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

Ureases vegetais e microbianas: efeitos na exocitose e participação de eicosanóides

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, para a obtenção do título de Doutor em Ciência.

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Porto Alegre
2006

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Este trabalho foi desenvolvido no Laboratório de Proteínas Tóxica, do Departamento de Biofísica, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, sob a orientação da Profª. Drª. Célia Regina Ribeiro da Silva Carlini. O trabalho foi realizado com suporte financeiro da CAPES, FAPERGS, CNPq e FINEP.

Agradecimentos:

Em primeiro lugar agradeço a minha família, meus pais Osorio e Maribel pelo amor, pela educação, pela participação na formação do meu caráter. Enfim, por tudo aquilo que apenas os pais podem dar a uma pessoa. À minha irmã Rita, pelo incentivo pelo amor pela admiração pela autenticidade.

Aos meu amigos, todos aqueles que acreditaram junto comigo que essa etapa se realizaria.

A todos e a cada um dos meus amigos do Laprotox, pelo companheirismo, troca de idéias, discussões, “pela amizade” *Valeu!!!!!* Vander (*Tinoko*), Martha, Arlete (*Lelé*), Fernanda (*Stanis*), Rafael, Diogo, Juliana (*Ju*), Melissa (*Mel*), Anne, Vanessa, Ângela P, Ângela M, Fábio e todos os outros que passaram e também deram um toque especial

Ao German, que tocou junto comigo este trabalho, *“valeu Alemão!!!!”*

Ao Luciano e a Silvia sempre dispostos a ajudar.

À Rita, minha esposa e companheira, quem me impulsiona a cada dia, mais uma conquista nossa. *Te amo!!!!!!!!*

À minha orientadora Profª.Drª. Célia Regina Carlini, exemplo de profissional e de ser humano, pela oportunidade, e confiança em meu trabalho, só posso agradecer mais uma vez, sinceramente. *“Obrigado Célia”*

Você me pergunta
Aonde eu quero chegar
Se há tantos caminhos na vida
E pouca esperança no ar
E até a gaivota que voa
Já tem seu caminho no ar
O caminho do fogo é a água
O caminho do barco é o porto
O do sangue é o chicote
O caminho do reto é o torto
O caminho do bruxo é a nuvem
O da nuvem é o espaço
O da luz é o túnel
O caminho da fera é o laço
O caminho da mão é o punhal
O do santo é o deserto
O do carro é o sinal
O do errado é o certo
O caminho do verde é o cinzento
O do amor é o destino
O do cesto é o cento
O caminho do velho é o menino
O da água é a sede
O caminho do frio é o inverno
O do peixe é a rede
O do pio é o inferno
O caminho do risco é o sucesso
O do acaso é a sorte
O da dor é o amigo
O caminho da vida é a morte...

Raus Seixas

ÍNDICE:

Lista de Abreviaturas:

°C graus célcius

ADP difosfato de adenosina

AMP monofosfato de adenosina

Ca^{2+} cálcio

CO Cicloxigenases

EET ácido epoxieicosatetraenóico

HEPTE ácido hidroxiperoxieicosatetraenóico

HETE ácido Hidroxieicosatetraenóico

kDA quilodaltons

Km constante de Michaelis

LO Lipoxigenases

μg microgramas

μg miligramas

Mg^{2+} magnésio

μL microlitros

μL mililitros

μM milimolar

PAF Fator agregador de plaquetas

PG prostaglandinas

PRP plasma rico em plaquetas

Esta tese é apresentada no seguinte formato:

A primeira parte é constituída por uma introdução que trata dos aspectos gerais do trabalho, sua justificativa e objetivos. Logo em seguida estão metodologia e materiais utilizados, em especial no capítulo IV dos resultados.

Os resultados foram divididos em capítulos. O Capítulo I é composto pelo artigo publicado no European Journal of Biochemistry (2004), intitulado, *Jackbean, soybean and Bacillus pasteurii ureases: Biological effects unrelated to ureolitic activity*. O Capítulo II apresenta o artigo intitulado, *Urease display biological effects independent of enzymatic activity. Is there connection to diseases causes by urease-producing bacteria?*; Brazilian Journal of Medical and Biological Research (2006) Review (no prelo); os dados de revisão bibliográfica deste artigo complementam o capítulo de introdução geral, além de apresentar alguns resultados experimentais. O Capítulo III contém o artigo *Bacillus pasteurii urease shares with plant ureases the ability to induce aggregation of blood platelets;* Archives of Biochemistry and Biophisics (no prelo). No capítulo IV são apresentados os resultados não submetidos à publicação até a presente data.

Na discussão geral há uma reflexão a respeito dos resultados obtidos neste trabalho e já previamente tratados nos artigos que compõem esta tese.

As referências bibliográficas apresentadas no final compreendem as referências presentes no corpo da tese (Introdução Geral, Materiais e Métodos, Discussão Geral), as demais são constantes nos artigos aqui apresentados.

1. RESUMO:

A urease de ***Canavalia ensiformis*** é de relevância histórica tendo sido a primeira proteína a ser cristalizada, feito realizado por Sumner em 1926 comprovando assim a natureza protéica das enzimas. Essa proteína também foi a primeira enzima contendo níquel a ser identificada. As ureases são amplamente encontradas em vegetais, bactérias e fungos, apresentando um alto grau de homologia entre si. Esta similaridade de seqüência primária indica um processo conservativo durante a Evolução e um papel fisiológico importante destas enzimas. As ureases microbianas estão envolvidas em processos patogênicos como formação de cálculos urinários, incrustação de catéter, pielonefrites, úlceras pépticas e possivelmente, na formação de tumores de estômago. Em vegetais, pouco se sabe a respeito da função biológica das ureases, embora sua presença em tecidos vegetais seja freqüente. Postula-se seu envolvimento na biodisponibilidade de nitrogênio; ou mesmo como mecanismo de defesa dos vegetais. A canatoxina, uma isoforma de urease isolada da ***C. ensiformis***, é tóxica para insetos e possui outras atividades biológicas de interesse, dentre as quais destacam-se: 1) atividade secretagoga e pró-agregante em plaquetas de coelho; 2) atividade hemaglutinante indireta para hemácias de coelho; 3) propriedade pró-inflamatória, evidenciada nos modelos de edema de pata e pleuresia em ratos. Este estudo propõe-se a investigar se ureases bacterianas apresentariam propriedades biológicas como as da canatoxina; e se essas propriedades são dependentes ou não da atividade enzimática dessas ureases.

2. INTRODUÇÃO GERAL

Ureases:

Ureases (EC 3.1.1.5) são hidrolases, níquel dependentes, que catalisam a reação de hidrólise da uréia à amônia e ácido carbônico. Sumner e colaboradores, em 1953 demonstraram que o carbamato de amônio, um dos produtos da hidrólise da uréia catalisada pela urease, forma-se a partir de CO_2 e NH_3. Assim, a uréia seria hidrolisada à ácido carbônico e amônia passando por uma intermediário de ácido carbâmico ou carbamato, e vários trabalhos posteriores estabeleceram que este é o primeiro intermediário livre na reação de hidrólise da uréia (Blakeley *et al*., 1969; Wang and Tarr, 1955). (FIGURA. 1).

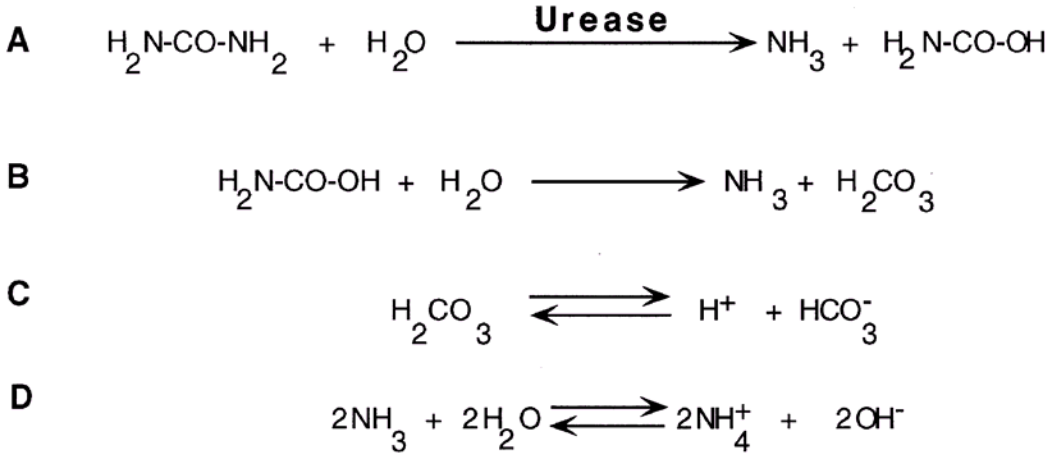

FIGURA 1. A reação catalisada pela urease. A ureia é clivada pela urease para produzir uma molécula de amônia e uma de carbamato (A). O carbamato decompõe-se espontâneamente à amônia e ácido carbônico (B). O ácido carbônico equilibrado em água (C), com as duas moléculas da amônia, que se tornam protonadas para render os íons do amonio e hidroxido (D). A reação resulta em uma ascensão no pH do meio da reação. Adaptado de Burne & Chen, 2000.

