense, assuming the released ammonia would have a deleterious effect upon predators. Altogether, our data reinforce the possibility of plant ureases having a protective role through an entirely different mechanism, unrelated to the release of ammonia.

We also compared the kinetic properties of these enzymes on the hydrolysis of urea and susceptibility to different urease inhibitors. Even the highly homologous JBU and SBU (86% identity and 92% similarity in a BLAST analysis) have different susceptibility to inhibition by *p*-HMB, AHA or *p*-BQ. Our data showed that JBU is fivefold more sensitive than SBU to inhibition by AHA, a Ni^{+2} and Zn^{+2} ions chelator [31], suggesting a different environment for the nickel atoms within the catalytic site. JBU and SBU also showed different susceptibility to two cysteine-reactive urease inhibitors, *p*-HMB [15,32] and *p*-BQ [33].

Taken together, our data show that ureases from plant and microbial sources belong to a group of multifunctional proteins with at least two distinctive domains: a thiol-dependent domain containing the ureolytic active site and a thiol-independent domain involved in toxic effects in insects (and mice, only for canatoxin) and the activation of blood platelets. Further elucidation of the 3D structures of plant enzymes should provide new insights for understanding the structural basis of the multiple biological effects displayed by ureases.

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Anexo 6.3

Becker-Ritt, A.B., Martinelli, A.H.S., Mitidieri, S., Feder, V., **Wassermann, G.E.**, Santi, L., Vainstein, M.H., Oliveira, J.T.A., Fiuza, L.M. , Pasquali, G and Carlini, C.R.

Antifungal activity of ureases.

Toxicon 50, 971- 983, 2007.

Nesse artigo nós demonstramos que a urease embrião-específico de soja (*Glycine max*), a urease majoritária do feijão-de-porco (*Canavalia ensiformis*) e uma recombinante de *Helicobacter pylori* urease têm efeito inibitório sobre o desenvolvimento de certos fungos fitopatogênicos filamentosos em concentrações sub-micromolares. A propriedade antifúngica das ureases não é afetada pelo tratamento das proteínas com um inibidor irreversível da atividade ureolítica. Microscopia eletrônica de varredura de fungos tratados com ureases sugerem plasmólise e injúrias à parede celular. Esses resultados reforçam um provável papel das ureases, somada à atividade entomotóxicas dessas proteínas, como parte do arsenal de compostos de defesas que a plantas usam contra predadores e fitopatógenos.

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Antifungal activity of plant and bacterial ureases ☆

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Abstract

Ureases (EC 3.5.1.5) are nickel-dependent metalloenzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide. Produced by plants, fungi and bacteria, but not by animals, ureases share significant homology and similar mechanisms of catalysis, although differing in quaternary structures. While fungal and plant ureases are homo-oligomeric proteins of 90 kDa subunits, bacterial ureases are multimers of two (e.g. *Helicobacter pylori*) or three subunit complexes. It has been proposed that in plants these enzymes are involved in nitrogen bioavailability and in protection against pathogens. Previous studies by our group have shown that plant ureases, but not a bacterial (*Bacillus pasteurii*) urease, display insecticidal activity. Herein we demonstrate that (*Glycine max*) embryo-specific soybean urease, jackbean (*Canavalia ensiformis*) major urease and a recombinant *H. pylori* urease impair growth of selected phytopathogenic fungi at sub-micromolar concentrations. This antifungal property of ureases is not affected by treatment of the proteins with an irreversible inhibitor of the ureolytic activity. Scanning electron microscopy of urease-treated fungi suggests plasmolysis and cell wall injuries. Altogether, our data indicate that ureases probably contribute to the plant arsenal of defense compounds against predators and phytopathogens and that the urease defense mechanism is independent of ammonia release from urea.
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Keywords: Antifungal protein; Plant pathogen; Urease; Soybean; Jackbean; *Helicobacter pylori*

☆ *Ethical statement*: The experimental protocols and procedures used to obtain the results were approved by the Research and Ethics Committee of the Institute of Biosciences, Federal University of Rio Grande do Sul.

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1. Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide (Dixon et al., 1975), widespread in plants, fungi and bacteria (Mobley and Hausinger, 1989). While fungal and plant ureases are homo-hexameric proteins of 90 kDa subunits, bacterial ureases are multimers of two or three subunit complexes. The sequence similarity of all ureases indicates that they are variants of the same ancestral protein, and are likely to possess highly conserved tertiary structures and similar catalytic mechanisms (Mobley et al., 1995; Sirko and Brodzik, 2000). Some bacterial ureases, such as those from *Proteus mirabilis* and *Helicobacter pylori* (Mobley et al., 1995; Olivera-Severo et al., 2006), play an important role in the pathogenesis of human and animal diseases. In the fungi *Coccidioides posadassi* (Mirbod-Donovan et al., 2006), *C. immitis* (Li et al., 2001), *Paracoccidioides brasiliensis* (Rappleye and Goldman, 2006) and *Cryptococcus neoformans* (Cox et al., 2000; Olszewski et al., 2004), urease production is also probably related to pathogenesis in humans.

Despite the abundance of ureases in plants, little has been revealed about their biological roles (Polacco and Holland, 1993; Sirko and Brodzik, 2000). Urease has been proposed to function coordinately with arginase in the utilization of seed protein reserves during germination (Thompson, 1980). A second proposed role is the assimilation of urea derived from ureide metabolism (Shelp and Ireland, 1985) or imported from the environment since urea is an effective foliar fertilizer (Zonia et al., 1995).

The best-studied urease derives from jackbean (*Canavalia ensiformis*) which was crystallized by Sumner (1926), representing the first crystals of a characterized enzyme. We described a family of urease-related genes in jackbean (Pires-Alves et al., 2003) and characterized an isoform of jackbean urease, named canatoxin (Carlini and Guimarães, 1981; Follmer et al., 2001, 2004a). Urease activity was found in all tissues of soybean plants (Polacco and Havir, 1979; Das et al., 2002). Soybean produces two urease isoenzymes (Holland et al., 1987) that share 87% identity at the amino acid level (Torisky and Polacco, 1990; Goldraij et al., 2003). The embryo-specific urease, encoded by the *Eu1* gene, is synthesized only in the developing embryo (Polacco and Havir, 1979; Polacco and Winkler, 1984), while the so-called "ubiquitous" urease, encoded by the *Eu4* gene, was found in all tissues examined (Polacco and Winkler, 1984; Stebbins et al., 1991). Although the ubiquitous urease is present in seeds at 0.001–0.01% levels of that of the embryo-specific urease, this isoenzyme is responsible for recycling metabolically derived urea (Polacco et al., 1985; Stebbins et al., 1991; Witte et al., 2002). It has been suggested that the embryo-specific urease does not play a urea-assimilatory role and that it may be involved in seed chemical defense (Polacco and Holland, 1993).