Embora, por muitos anos, tenha-se acreditado que urease reconhecesse especificamente a uréia, esta enzima também age sobre outros substratos. (Fishbein, 1969b) mostrou que hidroxiuréia e dihidroxiuréia são substratos para urease. Esta enzima catalisa também a hidrólise de algumas uréias substituídas e ésteres de ácido carbâmico, como a tiouréia e p-nitrofenilcarbamato (Bennett & Wren, 1977).

Ureases de diferentes fontes (bacterianas ou vegetais) apresentam diferentes afinidades por estes substratos. Da mesma forma, estas ureases possuem diferente suscetibilidade à inibidores, como ácido acetohidroxâmico (AHA) ou p-hidroximercuriobenzoato (Rosenstein, 1982). A urease da *Canavalia ensifomis* (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 níquel dependente (Dixon *et al*., 1975). Apesar de ser uma proteína de fácil cristalização, os cristais desta urease obtidos até a presente data apresentam 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*., 1995a). Portanto, ainda não se conhece a estrutura tridimensional dessa proteína.

Aspectos estruturais da urease da *Canavalia ensiformis*:

Ureases extraídas de diferentes sementes de *C. ensiformis* apresentam vários comportamentos no que diz respeito a seu estado conformacional (Fishbein, 1969). Esta enzima parece assumir formas de massas moleculares distintas (Sumner *et al*., 1938; Creeth *&* Nichol, 1960; Seghal, *et al*., 1966; Reither, *et al*., 1967, interconversíveis entre si. Esta série de diferentes formas é composta por isoenzimas poliméricas e funcionais, com diferentes propriedades catalíticas, e que interagem umas com as outras. Desta forma, a multiplicidade de isoenzimas de urease, juntamente com sua interconversibilidade, pode explicar muitas das complexidades catalíticas desta enzima. Lynn, 1962 e Lynn *&* Yankwich, 1964, utilizando estudos de

hidrólise de uréia marcada com C^{13}, deduziram que a urease não é uma entidade catalítica homogênea e que múltiplos sítios ativos, interativos e interconversíveis, são necessários para explicar os dados cinéticos obtidos. Já os dados cinéticos obtidos por (Fishbein, 1969a), indicaram que esta interação e interconversão ocorre entre diferentes espécies moleculares, ao invés de diferentes sítios ativos da mesma molécula. Estas diversas dúvidas sobre a forma molecular da urease foram parcialmente solucionadas com a dedução da seqüência de aminoácidos desta enzima a partir de cDNA (Riddles *et al*., 1991), seqüência esta que confirmou dados obtidos no seqüenciamento direto da proteína (Mamiya *et al*., 1985). A partir destes dados, mostrou-se que a unidade estrutural básica dessa urease é uma cadeia polipeptídica única com 840 resíduos de aminoácidos e uma massa molecular de 90,770 kDa. A urease da *C. ensiformis* (Zerner, 1991b; Jabri *et al*., 1995b) apresenta, em seu sítio ativo, dois átomos de níquel, sendo cada um deles coordenados por dois resíduos de histidinas e uma lisina carbamilada, compartilhada por ambos. A urease também tem um resíduo de cisteína importante localizado nas proximidades do sítio ativo, mas que não participa da reação de hidrólise de uréia. A forma mínima da proteína expressando atividade enzimática é a de um trímero de 270 kDa, sendo que a forma nativa é um hexâmero de 540 kDa (Zerner, 1991a).

Ureases estão amplamente distribuídas entre fungos, bactérias e plantas. Em bactérias, estas enzimas estão envolvidas em vários processos patogênicos, principalmente na formação de cálculos urinários, incrustação de catéter, pielonefrites, bem como em alguns casos de coma hepático. *Helicobacter pylori* está relacionada a casos de úlceras pépticas, e possivelmente na formação de tumores no estômago; estes processos, as ureases possuem um papel fundamental, principalmente na sobrevivência das bactérias em ambientes de pH desfavorável (FIGURA. 2). Apesar de inibidores de urease terem sido utilizados no tratamento de algumas destas patologias, seu uso foi descontinuado pelo fato de muitos pacientes apresentam reações colaterais adversas. O uso clínico de ácido acetohidroxâmico, um inibidor de

urease, causa depressão na síntese de DNA, afetando a medula óssea, além de ser teratogênico em doses elevadas.

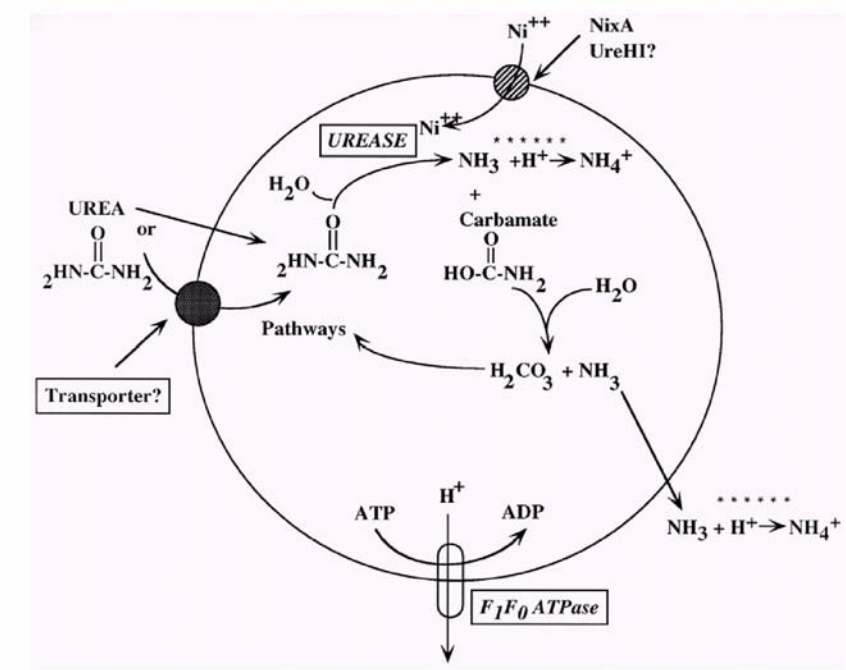

FIGURA 2.: rota metabólica da uréia e efeitos no pH do meio. No caso da ***H. pylori*** e talvez de outros organismos, a urease é um fator importante na sobrevivência em ambientes acidificados, provavelmente porque a neutralização do meio extracelular promovida pela urease, em torno das células é necessária para impedir danos irreversíveis à membrana celular. Adaptado de (Burne and Chen, 2000).

A primeira urease com sua estrutura 3D resolvida foi a de *Klebsiella aerogenes*, (1FWJ), sendo representativa da maioria das ureases bacterianas. Esta urease possui três cadeias polipeptídicas, denominadas subunidades α (60,3 kDa), β (11,7 kDa) e γ (11,1 kDa) (Mulrooney *&* Hausinger, 1990) com os dois átomos de níquel do sítio ativo localizados na subunidade α (Todd *&* Hausinger, 1989). Apesar da aparente variação na estrutura quaternária, a comparação da seqüência de aminoácidos mostra que estas ureases possuem mais de 50% de homologia. Os dados de seqüência primária mostram que as ureases de *C.ensiformis* e *K. aerogenes* são homólogas: as cadeias α, β e γ da urease da *K. aerogenes* correspondem, respectivamente, aos resíduos 1 a 101, 132 a 237 e 272 a 840, da urease da *C. ensiformis*. A clara correspondência de

aminoácidos entre uma urease de planta de cadeia única e as bacterianas, com duas ou três subunidades, indicam a ocorrência de uma fusão de genes, ou eventos de ruptura gênica, durante a evolução desta enzima (FIGURA. 3). Estudos de cristalografia de raio-X da urease da *K. aerogenes* (Jabri *et al*., 1995a) mostraram que esta enzima forma um trímero de unidades ($\alpha\beta\gamma$) em um arranjo triangular. Portanto a forma nativa desta proteína é $(\alpha\beta\gamma)_3$, diferenciando-se da oligomerização $(\alpha\beta_2\gamma_2)_3$, anteriormente proposta com base na análise da intensidade relativa das bandas correspondentes às subunidades α, β e γ, em gel de eletroforese (Todd & Hausinger, 1987).

DCH_{407}VHYI...GLK_{490}IH...NIH_{519}TD...TYH_{545}SE..MVCHHLDR....TID_{633}SQ	***Canavalia ensiformis***
DAH_{136}IHFI...GLK_{219}LH...AIH_{248}TD...TYH_{274}TE..MVCHHLDR....SSD_{362}SQ	***Bacillus sp.***
DCH_{136}VHYI...GLK_{219}LH...AIH_{248}SD...SFH_{274}VE..MVCHHLKR....TID_{362}AL	***Bacillus pasteurii***
DTH_{137}IHFI...GLK_{220}LH...AIH_{249}TD...TYH_{275}IE..MVCHHLDR....SSD_{363}SQ	***Bacillus subtilis***
DTH_{134}IHFI...GLK_{217}IH...AIH_{246}SD...VFH_{272}TE..MVCHHLDR....SSD_{360}SQ	***Proteus vulgaris***
DTH_{136}IHFI...GFK_{219}IH...AIH_{248}TD...TFH_{274}TE..MVCHHLDR....SSD_{362}SQ	***Helicobacter pylori***
DCH_{141}VHLI...GFK_{224}LH...ALH_{253}SD...AYH_{279}TE..MVCHHLNR....GSD_{367}SQ	***Mycobacterium tuberculosis***
DTH_{133}IHWI...GLK_{216}IH.. ALH_{245}SD...TFH_{271}TE..MVCHHLDR....SSD_{359}SQ	***Klebsiella aerogenes***

FIGURA. 3:Alinhamento da seqüência 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 liga-se a reagentes oxidantes como o para hidroximercuriobenzoato, resultando na inibição da reação por impedimento estéreo (dados coletados do Protein Data Bank).

A existência de ureases em tecidos vegetais é bastante comum, sendo em leguminosas particularmente ricas (Polacco & Holland, 1993b), entre as quais destaca-se a soja (***Glycine max***). Apesar de ampla distribuição desta proteína entre as plantas, pouco se conhece sobre sua função fisiológica. Postula-se que, em plantas superiores, a urease esteja envolvida com a biodisponibilidade de nitrogênio, e que este esteja disponível a partir da uréia apenas pela ação de ureases (Polacco & Holland, 1993b). Em plantas e culturas desprovidas de urease, quer induzidas

geneticamente (Meyer-Bothiling *et al*., 1989); (Polacco *et al*. ,1989), com o uso de inibidores de urease ou por remoção do níquel (Polacco *&* Holland, 1993a), observa-se um acúmulo de uréia ou um comprometimento do emprego de uréia como fonte de nitrogênio. A urease parece ser a única enzima níquel dependente nos vegetais, tendo sido observado que plantas de soja crescidas na ausência de níquel, apresentam o mesmo fenótipo de plantas nas quais a expressão da urease foi geneticamente bloqueada (Polacco *&* Holland, 1993a).

Uma questão ainda discutível sobre a função da urease nas plantas é fato da uréia ser uma forma de excreção de nitrogênio apenas em animais, ou seja, a uréia não é um metabólito majoritário nos vegetais onde esta enzima é abundante. Contudo é conveniente ressaltar que uréia não é o único substrato para as ureases. A descoberta de duas isoenzimas de urease na soja levantou uma série de dúvidas a respeito da função destas proteínas na planta (Polacco *&* Holland, 1993a). A urease ubíqua existente em todos os tecidos da planta, e a urease embrião-específica, sintetizada no embrião em desenvolvimento e acumulada na semente madura, onde a atividade ureásica é 1000 vezes maior que a da urease encontrada em toda a planta (Polacco *&* Winkler, 1984). Plantas mutantes com silenciamento da urease ubíqua apresentam anormalidades, como necroses nas extremidades das folhas e raízes, acúmulo de uréia nas folhas e sementes, e retardamento na germinação (Polacco *&* Holland, 1993a). Por outro lado, como a perda da urease embrião-específica não acarreta danos visíveis na planta, acredita-se que esta enzima não desempenha função fisiológica ligada ao metabolismo de nitrogênio na planta. Uma questão pertinente a este contexto é por que o embrião da soja em desenvolvimento "investiria" em uma enzima com grande atividade ureolítica, se a quantidade de uréia disponível é praticamente inexistente. Estes dados sugerem que esta urease parece não estar envolvida no metabolismo de nitrogênio da planta, mas sim em algum outro tipo de função, como por exemplo, a defesa da planta (Polacco *&* Holland, 1993a). Em outra leguminosa, a *C. ensiformis*, o fato da Canatoxina, uma isoforma de urease apresentar atividade tóxica para insetos e mamíferos (Carlini *et al*., 1997; Follmer *et*

al., 2001b; Follmer *et al*., 2001a; Stanisçuaski *et al*., 2005; Follmer *et al*., 2004), reforça a hipótese de que as ureases estariam envolvidas nos mecanismos de defesa das plantas.

A Canatoxina

Em 1981, Carlini & Guimarães isolaram a canatoxina, uma proteína tóxica de sementes da leguminosa *C. ensiformis*, que induz convulsão e morte de ratos e camundongos, com uma DL_{50} de 2 mg/kg, por via intraperitoneal. Esta proteína possui atividade inseticida (Carlini *et al*., 1997; Ferreira-DaSilva *et al*., 2000), e portanto, potencialidade para o desenvolvimento de plantas transgênicas resistentes a insetos que são pragas agrícolas. Além disso, determinou-se a existência de proteínas tóxicas, supostamente homólogas à canatoxina em várias outras leguminosas, dentre as quais, a soja (Carlini *et al*., 1988); (Vasconcelos *et al*., 1994).

A principal forma ativa da canatoxina apresenta uma massa molecular de 187 kDa quando analisada em gel-filtração em pH 7,5. Formas de massa molecular mais alto formam-se durante a estocagem prolongada da canatoxina a 4^{o}C. Em SDS-PAGE, em meio redutor ou não, sua massa molecular é de cerca. 95 kDa, sugerindo que a forma nativa da proteína é provavelmente um dímero. A canatoxina é uma metaloproteína contendo zinco e níquel (Follmer *et al*., 2001a).

Estudos de microsseqüenciamento indicaram que o resíduo N-terminal da canatoxina está bloqueado. Vários peptídeos internos da canatoxina já foram seqüenciados, obtidos por hidrólise tríptica ou por endoproteinase Lys-C, sendo que todos eles revelaram um alto grau de homologia com a seqüência primária de urease da *C. ensiformis*. A homologia da canatoxina com a urease revelou-se maior na porção N-terminal desta, sendo que peptídeos que se alinharam com a porção C-terminal da urease, mostrarem substituição de vários aminoácidos, algumas não

conservativas. Também a composição percentual de aminoácidos é indicativa de uma grande semelhança das duas proteínas.

O elevado grau de homologia encontrado levou a estudos comparativos das duas proteínas, para averiguar a hipótese de que a canatoxina fosse uma variante da urease. Follmer e colaboradores, 2001 e 2004 mostraram que era possível separar duas isoformas de urease a partir do extrato bruto de *C. ensiformis*, sendo que a canatoxina apresenta maior avidez por metais (Zn^{++} e Co^{++}) em cromatografia de afinidade em metal imobilizado, o que permitiu o estabelecimento de protocolos de purificação para a obtenção das isoformas altamente purificadas.