Our group has shown that plant and microbial ureases display several biological properties that are independent of their ureolytic activity, such as the activation of blood platelets, interaction with glycoconjugates and insecticidal activity (Follmer et al., 2001, 2004a). Besides these activities, jackbean canatoxin is also lethal to rats and mice by an intraperitoneal route (Carlini and Guimarães, 1981; Follmer et al., 2001) and inhibits the growth of phytopathogenic fungi (Oliveira et al., 1999). The entomotoxic effects of jackbean ureases and of the soybean embryo-specific soybean urease persisted after treatment of the enzymes with an irreversible inhibitor, demonstrating that protein domain(s) other than the active site might be involved (Follmer et al., 2004a, b). Actually, an internal entomotoxic peptide released upon digestion by insect cathepsins accounts for the insecticidal activity of canatoxin (Carlini et al., 1997; Ferreira-DaSilva et al., 2000). These findings reinforce the hypothesis that ureases might be involved in plant defense mechanisms (Carlini and Grossi-de-Sá, 2002). In the present work, we evaluated the inhibitory activities of two plant ureases and a bacterial urease on mycelial growth and/or spore germination of filamentous fungi, most of them important plant pathogens.

2. Material and methods

2.1. Embryo-specific soybean urease (SBU) and jack bean urease (JBU)

Embryo-specific urease was isolated from a local commercial variety of soybeans according to Follmer et al. (2004a), except for the last step in the purification protocol aimed to separate urease isoforms. The jackbean (major) urease was purified from *C. ensiformis* meal according to Follmer et al. (2004a). Homogeneity of purified proteins was checked by SDS-PAGE. The purified embryo-specific

soybean urease (SBU) and jackbean urease (JBU) were kept in 20 mM sodium phosphate (NaPB), pH 7.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol, at 4 °C.

2.2. *H. pylori recombinant urease (HPU)*

E. coli SE5000 cells expressing *H. pylori* urease (McGee et al., 1999) were kindly provided by Dr. Harry L. Mobley, University of Michigan Medical School, Ann Arbor, MI, USA. The crude extract obtained from an overnight culture was fractionated with ammonium sulfate at 30–70% saturation. The urease-enriched material was dialyzed against 20 mM NaPB, pH 7.8, 5 mM β-mercaptoethanol and 1 mM EDTA, and mixed with Q-Sepharose (Amersham-Biotech Pharmacia) (1 mL resin per 10 mg protein) equilibrated with the same buffer. Elution of urease-enriched fractions was achieved by adding 200 mM NaCl to the equilibrium buffer. The material was then applied into a Superose 6HR 10/30 column (Amersham-Biotech Pharmacia) equilibrated in the same buffer. Pooled fractions displaying urease activity were used in the experiments. Homogeneity of the purified protein was checked by SDS-PAGE.

2.3. *Chemical modifications of ureases*

Proteins were treated with 0.1 mM *p*-hydroxymercuribenzoate (*p*-HMB, Sigma Chemical Co.) for 24 h at 4 °C, and excess reagent was removed by exhaustive dialysis against 10 mM NaPB, pH 7.0, without β-mercaptoethanol (Follmer et al., 2004a). In the experiments using modified ureases, the last change of dialysis buffer was used as control.

2.4. *Protein content determination*

The protein content of fractions was determined by the method of Bradford (1976), using bovine serum albumin as standard.

2.5. *Urease activity*

Aliquots of 50 µL of 100 mM urea solution were mixed with protein samples and buffered with 20 mM NaPB, pH 7.5, to give a final volume of 500 µL. Reaction mixtures were incubated for 30 min at 37 °C and the ammonia released was determined colorimetrically (Weatherburn, 1967). One unit of urease activity was defined as the amount of enzyme required to release 1 µmol $NH_3\,min^{-1}$ at 37 °C and pH 7.5 under the conditions described.

2.6. *Fungi*

Rhizoctonia solani, *Fusarium solani*, *Fusarium oxysporum*, *Trichoderma* sp., *Trichoderma pseudokoningii*, *Trichoderma viride*, *Penicillium* sp., *Colletotrichum musae* and *Curvularia lunata* were from fungal collections maintained at the Department of Biochemistry and Molecular Biology, Universidade Federal do Ceará, Fortaleza, CE, Brazil, or at Universidade do Vale dos Sinos, São Leopoldo, RS, Brazil. *Penicillium herguei*, *Colletotrichum gloeosporioides* and *Aspergillus glaucus* were kindly provided by Dr. Valdirene Gomes, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil.

2.7. *Antifungal activity*

(1) *Disc plate diffusion assay*: Spores (10^6 in 100 µL) were spread over the surface of Petri dishes (86-mm internal diameter) containing potato-dextrose-agar (PDA, Acumedia Manufacturer, Inc.). Sterile filter paper discs (5-mm diameter, Whatman 3MM) loaded with about 40 µL of protein solutions (12.5 µg protein μL^{-1} in 20 mM NaPB, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.5) were placed in the center onto previously inoculated PDA plates. The dishes were inverted and then incubated at 28 °C. Antifungal activity was visualized as a zone of inhibition of fungal growth around the paper disc. Alternatively, to evaluate spore germination, 10^6 spores were suspended in 40 µL of protein solutions (12.5 µg protein μL^{-1}), incubated for 2 h at 28 °C and then inoculated onto the PDA-containing Petri dishes. The toxic effect of ureases was assessed by visual comparison of the mycelial development of protein-treated fungi and untreated controls.
(2) *Turbidimetric evaluation of fungal growth*: Spores (10^3 in 10 µL) were inoculated onto 96-well plates containing 110 µL potato dextrose broth (PDB, Becton Dickenson Co.) buffered to pH 7.0 with 10 mM NaPB (to avoid precipitation of urease), containing 1 mM β-mercaptoethanol, and incubated at 28 °C for 16 h, followed by addition of 50 µL of protein solutions in the same buffer (time zero). As controls, 50 µL buffer alone or 9.5% v/v H_2O_2

was used. The plates were incubated at 28 °C without shaking and the absorbance at 430 nm (A_{430}) was followed on a plate reader (Spectramax, Molecular Devices) at 12 h intervals up to 72 h (Broekaert et al., 1990).

2.8. Determination of antifungal IC_{50}

Concentrations of SBU causing 50% inhibition of growth (IC_{50}) were calculated for some fungi. Fungal growth of triplicates was measured turbidimetrically (as described above) in the presence of buffer (100% growth) or six different concentrations (from 0.05 to 1.15 μM) of SBU. The protein concentrations reducing growth to 50% of control values after 72 h were taken as IC_{50}.