Ensaios de atividade enzimática sobre uréia indicaram que a canatoxina purificada apresenta cerca de 25% da atividade ureolítica da própria urease (Tipo C-3). A canatoxina apresenta Km de 1,50 a 7,70 mM, na faixa de pH 6,5 a 8,5, semelhantes aos valores obtidos para a urease C-3 e citados na literatura (Carlini *et al*., 1997; Follmer *et al*., 2001b; Follmer *et al*., 2001a; Zerner, 1991a). Os valores das constantes catalíticas calculadas para a urease e a canatoxina foram de 61,0 mM/s (trímero) e 18,09 mM/s (dímero), respectivamente, Com eficiência catalítica (Kcat/Km) de $27x10^3$ /s e $6x\ 10^{3,}$ para urease e canatoxina, respectivamente.

Estudos adicionais mostraram que as várias atividades biológicas descritas para a canatoxina não são dependentes da atividade ureásica da molécula. Assim, a canatoxina tratada com 200 μM de *p*-hidroximercuriobenzoato perde totalmente a atividade ureásica, mas mantém inalterada a sua atividade tóxica em camundongos, ainda induz agregação plaquetária, produz hemaglutinação indireta e mantém sua atividade inseticida. As mesmas observações foram feitas para a urease tratada com p-hidroximercuribenzoato (Follmer *et al*., 2001a), A Tabela 1 resume os dados comparativos disponíveis para as ureases de *C ensiformis.*

Estes resultados indicam que a canatoxina é uma variante da urease da mesma semente; contudo, um dado importante obtido nesses trabalhos diz respeito à existência de domínios protéicos distintos, nestas proteínas, os quais são

responsáveis por atividades biológicas diferentes: um domínio com atividade hidrolítica sobre uréia, suscetível de inibição por agentes quelantes e oxidantes; e pelo menos mais um outro domínio, níquel e tiol independentes, que seria responsável pela toxicidade intraperitoneal da canatoxina, pelo efeito pró-agregante, pela capacidade de interação com glicoconjugados e pela atividade inseticida.

Tabela 1: Comparação fisico-química e atividades biológicas entre canatoxina e urease (ECC3.5.1.5), adaptado de Carlini and Grossi-de-Sa, 2002.

Comparative data on physicochemical and biological properties of canatoxin and jack bean urease (EC 3.5.1.5)

	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	Nickel-2 mol/mol
	Zinc-1 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)

Mecanismo De Ação da Canatoxina Em Modelos Mamíferos

Estudos *in vivo* mostram que canatoxina induz em ratos bradicardia, hipertensão e hipotermia, que precedem o fenômeno convulsivo tônico-clônico característico de sua ação tóxica. Em camundongos tratados com a toxina em doses convulsivantes ou subconvulsivantes, não foram observadas alterações dos níveis cerebrais e medulares de vários neurotransmissores. Preparações de músculos lisos e estriados foram insensíveis à canatoxina. O fenômeno convulsivo induzido por esta proteína tem provavelmente origem medular, com modulação dos centros superiores do encéfalo (Carlini *et al*., 1984).

Alterações dos níveis plasmáticos de gonadotrofinas e de prolactina (Ribeiro-daSilva *et al*., 1989) são observadas em ratas tratadas cronicamente com doses sub-letais da proteína, reforçando os dados que apontam uma ação central para a canatoxina. A canatoxina altera também a insulinemia e glicemia de ratos de maneira dose- e sexo-dependente (Ribeiro-daSilva *et al*., 1986) (Ribeiro-daSilva *&* Prado, 1993).

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. Tal efeito secretagogo da canatoxina envolve mediação por metabólitos do ácido araquidônico via lipoxigenases (Tabela 2).

TABELA 2. Efeito secretagogo da canatoxina: modulação por inibidores de lipoxigenase.

MODELO	EFEITO	DE_{50}	INIBIDOR	DOSE	INIBIÇÃO	Ref
Plaquetas, coelho	agregação	300 nM	NDGA	520 μM	50	(a)
			ETYA	19 μM	50	
			BW755C	50 μM	50	
	secreção: serotonina	300 nM	NDGA	500 μM	75	(b)
			Esculetina	100 μM	87	
Sinaptossomas, rato	secreção: serotonina	500 nM	NDGA	200 μM	90	(b)
			Esculetina	100 μM	90	
	secreção: dopamina	2 μM	NDGA	200 μM	42	
Ilhotas pancreáticas, rato	secreção de insulina	500 nM	NDGA	200 μM	76	(b,c)
			Esculetina	100 μM	36	
Mastócitos: rato	secreção de histamina	500 nM	não testado			(d)
macrófagos, camundongo	secreção: enzimas	200 nM	NDGA	150 μM	não inibe	(e)
Rato, *in vivo*	hipoglicemia	0,4 mg/Kg	NDGA	125 mg/Kg	100	(f)
			Esculetina	125 mg/Kg	100	
Rato, *in vivo*	hiperinsulinemia	0,4 mg/Kg	NDGA	125 mg/Kg	100	(g)
Rato, *in vivo*	hipoxia	0,4 mg/Kg	NDGA	125 mg/Kg	72	(h)
			Esculetina	125 mg/Kg	50	
Rato, *in vivo*	Edema de pata	0,4 mg/Kg	NDGA	125 mg/Kg	66	(i)
			Esculetina	125 mg/Kg	50	
Rato, *in vivo*	convulsões	0,4 mg/Kg	NDGA	125 mg/Kg	75	(h)

(a)(Carlini *et al*., 1985); (b) (Barja-Fidalgo *et al*., 1991a); (c) (Barja-Fidalgo *et al*., 1991b); (d) (Grassi-Kassisse & Ribeiro-daSilva, 1992); (e) (Ghazaleh *et al*., 1992); (f) (Ribeiro-daSilva *et al*., 1986); (g) (Ribeiro-daSilva & Prado, 1993); (h) (Ribeiro-daSilva *et al*., 1992); (i) (Benjamin *et al*., 1992; Ribeiro-daSilva *et al*., 1992).

Conforme esses resultados, a canatoxina é capaz de induzir secreção de dopamina e de serotonina em sinaptosomas de cérebro total de rato (Barja-Fidalgo *et al*., 1991b), dados que sugerem que a ação convulsivante da canatoxina poderia ser conseqüente à liberação maciça de neurotransmissores excitatórios.

Em outro modelo estudado, a canatoxina mostrou-se capaz de induzir agregação de plaquetas de diferentes espécies animais, atuando na faixa nanomolar. Este efeito se deve à uma ação secretagoga da toxina, promovendo exocitose dos grânulos densos plaquetários, ricos em serotonina e ADP, que desencadeiam a reação de agregação (Carlini *et al*., 1985; Barja-Fidalgo *et al*., 1991b; Carlini *et al*., 1985) Estudos com inibidores específicos indicam que nenhuma das vias clássicas da agregação plaquetária (ADP, Tromboxana A2 ou *PAF-acether*) está envolvida na resposta das plaquetas à canatoxina. O efeito agregante da canatoxina envolve ativação da 12-lipoxigenase endógena da plaqueta, uma vez que plaquetas pré-tratadas com diferentes inibidores desta enzima, passam a não mais responder à toxina (Carlini *et al*., 1985; Barja-Fidalgo *et al*., 1991b).

A hiperinsulinemia e a conseqüente hipoglicemia observadas em ratos tratados com canatoxina (Ribeiro-daSilva *et al*., 1986; Ribeiro-daSilva *&* Prado, 1993) também poderiam resultar de uma ativação da 12-lipoxigenase, uma vez que a modulação da secreção de insulina nas ilhotas pancreáticas depende desta enzima (Metz *et al*., 1983d; Metz, 1985a). De fato, ilhotas pancreáticas isoladas de rato secretam insulina em presença da canatoxina de modo dose-, tempo e temperatura-dependente. A canatoxina (1 μM) é 20.000 vezes mais potente que a glicose (20 mM) para a indução da resposta máxima de secreção de insulina. Também aqui, através do uso de inibidores específicos, constatou-se o envolvimento do sistema da 12-lipoxigenase na resposta das ilhotas à toxina, quer in vivo como in *vitro* (Barja-Fidalgo *et al*., 1991a; Barja-Fidalgo *et al*., 1991b; Ribeiro *et al*., 1993.

Os macrófagos peritoneais de camundongo são também susceptíveis ao efeito secretagogo da canatoxina, em concentrações nanomolares, liberando enzimas lisossomais como a N-acetil-ß-D-glicosaminidase, fosfatase ácida e α-manosidase (Ghazaleh *et al*., 1992). No entanto, o efeito secretagogo da canatoxina em macrófagos não parece ser mediado por metabólitos do ácido araquidônico, quer via lipo ou cicloxigenase, e envolve a ativação da guanilato ciclase e da NO sintase. Estudos adicionais indicaram ainda que, em presença da canatoxina, os macrófagos apresentam-se ativados, provavelmente exercendo ação tumoricida (Barja-Fidalgo *et al*., 1992).

A canatoxina apresenta atividades pró-inflamatórias: é capaz de induzir migração de neutrófilos e monócitos nas cavidades peritoneal e pleural, além de apresentar ação no modelo *"skin air pouches"*, mediada pela liberação de fatores quimiotáxicos de macrófagos peritoneais (Barja-Fidalgo *et al*., 1992). O edema de pata em ratos é dose dependente com pico máximo após 6 horas da injeção intraplantar e com redução total em 48 horas, em doses de 50 μg e 100 μg de canatoxina por pata, fenômeno este que parece ser mediado por metabólitos de lipoxigenase (Benjamin *et al*., 1992).

Outros estudos abordaram o efeito inibitório da canatoxina sobre a acumulação de Ca^{2+} em vesículas de membranas do retículo sarco-plasmático, resultante da atividade enzimática de uma Ca^{2+} Mg^{2+}-ATPase presente neste tecido. A toxina parece desacoplar o transporte de cálcio, através da membrana, da atividade hidrolítica da enzima sobre o ATP, um dado relevante para o entendimento das propriedades secretagogas desta proteína (Alves *et al*., 1992)

Os fluxos e níveis de Ca^{2+} intracelulares também foram estudados em plaquetas de coelhos ativadas por canatoxina. Verificou-se que o aumento dos níveis de Ca^{2+} intracelular, decorrente da ativação plaquetária, não está associado com a formação de inositol trifosfato ou mobilização dos pools internos de Ca^{2+} na plaqueta. Por outro

lado, ocorre influxo de Ca^{2+} do meio externo, através de canais susceptíveis a nitrendipina e verapamil. Este influxo ocorre nos primeiros 15 segundos após exposição das plaquetas a canatoxina, precedendo a secreção e agregação plaquetárias, bem como a mobilização de ácido araquidônico endógeno da plaqueta. Nestes estudos, mostrou-se também que a ativação desses canais de Ca^{2+} é modulada por aumento nos níveis de GMP cíclico (Ghazaleh *et al*., 1997).

***Helicobacter pylori*:**

A bactéria *H. pylori*, é uma bactéria, aeróbia, gram-negativa, 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 *et al*, 1987; Goodwin *&* Armstrong, 1990)), essenciais para mobilidade da bactéria. Esse microrganismo foi isolado pela primeira vez em 1982 por Marshall e Warren, conduzindo a uma nova era na microbiologia gástrica (Marshall *&* Warren, 1984). Estes microrganismos não invadem a mucosa gástrica, mas estão presentes entre a camada de muco e de tecido (Marshall, 1989, Warren & Marshall, 1983). Inicialmente essa bactéria recebeu denominação científica de *Campylobacter pyloridis*, mais adiante passando a *Helicobacter pylori*. Após os relatos de Marshall e Warren, investigadores do mundo todo confirmaram a presença destes organismos na mucosa gástrica (Jones *et al*., 1984; Langenberg *et al*., 1984; McNulty *&* Watson, 1984).

Em torno de 1984, associou-se fortemente a presença de *H. pylori* com a presença de inflamação na mucosa gástrica (gastrites superficiais crônicas), e especialmente, com a infiltração de células polimorfonucleares (gastrites ativas crônicas). Em 1991, quatro artigos mostraram pela primeira vez a associação entre infecção por *H. pylori* e a presença (Talley *et al*., 1991) ou o desenvolvimento (Forman *et al*., 1991; Nomura *et al*., 1991; Parsonnet *et al*., 1991a) de câncer gástrico.

Em 1994, a Agência Internacional para Pesquisa de Câncer, um braço da Organização de Saúde Mundial, revisou as evidências disponíveis e declarou que *H. pylori* era um carcinógeno para a espécie humana.

Em 1994, uma conferência no “National Institutes of Health” (NIH), concluiu que *H. pylori* é importante causador de úlcera péptica, recomendando aos indivíduos com úlceras tratamentos para a erradicação do organismo (NIH Consensus Conferense, 1994). Taylor & Personnet, em 1995 revelaram que, em países em desenvolvimento, de 70 a 90% da população são portadores de *H. pylori*, e que a maioria destes adquirem a infecção antes dos 10 anos de idade. Em países desenvolvidos, a freqüência da infecção está entre 25 a 50% da população, também adquirida durante a infância. A maioria dos estudos sugerem que a freqüência da infecção entre homens e mulheres é a mesma, e não há indícios de que a infecção por ***H. pylori*** seja transmitida sexualmente (Perez-Perez *et al*., 1991; Polish *et al*., 1991).

Uma das questões cruciais na transmissão do ***H. pylori*** é como o microrganismo é transportado do estômago de uma pessoa para outra. Três caminhos estão bem descritos:

1) pouco comum, um meio de transmissão dá-se através do uso de instrumentos contaminados utilizados em exames endoscópicos introduzidos em uma outra pessoa (Akamatsu *et al*., 1996), podendo o risco ser reduzido com a esterilização dos materiais (Katoh *et al*., 1993; Tytgat, 1995).

2) a transmissão fecal-oral parece ser importante, embora o *H. pylori* tenha sido isolado das fezes de crianças infectadas com o microrganismo (Thomas *et al*., 1992). Contudo o isolamento a partir de material fecal não é comum, o que pode indicar que a descamação gástrica deva ser intermitente. Água contaminada por fezes também pode sim ser uma fonte de infecção (Klein *et al*., 1991), embora o microrganismo ainda não tenha sido isolado de água.

3) a transmissão oral-oral foi identificada no caso de mulheres africanas que pré-mastigam os alimentos de suas crianças (Megraud, 1995) Todos os pacientes

infectados por *H. pylori* desenvolvem inflamação gástrica crônica (Blaser, 1990a), contudo esta condição normalmente é assintomática. A presença de *H. pylori* também está fortemente associada com o risco de desenvolvimento de gastrite atrófica (Blaser, 1990b), que é uma lesão precursora de câncer gástrico. Dessa forma, não é surpreendente que a presença de *H. pylori* também esteja associada com adenocarcinoma distal, mas não proximal, de estômago (Nomura *et al*., 1991; (Parsonnet *et al*., 1991b; Talley *et al*., 1991). A associação da infecção por *H. pylori* com o desenvolvimento de tumores é extremamente importante, tendo em vista que o câncer gástrico é a segunda principal causa de morte de câncer no mundo (Neugut *et al*., 1996).

H. pylori associa-se exclusivamente às células da mucosa gástrica, e não aparece associado ao epitélio do intestino delgado ou em metaplasia intestinal. O *H. pylori* é visto no bulbo duodenal só em associação com metaplasia gástrica. A presença de *H. pylori* é acompanhada por infiltração de células inflamatórias no epitélio, o que não é associado especificamente com modificações metaplásicas e formação de granuloma (Dixon, 1995b). A importância da inflamação é altamente variável, indo de uma infiltração mínima superficial sem comprometimento do tecido glandular, até uma inflamação severa, com formação de microabscessos (Genta *&* Graham, 1994). Em paralelo, freqüentemente há mudanças degenerativas de células da superfície epitelial, inclusive com depleção de mucina, vacuolização citoplasmática, e desorganização de glândulas da mucosa. Depois da erradicação de ***H. pylori*** por agentes antimicrobianos, a maioria destas características desaparecem rapidamente (Dixon, 1995a), porém a presença de leucócitos pode persistir durante vários meses.

Gastrites superficiais, caracterizadas por infiltração da mucosa gástrica por células polimorfonucleares e/ou demais leucócitos, invariavelmente é vista em pacientes infetados com *H. pylori*, sugerindo que a presença dessas células podem ser importantes para a sobrevivência de *H. pylori* em vivo (Blaser, 1992).