2.9. Scanning electron microscopy

Preparation of samples for scanning electron microscopy (SEM) analysis was done according to Faganello et al. (2006). *P. herguei* mycelia cultured for 48 h as in the turbidimetric assay in the absence or presence of 0.57 μM SBU were recovered by filtration in paper filters (Whatmann no. 1). The retained mycelial mass was fixed for 4 days at 4 °C with 2% (v/v) glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. Post-fixation was carried out in 1% (w/v) osmium tetroxide in the same buffer. The specimens were rinsed in buffer, dehydrated in a series of 30–100% acetone solutions, dried at critical point in CO_2 (CPD 030 BALTEC), and coated with gold in a sputter-coater (SCD 050 BALTEC). The material was examined in a Jeol JSM 6060 scanning electron microscope at the Centro de Microscopia Eletrônica da Universidade Federal do Rio Grande do Sul (CME/UFRGS, Porto Alegre, RS, Brazil).

2.10. Statistical analysis

The results were subjected to analysis of variance (ANOVA) and the significance of differences among means was determined by the Tukey test, with $p \leqslant 0.05$ considered statistically significant.

3. Results

3.1. Native and modified ureases

The ureolytic specific activities of soybean embryo-specific urease (SBU), jackbean major urease (JBU) and *H. pylori* urease (HPU) used in the experiments were approximately 0.525, 1.62 and 2.52 U mg^{-1} protein min^{-1} at pH 7.5, respectively. After treatment with ρ-hydroxy-mercuribenzoate, which irreversibly blocks the enzymatic activity of ureases by alkylating a cysteine residue near the active site (Jabri et al., 1995), modified SBU or JBU showed less than 5% of their initial enzymatic activities.

3.2. Effect of ureases on fungal growth

As a first approach to test the antifungal properties of ureases, the ability of embryo-specific soybean urease (SBU) to inhibit hyphal growth and the germination of fungal spores was investigated using a disc plate diffusion assay. Table 1 shows that SBU suppressed mycelial growth and/or inhibited spore germination of *F. solani*, *P. herguei*, *C. lunata* and three species of *Trichoderma*. To obtain more quantitative data, inhibition of fungal growth was analyzed turbidimetrically in microwell plates. Fig. 1 shows dose curves for the inhibitory effects of SBU upon the ascomycetes phytopathogens *C. musae*, the etiological agent of antracnose in bananas and the foliar blight on *Zoysia* grass, and *C. lunata*, the most commonly associated organism with the crown rot disease, an important problem in export bananas. Table 2 shows that SBU antifungal activity (IC_{50}) in the turbidimetric assay is within the 0.1–1.0 μM range for 4 out of 7 species of the tested fungi, including against *F. oxysporum*, a phytopathogen that causes wilt disease in more than a hundred species of plants. However, no antifungal activity was observed for *T. viride* or *F. solani*. Fig. 2 compares the growth of seven different fungi after 48 and 72 h of exposure to 0.57 μM of SBU or JBU. *C. musae* and *P. herquei* (a maize pathogen) were affected by both ureases. SBU inhibited also the growth of *F. oxysporum*, but even the highest concentration tested for this protein (1.15 μM) did not affect the growth of *F. solani*. Although *T. viride* was inhibited by SBU in the agar plate tests (Table 1), it was not affected in liquid medium assay at the maximal dose tested (1.15 μM). On the other hand, *F. solani* and *F. oxysporum* were inhibited only by JBU. Fig. 2 also shows that the antifungal effect of 0.57 μM SBU was more persistent, with little or no change at 72 h as compared to 48 h exposition. In contrast, the inhibitory effect of JBU was short-lasting as the inhibition seen at 48 h decreased or was overcome

Table 1
Antifungal activity of soybean seed urease on mycelial growth and spore germination of filamentous fungi

Fungi	Phylum/class/pathogenicity	Antifungal activity	
		Mycelial growth[a]	Spore germination[b]
Aspergillus glaucus	Ascomycota/Eurotiomycetes Potential opportunist or pathogen, allergenic	–	–
Colletotrichum gloeosporioides	Ascomycota/Sordariomycetes Plant pathogen	–	–
Curvularia lunata	Ascomycota/Euascomycetes Plant pathogen	3.20±0.70	+
Fusarium oxysporum	Ascomycota/Euascomycetes Plant pathogen	–	–
Fusarium solani	Ascomycota/Euascomycetes Plant pathogen	–	+
Penicillium herquei	Ascomycota/Eurotiomycetes Plant pathogen, Saprophytic	3.81±0.95	+
Penicillium sp.	Ascomycetes Soilborne fungus, Saprophytic	–	–
Rhizoctonia solani	Basidiomycota/Homobasidiomycetes Plant pathogen	–	–
Trichoderma pseudokoningii	Ascomycota/Sordariomycetes Plant pathogen	4.02±0.66	+
Trichoderma sp.	*Ascomycetes*	+	+
Trichoderma viride	Ascomycota/Sordariomycetes Saprophytic	2.75±0.67	+

Solutions of soybean seed urease (500 μg/assay) were applied onto disc papers for the assay of hyphal growth inhibition or incubated with spore suspensions subsequently tested for germination.
[a]Diameter in centimeters of inhibition zone after 48 h at 28 °C (mean±SD, $N = 3$);
[b]+, inhibition; –, no inhibition.