A infecção por *H. pylori* pode causar injúria da mucosa gástrica pela indução, direta ou indireta, de incremento na secreção de ácido gástrico. Essa indução dá-se devido a capacidade deste microrganismo em metabolizar um precursor não ulcerogênico, o “Lyso-PAF”, produzido por células da mucosa gástrica em condições basais (em pessoas saudáveis), em resposta à gastrina, a PAF um potente agente ulcerogênico. O PAF (1-O-alquil-2-acetil-sn-glicero3-fosforilcolina) estimula a secreção de ácido gástrico via receptores celulares específicos (Sobhani *et al*., 1992; Sobhani *et al*., 1996). Outro aspecto a ser considerado, é o fato dessa bactéria inibir, *in vitro*, a secreção de muco, afetando o mecanismo primário de defesa da mucosa gástrica (Micots *et al*., 1993).

Urease de *Helicobacter pylori*:

A urease nativa de *H. pylori* é um hexâmero de 540 kDa, cujo monômero é formado por duas subunidades, UreA de 30 kDa e UreB de 60 kDa, contendo átomos de níquel, na proporção estequiométrica de 2 Ni para cada monômero UreA/UreB, (Dunn *et al*., 1990; Hu *&* Mobley, 1990b). Essa enzima tem grande afinidade pelo substrato uréia com um Km de ~ 0,3 mM, o que permite sua atividade em concentrações submilimolares de uréia (Dunn *et al*., 1990); (Hu *&* Mobley, 1990a), normalmente presente no plasma e nos tecidos.

A urease de *H. pylori* é considerada um fator de virulência importante, tanto na colonização quanto na manutenção da infecção, por esse microrganismo. Mutantes urease negativos são incapazes de colonizar porcos gnotobióticos e tampouco camundongos “*nude*”. (Eaton *et al*., 1991; Tsuda *et al*., 1994). Presume-se que a urease de *H. pylori* contribui para a produção de um microclima neutro para o microrganismo no lúmen gástrico. Sabe-se ainda que essa proteína está envolvida na ativação de fagócitos e na produção de citocinas inflamatórias (Harris *et al*., 1996). *H. pylori* induz em macrófagos *in vitro* aumento da óxido nítrico sintase induzível (iNOS)

(Wilson *et al*., 1996) resultando em uma grande liberação de oxido, nítrico o qual está associado à ativação de células do sistema imune no tecido lesado.

Plaquetas:

Plaquetas são microcélulas anucleadas com diâmetro aproximado de 2 – 3 μm que circulam em torno de 10 dias no sangue. No homem, a concentração plasmática normal de plaquetas é de 150 – 400 X 10^9/L. As plaquetas podem ser ativadas por vários agentes fisiológicos, incluindo adenosina difosfato (ADP), noradrenalina, colágeno, trombina e Fator Ativador de Plaquetas (PAF). Esse tipo de célula também pode ser ativado por complexos imunes gerados durante infecções, toxinas protéicas de várias origens (animais, vegetais e microbianas), e também por estresse mecânico, causado pela força tangencial aplicada às células pelo fluxo sanguíneo.

As características de alta reatividade plaquetária fazem desse tipo celular um modelo experimental interessante, pela gama de resultados que podem ser obtidos a partir de ensaios de agregação, pela facilidade de obtenção do material bem como simplicidade e robustez do tipo de ensaio realizado, tornando-se assim uma ferramenta importante no estudo de mecanismos de ação de diferentes moléculas em nível celular. A canatoxina, como citado anteriormente apresenta atividade biológica sobre plaquetas, induzindo a liberação de seus grânulos densos e conseqüente agregação, via ativação da 12-lipoxigenase, (Carlini *et al*., 1985).

Receptores plaquetários desencadeiam então a ativação de fosfolipases de membrana das plaquetas, as quais hidrolisam fosfolipídios de membrana liberando ácido araquidônico, o qual é metabolizado via cicloxigenase ou lipoxigenase, com liberação de eicosanóides mediadores. A maioria dos ativadores plaquetários atua pela ligação a receptores específicos na superfície da membrana das plaquetas. Após a estimulação do receptor, várias rotas de ativação de plaquetas podem ser iniciadas, resultando em várias atividades biológicas:

- Mudança na forma das plaquetas ou "*shape change*": as plaquetas mudam sua forma de discóide para esférica, com emissão de pseudópodes, o que facilita a agregação e a sua atividade coagulante.

- Adesão à parede do vaso sanguíneo: Através da exposição da glicoproteína GPIb-IX, a qual liga-se ao Fator de von Willebrand (vWF), fazendo a ponte entre as plaquetas e o colágeno sub-endotelial.

- Liberação de componentes envolvidos na hemostase: liberação de ADP, serotonina tromboxana A_2 (TXA_2) e (vWF) a partir de grânulos intracelulares das plaquetas.

- Agregação via exposição da glicoproteína GPIb-IX, um receptor para (vWF), e pela exposição de outra glicoproteína de membrana, a GPIIa-IIIb, um receptor de ligação a fibrinogênio. A interação entre este receptor e o fibrinogênio propicia a formação de pontes entre as plaquetas, a ativação desse receptor sinaliza para a mobilização de Ca^{++}, fosforilação da tirosina, ativação do metabolismo do fosfatidato e reorganização do citoesqueleto; essa série de eventos conduz a resposta de agregação das plaquetas de uma fase reversível à fase irreversível do processo.

A agregação plaquetária representa o mais importante evento durante a formação do tampão plaquetário (Ruggeri, 1997). Dois tipos de agregação podem ser distinguidos in vitro: a agregação primária é reversível e ocorre sem reação de liberação, e a segunda agregação, que é irreversível e associada à reação de liberação (Born, 1962). Esta segunda agregação é principalmente mediada por endoperóxidos (Eps) e (TXA_2), eicosanóides derivados do ácido araquidônico, e ADP e Ca^{++} secretados dos grânulos densos plaquetários (Hamberg *et al*., 1975, Gachet, 2000). A eficiência desse fenômeno depende do agonista, e de sua concentração, para a indução da agregação *in vitro*.

O método mais utilizado para avaliação da atividade das plaquetas é a medida da agregação plaquetária em Plasma Rico em Plaquetas (PRP), obtido por

centrifugação a partir de sangue na presença de anticoagulantes, por turbidimetria (Born *&* Cross, 1963). Os agentes de agregação freqüentemente utilizados são ADP, noradrenalina, colágeno, ácido araquidônico, U46619 (que mimetiza TXA_2) ou, um peptídeo ativador de receptor de trombina, como SFLLRN (a trombina não pode ser usada em PRP por causa de sua ação coagulante). Em condições normais, em PRP citratado a agregação causada por todos esses agonistas é associada à formação de TXA_2, secreção do conteúdo dos grânulos plaquetários e ao aparecimento da P-selectina na superfície das plaquetas. Em PRP citratado, baixas concentrações de ADP causam somente a fase primária reversível da agregação (FIGURA.5), mas em concentrações superiores a 1 – 3 μM, a agregação em plaquetas humanas evolui para a segunda fase de agregação irreversível, dependente da formação de TXA_2. Em altas concentrações de ADP as duas fases se fundem, resultando em uma curva de agregação contínua (FIGURA.5), efeito semelhante ao que acontece quando as plaquetas são ativadas por colágeno ou trombina. Na presença de concentrações fisiológicas de Ca^{++} apenas a primeira fase da agregação acontece (Packham *et al*., 1989). Um agonista fraco, a noradrenalina, em concentrações entre 5 – 10μM agrega plaquetas em PRP citratado sem uma modificação inicial no formato das plaquetas *"shape change"*; contudo a noradrenalina é agonista pouco potente. Se for utilizado algum agente com capacidade de inibição na formação de TXA_2, não há formação de agregados em resposta a qualquer concentração de noradrenalina.