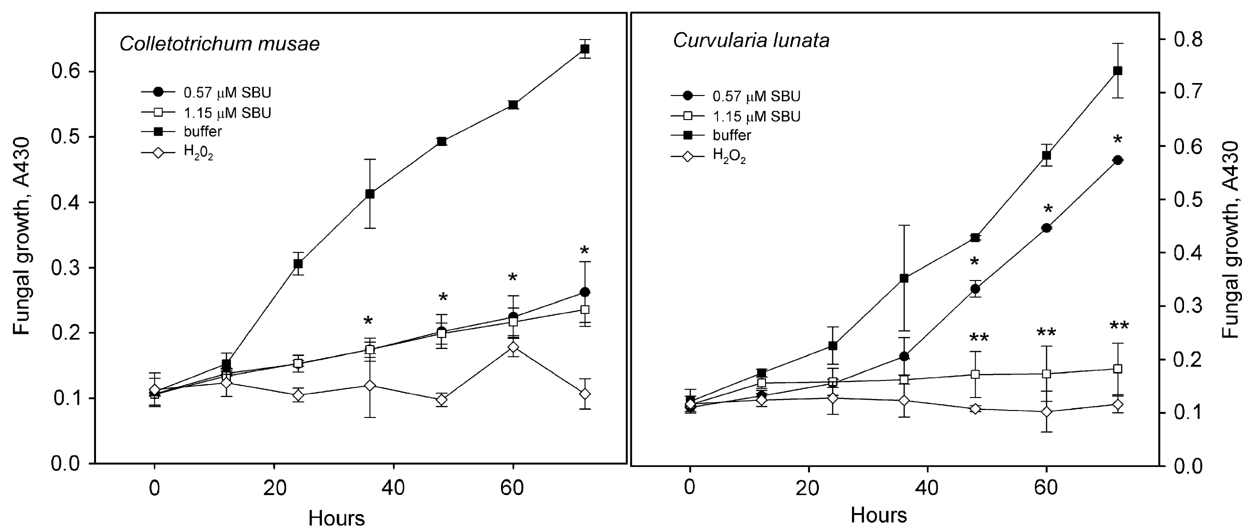

Fig. 1. Inhibitory effect of soybean urease (SBU) on growth of *C. musae* (left panel) and *C. lunata* (right panel). Spores (10^3 in 10 μL) of each fungus were inoculated onto 96-well plates containing 110 μL of potato dextrose broth buffered to pH 7.0 with 10 mM NaPB containing 1 mM β-mercaptoethanol. After incubation at 28 °C for 16 h, 50 μL of SBU solutions in the same buffer were added to the wells. As controls, 50 μL buffer alone or 9.5% (v/v) H_2O_2 was used. The plates were further incubated at 28 °C and the absorbance at 430 nm (A_{430}) was read every 12 h up to 72 h. Data shown (mean±SD, triplicate points) are from one experiment out of at least three with similar results. Means indicated by (*) or (**) are statistically different from fungal growth in the presence of buffer according to Tukey's test ($p<0.05$ or 0.02, respectively).

Table 2
Antifungal activity (IC_{50}) of soybean seed urease (SBU)

Fungus	SBU-IC_{50} (μM)
Fusarium solani	NI
Colletotrichum musae	0.81 ± 0.04
Curvularia lunata	0.61 ± 0.02
Trichoderma viride	NI
Penicillium herguei	0.27 ± 0.02
Fusarium oxysporum	0.61 ± 0.03

Fungal growth of triplicates was measured turbidimetrically (as described above) in the presence of buffer (100% growth) or six different concentrations (0.05–1.15 μM) of SBU. The protein concentrations reducing growth to 50% of control values after 72 h were taken as IC_{50}. Values are mean ± SD of triplicate points ($N = 6$). NI: no-inhibition at the maximal dose tested.

by most fungi at 72 h. For *C. lunata* and *P. herguei,* inhibition caused by 0.57 μM JBU at 48 h gives place to an enhanced growth after 72 h. Control experiments in which SBU and JBU were submitted to the same incubation time (72 h) and temperature (28 °C) in the absence of fungi indicated that the proteins resisted these conditions without appreciable loss of enzymatic activity.

Fig. 3 shows that the recombinant *H. pylori* urease also inhibited the mycelial growth of *P. herguei* and *C. lunata*. Although the microbial enzyme shows 2- to 5-fold higher ureolytic activity as compared to JBU or SBU, respectively, it was less effective to inhibit fungal growth when the same dose of the three proteins was used.

3.3. Antifungal effect of ureases is not related to the enzymatic activity

The lower antifungal activity of HPU suggested that ammonia release is not important for the protein's fungicidal/fungistactic property. To assess the involvement of the enzymatic activity of SBU and JBU in their antifungal property, *p*-hydroxymercuribenzoate inactivated enzymes (mSBU and mJBU, respectively) were tested on *P. herguei* (highly sensitive) and *C. lunata* (moderately sensitive) development. Fig. 4 shows that modified JBU, although devoid of ureolytic activity, was fungal growth. The same result was obtained with modified SBU (not shown), indicating that the ureolytic activity of the proteins does not contribute to their antifungal effect.

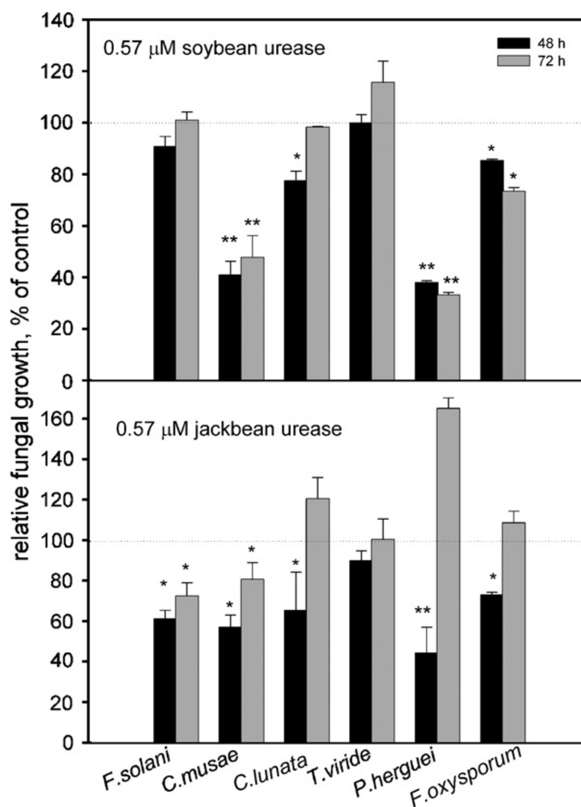

Fig. 2. Susceptibility of different fungi to soybean (upper panel) and jackbean (lower panel) ureases. Spores (10^3 in 10 μL) were inoculated onto 96-well plates containing 110 μL of PDB buffered to pH 7.0 with 10 mM NaPB, 1 mM β-mercaptoethanol, incubated at 28 °C for 16 h, and then 50 μL of the protein solutions in the same buffer were added to the wells. The plates were incubated at 28 °C and the absorbance at 430 nm (A_{430}) after 72 h was recorded. Data (mean ± SD of triplicate points) are expressed as percentage of correspondent control growth in the presence of buffer alone. One experiment out of at least three with similar results is shown. Means indicated by (*) or (**) are statistically different from fungal growth in the presence of buffer (100%) according to Tukey's test ($p < 0.05$ or 0.02, respectively).

3.4. Scanning electron microscopy analysis

Marked reduction in the turbidity of the growing cultures was seen under the present experimental conditions. In control *P. herguei*, hyphae appeared linear, elongated and with smooth surfaces (Fig. 5). In contrast, fungi treated with SBU or JBU showed vegetative hyphae appearing branched, disorganized and collapsed, some with distorted ends. Other important alterations of *P. herguei* in the presence of ureases include cell enlargement (distended balloon-shaped cells), irregularities of the hyphal surface, abnormal hyphal outgrowth and ruptures of the cell wall, suggestive of plasmolysis.