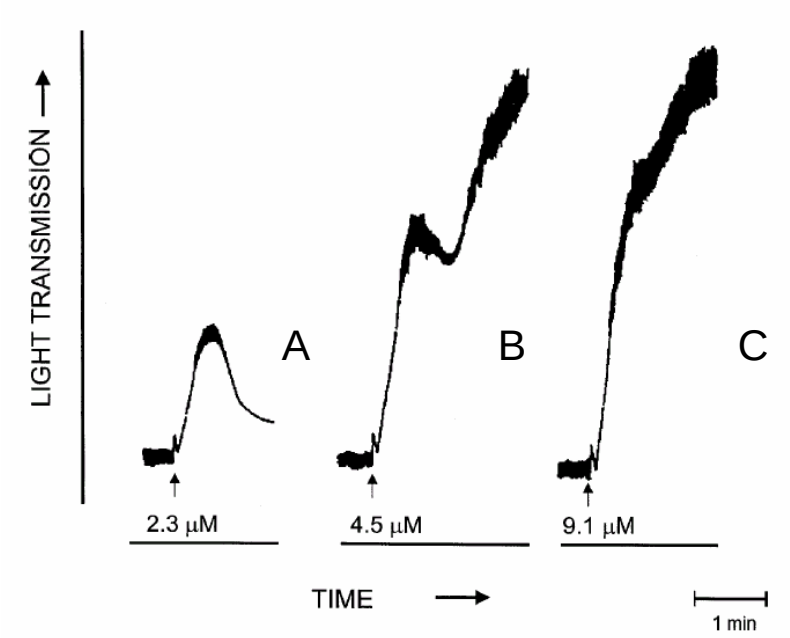

FIGURA.5 Agregação induzida por ADP em PRP citratado, mostrando a fase primária de agregação induzida por uma baixa concentração de ADP (A), agregação primária seguida pela segunda onda, induzida por uma concentração ligeiramente mais alta de ADP (B), e fusão das duas fases de agregação com uma alta concentração de ADP (C). Adaptado de Rand *et al*., 2003.

Fibras de colágeno (1 – 5 μg/mL) provocam, antes do início da agregação, um característico prolongamento de até 1 minuto, a fase "*lag*". A agregação induzida por colágeno requer formação de TXA_2 e secreção do conteúdo dos grânulos plaquetários, e assim sendo, é essencialmente irreversível. Inibidores da formação de TXA_2 bloqueiam a agregação induzida por baixas concentrações de colágeno, embora haja "*shape* change". Em uma rara deficiência da glicoproteína GPIV, as plaquetas não agregam em resposta ao colágeno, mas agregam sob estímulo de outros antagonistas (Moroi *&* Jung, 1997).

Na medicina humana as plaquetas têm participação na fisiopatologia de doenças como aterosclerose, trombose, metástase, formação de tumores malignos, rejeição de órgãos transplantados, inflamação e coagulação intravascular disseminada (Fuse, 1996).

Papel fisiológico dos eicosanóides:

Os eicosanóides, metabólitos derivados do ácido araquidônico por rotas metabólicas distintas, entre as quais a via das cicloxigenases e a via das lipoxigenases (FIGURA. 4), são hoje reconhecidos como segundo mensageiros envolvidos na transdução de sinais numa vasta gama de fenômenos fisiológicos e patológicos. Diversas linhas de pesquisas têm demonstrado que produtos de lipoxigenases, como leucotrienos, estariam envolvidos nos processos secretórios de diferentes tipos celulares (Snider *et al*., 1984; Metz, 1985b;Metz *et al*., 1983a; Metz *et al*., 1983b; Metz *et al*., 1983c); Sasakawa *et al*., 1984; Piomelli *et al*., 1987; Naor *et al*., 1985). O envolvimento de metabólitos da 12-lipoxigenase na secreção de alguns neurotransmissores e hormônios hipotalâmicos também já foi caracterizada, sendo que a 12-lipoxigenase é a isoenzima predominante em tecido cerebral (Adesuyi *et al*., 1985), bem como em plaquetas (Nugteren, 1982)

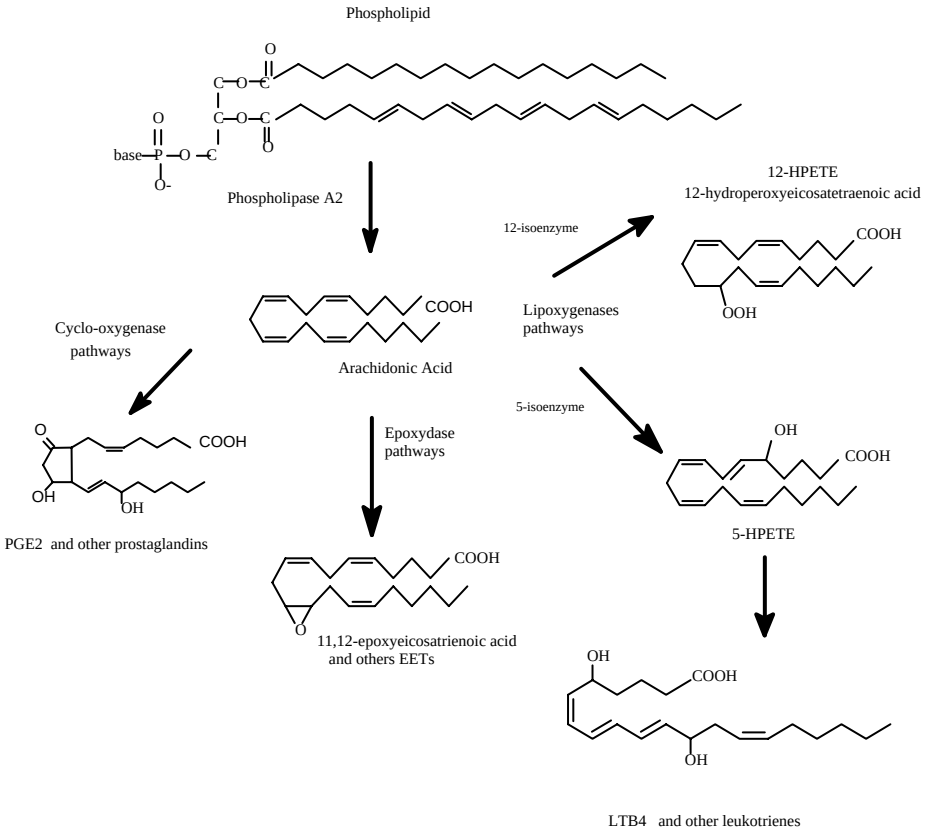

FIGURA. 4: Principais vias do metabolismo dos eicosanóides. A liberação de ácido araquidônico, constituinte minoritário dos fosfolipídios de membranas, ocorre por hidrólise catalisada por fosfolipases tipo A2, em decorrência da ativação celular. Uma vez liberado, o ácido araquidônico será substrato para diferentes rotas metabólicas, particulares para cada tipo celular. Através da via da cicloxigenase formar-se-ão prostaglandinas e tromboxanas, enquanto que a ação das diferentes lipoxigenases levará á formação dos hidroperóxidos correspondentes. No caso do 5-HPETE, este poderá ainda ser transformado em leucotrienos ou lipoxinas. Adaptado de Stanley-Samuelson *&* Ogg, 1994.

O ácido araquidônico, atuando diretamente ou na forma de metabólitos eicosanóides, regula uma série de funções celulares (Sakata *et al*., 1987); (Sumida *et al*., 1993). Além disso, modificações no metabolismo do ácido araquidônico estão envolvidas em muitas alterações patológicas (Goetzl *et al*., 1995).

A mobilização do ácido araquidônico esterificado em fosfolipídios de membrana dá-se, principalmente a partir da ação da fosfolipase A_2 (PLA_2). Após essa liberação, o ácido araquidônico pode participar de múltiplas rotas metabólicas, inclusive ser reincorporado aos fosfolipídios de membrana (Glatz *et al*., 1997). Como uma alternativa à reincorporação, o ácido araquidônico pode ser metabolizado nas rotas da cicloxigenases, lipoxigenases ou citocromo P450, formando metabólitos chamados eicosanóides, os quais possuem potentes atividades biológicas.

Os produtos de cicloxigenase estão envolvimos na patogênese de várias doença doenças inflamatórias como aterosclerose devido ao potencial inflamatório de PGE2 e tromboxana A2, (Linton *et al*., 2004) bem como seu envolvimento no desenvolvimento de problemas vasculares decurrentes da diabetes (Natarajan & Nadle, 2004), em contrapartida os produtos das lipoxigenases também estão implicados na patogênese da aterosclerose e em problemas vasculares ligados à diabetes (Natarajan & Nadle, 2004).