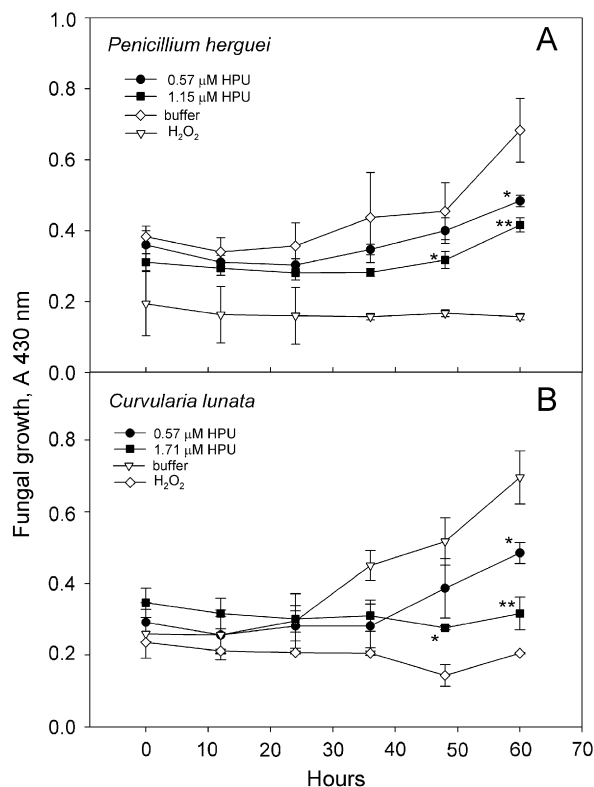

Fig. 3. Effect of *H. pylori* urease (HPU) on fungal growth. Spores (10^3 in 10 μL) of *P. herguei* (panel A) or *C. lunata* (panel B) were inoculated onto 96-well plates containing 110 μL of PDB buffered to pH 7.0 with 10 mM NaPB, 1 mM β-mercaptoethanol, incubated at 28 °C for 16 h, and then 50 μL of HPU solution in the same buffer was added to the wells. As control, 50 μL buffer alone or 9.5% (v/v) H_2O_2 was used. The plates were incubated at 28 °C and the absorbance at 430 nm (A_{430}) was read every 12 h up to 60 h. Data shown (mean ± SD of triplicate points) are from one experiment out of at least three with similar results. Means indicated by (*) or (**) are statistically different from fungal growth in the presence of buffer according to Tukey's test ($p < 0.05$ or 0.02, respectively).

4. Discussion

Antifungal peptides and proteins are among the arsenal of compounds plants produce to combat phytopathogenic fungi (Broekaert et al., 1997; Selitrennikoff, 2001; Thomma et al., 2002; De Lucca et al., 2005). Transgenic plants expressing antifungal proteins are expected to be resistant to the devastating damage caused by fungal infections (De Lucca, 2000, 2005). Several families of antifungal plant proteins have been isolated from different organs (seeds, leaves and flowers) and intercellular fluids. Major groups of plants proteins with antifungal activity comprise thaumatin-like proteins, chitinases, chitin-binding proteins, defensins, defensin-like proteins, ribosome-inactivating proteins, lipid transfer protein-like proteins, protease inhibitors and lectins, among others (Broekaert et al., 1997; De Lucca, 2000, 2005; Selitrennikoff, 2001).

To our knowledge, there are no reports so far on the isolation of antifungal proteins from plants of the *Canavalia* genus. The glucose–mannose lectins from *C. ensiformis* (concanavalin A) and from the closely related *C. gladiata* are devoid of antifungal activity (Wang and Ng, 2007; Wong and Ng, 2005). An endochitinase (Schlesier et al., 1998) and an inactivated chitinase-like protein (concanavalin B, (Hennig et al., 1995) were isolated from *C. ensiformis,* but they were not assayed for antifungal properties.

In soybeans, volatiles derived from the enzymatic action of lipoxygenase are able to inhibit the growth of *Aspergillus flavus* and the production of aflatoxins (Boue et al., 2005). Glysojanin, a protein (25 kDa) homologous to chitin synthase isolated from the black soybean *Glycine soja*, showed potent antifungal activity against the fungi *F. oxysporum* and *Mycosphaerella arachidicola* (Ngai and Ng, 2003).

Regarding *H. pylori*, it has been shown that HP (2–20), a cecropin-like peptide derived from the N-terminal region of the ribosomal protein L1, possesses antimicrobial activity and exerts antifungal effects by damaging the plasma membranes of *Candida albicans* (Ribeiro and Medina-Acosta, 2003).

In this work, we showed that the major jackbean urease and the soybean seed urease were able to inhibit the vegetative growth and/or germination of several filamentous fungi at sub-micromolar concentrations. Although less active, the two-chain urease of *H. pylori* was also detrimental to fungal growth. Canatoxin, an isoform of jackbean urease, was previously shown to inhibit the growth of the phytopathogenic fungi *Macrophamina phaseolina, Sclerotium rofstii* and *Colletotrichum gloesporioides* using 1 mg purified protein in a disc plate diffusion assay (Oliveira et al., 1999).

Little is known about the physiological role of ureases, especially in plants. A perception shared by many is that urease plays no major role in plants because its substrate, urea, is not a major plant metabolite (Polacco and Holland, 1993).

The distribution and accumulation pattern of ureases in leguminous seeds during embryo maturation are

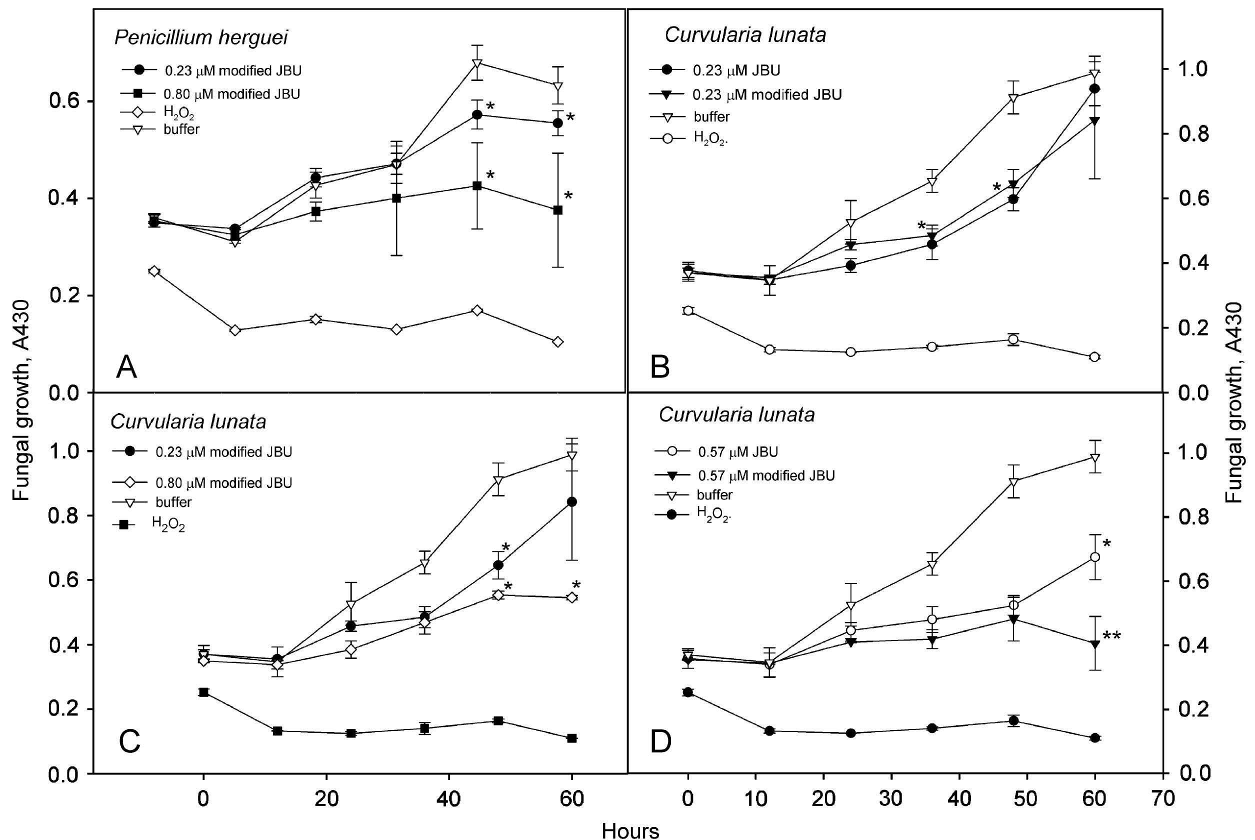

Fig. 4. Effect of *p*-hydroxymercuribenzoate modified jackbean urease (mJBU) on fungal growth. Spores (10^3 in 10 μL) of *P. herguei* (panel A) or *C. lunata* (panels B–D) were inoculated onto 96-well plates containing 110 μL of PDB buffered to pH 7.0 with 10 mM NaPB, 1 mM *β*-mercaptoethanol, incubated at 28 °C for 16 h, and then 50 μL of native or modified JBU solution was added to the wells. As control, 50 μL buffer or 9.5% v/v H_2O_2 was used. The plates were incubated at 28 °C and the absorbance at 430 nm (A_{430}) was read every 12 h up to 60 h. Data shown (mean ± SD of triplicate points) are from one experiment out of at least three with similar results. Means indicated by (*) or (**) are statistically different from fungal growth in the presence of buffer according to Tukey's test ($p<0.05$ or 0.02, respectively).

suggestive of an important physiological role. Both jackbean urease isoforms and the embryo-specific soybean urease present entomotoxic activity, but not the tri-chain bacterial enzyme from *Bacillus pasteurii* (Follmer et al., 2004a, b). The entomotoxicity relies on an internal fragment of the proteins released upon hydrolysis by insect midgut proteases (Ferreira-DaSilva et al., 2000). The lack of insecticidal properties of *B. pasteurii* urease is probably related to the absence of part of this entomotoxic peptide, whose amino acid sequence corresponds to a gap between the C-terminal of B chain and the N-terminal of the C chain of the bacterial enzyme (Follmer et al., 2004a). Here we show that *H. pylori* urease has fungicidal activity. This two-chain bacterial urease lacks the same part of the protein molecule as the *B. pasteurii* enzyme when both are compared to one-chain plant ureases (Fig. 6). Thus, it can be concluded that the internal fragment of plant ureases displaying entomotoxic activity is not involved in the fungicidal property of plant and *H. pylori* enzymes. Confirming this hypothesis, we observed that a recombinant insecticidal peptide derived from an isoform of jackbean urease (jaburetox-2Ec) did not affect the growth of different fungi (unpublished data).

The antifungal activity of ureases is not related to their enzymatic activity. This was shown by the lower antifungal effect of the more enzymatically active *H. pylori* enzyme, as well as by the persistence of the antifungal properties of plant ureases irreversibly inactivated by *p*-hydroxy-mercuribenzoate. Canatoxin is devoid of chitinase and proteinase inhibitory activities (Carlini et al., 1997), which could account for the antifungal property of the jackbean ureases. Preliminary results showed that

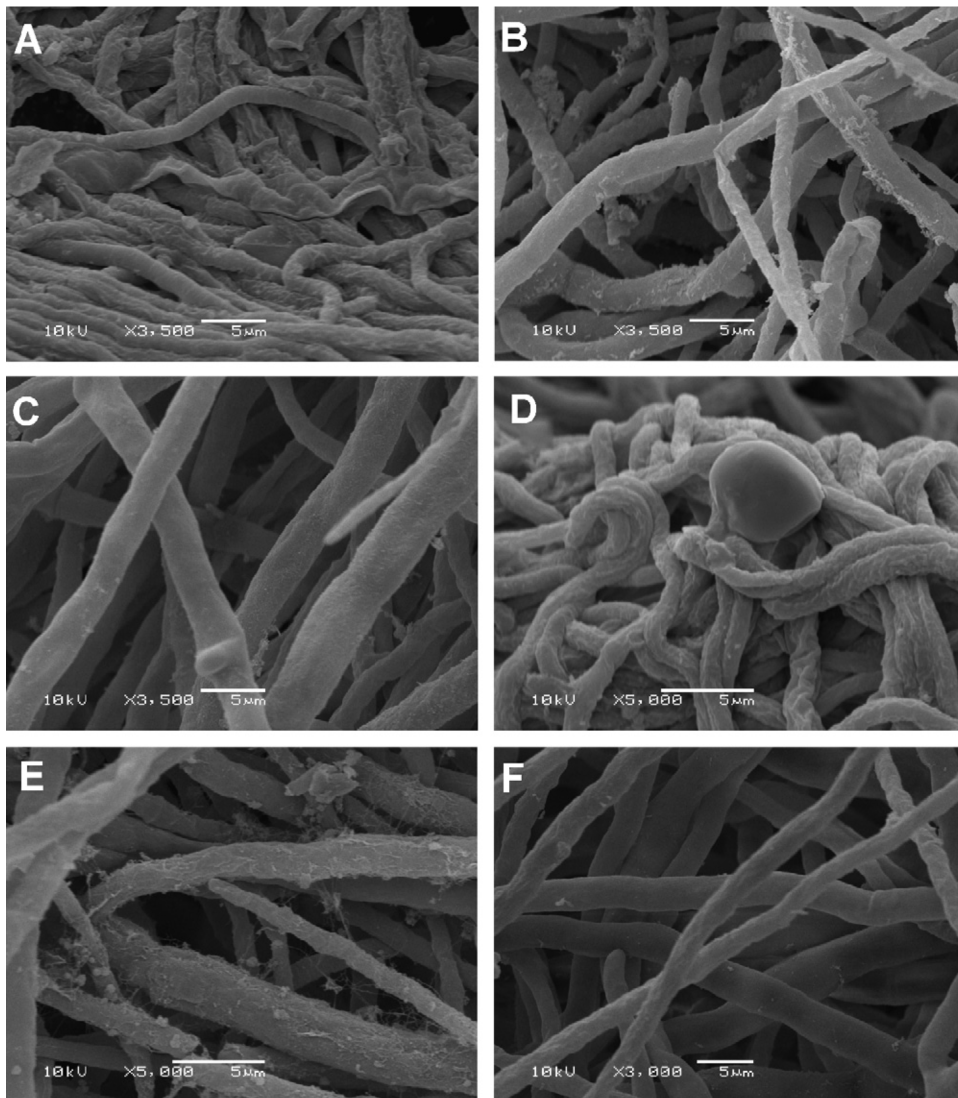

Fig. 5. Scanning electron microscopy of *P. herguei*. The fungi were fixed after 48 h of culture at 28 °C in the absence (panels C and F) or presence of 0.57 µM of soybean urease (panels A and D) or jackbean urease (panels B and E). White bars: 5 µm.

JBU is also able to inhibit the growth of the oomycete *Pythium oligandrum* whose cell walls lack chitin, reinforcing that the antifungal property of ureases probably does not involve a chitin-binding or chitinase activity.

The antifungal properties of ureases affected fungi pertaining to Ascomycota and Basidiomycota. On the other hand, not all fungi tested were affected under our experimental conditions, with contrasting responses seen even at genus level. We have no clues yet to the factors that dictate(s) the sensitivity of different fungi to urease antifungal activity.

The antifungal activity of plant defensins, such as Psd1 and Psd2, isolated from *Pisum sativum* (Almeida et al., 2000) appears to require specific binding to membrane targets, particularly glycosphingo lipids, resulting in fungal growth arrest (Thevissen et al., 2004, 2005). Carbohydrate-binding properties are also a common feature of *H. pylori* urease (Icatlo et al., 2000; Aspholm-Hurtig et al., 2004), jackbean ureases (Follmer et al., 2001) and some antifungal plant lectins (Lungu et al., 1990), such as that of the stinging needle agglutinin (Does et al., 1999) or from the legume *Luetzelburigia auriculata* (Melo et al., 2005). Thus,

```
M65260      MKLSPREVEKLGLHNAGYLAQKRLARGVRLNYTEAVALIASQIMEYARDGEKTVAQLMCL 60
AY230157    MKLSPREVEKLGLHNAGYLAQKRLARGLRLNYTEAVALIATQIMEFARDGEKTVAQLMCI 60
Hpylori     MKLTPKELDKLMLHYAGELAKKRKEKGIKLNYVEAVRLISAHIMEEARRGKKTAAELMQE 60
            ***:*:*::** ** ** **:**  :*::***.*** **:::*** ** *:**.*:**

M65260      GQHLLGRRQVLPAVPHLLNAVQVEATFPDGTKLVTVHDPISRENGELQEALFGSLLPVPS 120
AY230157    GKHLLGRRQVLPEVQHLLNAVQVEATFPDGTKLVTVHDPISCEHGDLGQALFGSFLPVPS 120
Hpylori     GRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIEAN----------------- 103
            *: **   :*:  *  ::: * :** *********** **. :

M65260      LDKFAETKEDNRIPGEILCEDECLTLNIGRKAVILKVTSKGDRPIQVGSHYHFIEVNPYL 180
AY230157    LDKFAENKEDNRIPGEIIYGDGSLVLNPGKNAVILKVVSNGDRPIQVGSHYHFIEVNPYL 180
Hpylori     ---------GKLVPGELFLKNEDITINEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCL 154
                     .: :***::  :  ::* *::** :** . ****:*:***:**:***  *

M65260      TFDRRKAYGMRLNIAAGTAVRFEPGDCKSVTLVSIEGNKVIRGGNAIADGPVNETNLEAA 240
AY230157    TFDRRKAYGMRLNIAAGNATRFEPGECKSVVLVSIGGNKVIRGGNNIADGPVNDSNCRAA 240
Hpylori     DFDREKTFGKRLDIASGTAVRFEPGEEKSVELIDIGGNRRIFGFNALVDRQADNESKKIA 214
             ***.*::* **:**:*.*.*****: *** *:.* **: * * * :.*  .:: . . *

M65260      MHAVRSKGFGHEEEKDASEGFTKEDPNCPFNTFIHRKEYANKYGPTTGDKIRLGDTNLLA 300
AY230157    MKAVVTRGFGHVEEENAREGVTGED--YSLTTVISREEYAHKYGPTTGDKIRLGDTDLFA 298
Hpylori     LHRAKERGFHGAKSDDNYVKTIKEM------KKISRKEYASMYGPTTGDKVRLGDTDLIA 268
            :: .  :**   :..:       *       . * *:***  ********:*****:*:*

M65260      EIEKDYALYGDECVFGGGKVIRDGMGQSCGHPPAISLDTVITNAVIIDYTGIIKADIGIK 360
AY230157    EIEKDFALYGDECVFGGGKVLRDGMGQSCGDPPAISLDTVITNAVIIDYSGIIKADIGIK 358
Hpylori     EVEHDYTIYGEELKFGGGKTLREGMSQSN-NPSKEELDLIITNALIVDYTGIYKADIGIK 327
            *:*:*:::**:*  *****.:*:**.**  .*.  .** :****:*:**:** *******

M65260      DGLIASIGKAGNPDIMNGVFSNMIIGANTEVIAGEGLIVTAGAIDCHVHYICPQLVYEAI 420
AY230157    DGLIVSIGKAGNPDIMDDVFFNMIIGANTEVIAGEGLIVTAGAIDCHVHYICPQLVDEAI 418
Hpylori     DGKIAGIGKGGNKDMQDGVKNNLSVGPATEALAGEGLIVTAGGIDTHIHFISPQQIPTAF 387
            ** *..***.** *: :.*  *: :*. **.:*********.** *:*:*.** :  *:

M65260      SSGITTLVGGGTGPAAGTRATTCTPSPTQMRLMLQSTDYLPLNFGFTGKGSSSKPDELHE 480
AY230157    SSGITTLVGGGTGPTAGTRATTCTPAPSQMKLMLQSTDDLPLNFGFTGKGSSSKPDELHD 478
Hpylori     ASGVTTMIGGGTGPADGTNATTITPGRRNLKWMLRAAEEYSMNLGFLAKGNASNDASLAD 447
            :**:**::******: **.*** **.  ::: **::::  .:*:** .**.:*:  .* :

M65260      IIKAGAMGLKLHEDWGSTPAAIDNCLTIAEHHDIQINIHTDTLNEAGFVEHSIAAFKGRT 540
AY230157    IIKAGAMGLKLHEDWGSTPAAIDSCLTVADQYDIQINIHTDTLNEAGFVEHSIAAFKGRT 538
Hpylori     QIEAGAIGFKIHEDWGTTPSAINHALDVADKYDVQVAIHTDTLNEAGCVEDTMAAIAGRT 507
             *:***:*:*:*****:**:**: .* .* :*:::*:*: ********** **.::**: ***

M65260      IHTYHSEGAGGGHAPDIIKVCGIKNVLPSSTNPTRPLTSNTIDEHLDMLMVCHHLDREIP 600
AY230157    IHTYHSEGAGGGHAPDIIKVCGMKNVLPSSTNPTRPLTLNTIDEHLDMLMVCHHLNREIP 598
Hpylori     MHTFHTEGAGGGHAPDIIKVAGEHNILPASTNPTIPFTVNTEAEHMDMLMVCHHLDKSIK 567
            :**:*:**************.* :*:**:****** *:* **  **:*********::.*

M65260      EDLAFAHSRIRKKTIAAEDVLNDIGAISIISSDSQAMGRVGEVISRTWQTADPMKAQTGP 660
AY230157    EDLAFACSRIREGTIAAEDILHDIGAISIISSDSQAMGRVGEVISRTWQTANKMKVQRGP 658
Hpylori     EDVQFADSRIRPQTIAAEDTLHDMGIFSITSSDSQAMGRVGEVITRTWQTADKNKKEFGR 627
            **: ** ****  ****** *:*:* :** **************:******:  * : *

M65260      LKCDSSDNDNFRIRRYIAKYTINPAIANGFSQYVGSVEVGKLADLVMWKPSFFGTKPEMV 720
AY230157    LQPGESDNDNFRIKRYIAKYTINPAIANGFSQYVGSVEVGKLADLVMWKPSFFGAKPEMV 718
Hpylori     LKEEKGDNDNFRIKRYLSKYTINPAIAHGISEYVGSVEVGKVADLVLWSPAFFGVKPNMI 687
            *:  ..*******:**::*********:*:*:*********:****:*.*:***.**:*:

M65260      IKGGMVAWADIGDPNASIPTPEPVKMRPMYGTLGKAGGALSIAFVSKAALDQRVNVLYGL 780
AY230157    IKGGVVAWADMGDPNASIPTPEPVKMRPMFGTLGKAGGALSIAFVSKAAVDQRVHALYGL 778
Hpylori     IKGGFIALSQMGDANASIPTPQPVYYREMFAHHGKAKYDANITFVSQAAYDKGIKEELGL 747
            ****.:* :::**.*******:**  * *:.  ***    .*:***:** *: ::   **

M65260      NKRVEAVSNVRKLTKLDMKLNDALPEITVDPESYTVKADGKLLCVSEATTVPLSRNYFLF 840
AY230157    NKRVEAVGNVRKLTKLDMKLNDSLPQITVDPDNYTVTADGEVLTSFATTFVPLSRNYFLF 838
Hpylori     ERQVLPVKNCRNITKKDMQFNDTTAHIEVNPETYHVFVDGKEVTLNQSIK---------- 797
            :::* .* * *::** **::**: ..* *:*:.* * .**: :    :
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Fig. 6. Alignment of primary sequences of jackbean major urease (accession code M65260), embryo-specific urease (AY230157) and *H. pylori* urease (subunit A-AAO34402; subunit B-AAO34403). Amino acids participating in the ureolytic active site are shadowed in gray. The Cys residues modified by *p*-hydroximercurybenzoate (Jabri et al., 1995) are shown in a box.

the sugar composition of fungal cells might be implicated in the difference of their susceptibility to ureases.

The effect of ureases on the morphology of *P. herguei*, a maize pathogen, was investigated. Scanning electron microscopy of urease-treated fungi revealed that cell wall is damaged, suggesting that ureases may interfere with the osmotic balance, as described for micafungin on *Aspergillus fumigatus* (Nishiyama et al., 2005).

Most fungi are able to hydrolyze urea into ammonia. In some fungi, urea is hydrolyzed by urease, a nickel-containing enzyme homologous to the plant and bacterial enzymes studied in this work. In other fungi, urea is first carboxylated to yield allophanate by an ATP-hydrolyzing urease or urea amidolyase (Nishiya and Imanaka, 1993; Sumrada et al., 1982). We are presently testing fungal ureases for the ureolysis-independent properties displayed by plant and microbial ureases.

In conclusion, the antifungal properties of plant and bacterial ureases we described here may shed some light on the physiological roles of these proteins. The data presented further reinforce the possibility of a protective role of plant ureases besides phytophagous insects also against phytopathogenic fungi.

Acknowledgments

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Anexo 6.4.

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Curriculum Vitae

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Curriculum Vitae

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Atividades

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Estágio:
estágio de IC - bolsa da FAPERGS

07/1999 - Atual Projetos de pesquisa, Instituto de Biociências, Departamento de Biofísica
Participação em projetos:
Proteínas Tóxicas de Origem Vegetal

08/2001 - 08/2002 Estágio, Instituto de Biociências, Departamento de Biofísica
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Projetos

1999 - Atual Proteínas Tóxicas de Origem Vegetal

Descrição: Objetivo: Apesar da alta homologia entre isoformas de urease, várias atividades biológicas e propriedades fisico-químicas são características de uma isoforma em particular. Por análises cromatográficas, eletroforética, espalhamento de luz, difração de raios-X, modelagem molecular, estudamos relações estrutura X atividade biológica de ureases de vegetais (Canavalia ensiformis; Glycine max; Enterolobium contortisiliquum); e ureases de bacterias patogenicas(Helicobacter pylori; Proteus mirabilis).
Situação: Em Andamento Natureza: Pesquisa
Alunos envolvidos: Graduação (4); Especialização (0); Mestrado acadêmico (3); Mestrado profissionalizante (0); Doutorado (5);
Integrantes: German Enrique WassermannCélia Regina Carlini (Responsável); Deiber Olivera Severo; Rafael Real Guerra; Juliana Boeira de Barcelos
Financiador(es):
Número de produções C,T & A: 9/

Produção em C, T & A

Produção bibliográfica
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