As cicloxigenases convertem o ácido araquidônico livre em prostaglandinas (PG) e endoperóxidos, que são metabolizado à TXA_2, $TXB_{2,}$ e outras prostaglandinas. Na rota da lipoxigenase (LO), vários produtos diferentes são formados em função da isoenzima envolvida na reação. A 5-lipoxigenase (5-LO) transforma ácido araquidônico em ácido 5-hidroxiperoxieicosatetraenóico (5-HPETE), e posteriormente levando ao leucotrieno A_4. As isoenzimas 12 e 14-lipoxigenases são responsáveis pela conversão do ácido araquidônico em 12 e 15-ácido hidroxieicosatetraenóico (HETE) respectivamente. Pela via do citocromo P450 são gerados a partir do ácido araquidônico, vários metabólitos oxigenados, incluindo (HETE) e ácido epoxieicosatetraenóico (EET) (Needleman *et al*., 1986). Em plaquetas, o ácido

araquidônico é metabolizado pelas cicloxigenases e 12-lipoxigenase, obtendo-se como metabólito final TXA_2 e 12-HETE, respectivamente.

A síntese de PGs através das cicloxigenases e liberação de TXA_2 pelas plaquetas ocorre sob estímulo de agonistas como trombina, colágeno ou ADP (Hammarstrom *et al*., 1975 Patrono *&* Salvati, 1989). A TXA_2 liberada pelas plaquetas estimula receptores de TXA_2 nas próprias plaquetas (Coleman *et al*., 1994; Halushka, 1989). A ativação dos receptores de TXA_2, resulta em ativação da fosfolipase C (PLC), liberando cálcio de depósitos intracelulares. O aumento do cálcio intracelular amplifica a agregação plaquetária, resultando na síntese e liberação de mais TXA_2 e ADP das plaquetas ativadas (Kroll & Schafer, 1989a). O ADP secretado dos grânulos densos plaquetários induz um rápido influxo de cálcio externo , a mobilização de cálcio intracelular, bem como a inibição da adenilato ciclase (Mills, 1996). Ambos, TXA_2 e ADP, participam do “feedback” positivo que leva à segunda e irreversível fase de agregação plaquetária (Kroll & Schafer, 1989b).

O metabolismo do ácido araquidônico via 12-lipoxigenase em plaquetas, leva a formação de 12-HPETE, o qual é reduzido a 12(S)-hidroxieicosatetraenóico [12(S)-HETE], acredita-se que este tenha função na regulação da agregação plaquetária; o 12-HETE potencializa a agregação plaquetária in vitro (Sekiya *et al*., 1989; Sekiya *et al*., 1991). O uso de OPC-29030, inibidor específico para 12-HETE, resultou em inibição de agregação plaquetária induzida por ADP. O OPC-29030, *in vitro*, também inibe a ativação do receptor para fibrinogênio, a glicoproteína (GPIIb/IIIa), por conseguinte a inibição de (GPIIb/IIIA) por OPC-29030 sugere a participação do metabólito plaquetário 12-(HETE) como mediador de agregação (Katoh *et al*., 1998).

3. JUSTIFICATIVA:

Sendo a urease uma enzima de interesse bioquímico e biotecnológico e importância histórica no estudo de proteínas e sabendo-se de sua conservação e ocorrência em diferentes organismos, é pertinente a investigação das relações existentes entre as diferentes ureases encontradas em organismos evolutivamente distantes, tais como plantas e bactérias.

H. pylori, é uma bactéria produtora de urease é de conhecido interesse médico pelo fato de estar associada ao desenvolvimento de estados patológicos em seres humanos, sendo sua urease reconhecida como um importante fator de virulência para esse patógeno. Contudo a presença de urease em microrganismos não é exclusividade apenas de organismos patogênicos; outras bactérias não patogênicas, como *Bacillus pasteurii*, também produzem essa enzima.

Em vegetais, a presença de ureases é relativamente comum, especialmente em leguminosas. Uma isoforma dessa proteína, encontrada em *C. ensiformis*, a Canatoxina (Carlini & Guimaraes, 1981), é tóxica para mamíferos e insetos, apresentando propriedades farmacológicas interessantes, como indução de agregação plaquetária e ação pró-inflamatória não relacionadas à sua atividade enzimática sobre uréia (Follmer *et al*., 2001b).

Pelo exposto acima, o interesse científico em estudar ureases microbianas (bactérias e fungos), justifica-se não só por sua associação em casos clínicos de interesse médico, mas principalmente por constituírem, juntamente com ureases vegetais, um conjunto de proteínas homólogas para as quais ainda há muitas questões estruturais e biológicas pouco compreendidas.

Resultados obtidos em estudos comparativos com ureases de diferentes fontes podem contribuir, não só para o entendimento das relações entre a estrutura tridimensional e propriedades biológicas dessas enzimas, mas também podem trazer

subsídios importantes para a terapêutica e compreensão da etiologia de patologias causadas por organismos produtores de ureases.

4. OBJETIVOS:

Objetivos Gerais

Neste trabalho visamos realizar estudos comparativos de propriedades farmacológicas independentes da atividade ureolítica entre uma urease vegetal, a canatoxina, e as ureases bacterianas de *H. pylori* e *B. pasteurii*.

Objetivos específicos:

- Desenvolver protocolo para purificação da urease de *H. pylori* recombinante;
- Investigar a capacidade das ureases bacterianas em induzir agregação plaquetária e caracterizar os mecanismos de ação envolvidos, comparando-os com a canatoxina;
- Testar o envolvimento das rotas mediadas por ADP, Fosfolipase A_2, PAF na resposta das plaquetas à urease;
- Testar vias metabólicas/sinalização: Cicloxigenase e Lipoxigenase na resposta das plaquetas à urease;
- Testar o influxo de cálcio na resposta das plaquetas à urease;
- Investigar se a urease de *H. pylori* apresenta propriedades pró-inflamatórias utilizando o modelo *in vivo* de edema de pata em camundongo, comparando com a canatoxina.

5. Materiais e Métodos:

Materiais:

Os compostos utilizados nestes experimentos foram adquiridos junto a Sigma Chemical Co., St Louis, EUA.; monofosfato de adenosina (AMP) difosfato de adenosina (ADP), Esculetina, dexametasona, Indometacina, cloranfenicol.

- Urease de *B. pasteurii* (Sigma Chem. Co)

- Cepa Se 5000. *Escherichia coli* gentilmente cedida pelo Prof. PhD. Harry l.T Mobley (University of Michigan Medical School)

- Plasmídio pHP8080. gentilmente cedido pelo Prof. PhD. Harry l.T Mobley (University of Michigan Medical School)

- Coelhos Nova Zelândia mantidos em ambiente climatizado, fotoperíodo controlado, com água e comida *ad libitum* até o momento dos experimentos.

- Camundongos "*Swiss"* mantidos em ambiente climatizado, com fotoperíodo controlado, com água e comida *ad libitum* até o momento dos experimentos.

Métodos:

- **Preparação de *Células* competentes e transformação de *Escherichia coli*:** conforme (Sambrook *et al* 1989).

- ***Urea segregation Agar:*** conforme (Mobley *et al*, 1999).

Cultivo das Cepas de *Escherichia coli* recombinantes:

- Cepa SE5000: ***E. coli***, recombinante, contendo o plasmídio (pHP8080), para expressão de urease de ***H. pylori***, com marca de seleção para cloranfenicol, gentilmente cedida pelo Profº.Dr. Harry L.T. Mobley (departament of Microbiology and

Immunology – University of Maryland School of Medicine) As células foram armazenadas a -80 em 50% (v/v) de glicerol.

- Pré cultura: pré-inóculos foram preparados na proporção de 1:50 em meio "LB" estéril, líquido, na presença de cloranfenicol, em capela de fluxo laminar. O desenvolvimento dos microrganismos em pré-inóculo foi feito em tubos de ensaio por um período de 18 horas a 37ºC sob agitação.

- Cultura: pré-inóculos preparados na proporção 1:50 em meio "LB" estéril, líquido, na presença de cloranfenicol 20 μg/mL, em capela de fluxo laminar. O desenvolvimento dos microrganismos em pré-inóculo foi feito em tubos Erlenmayer por um período de 18 horas a 37ºC sob agitação.

Preparação de extratos brutos a partir dos cultivos bacterianos:

- Após o desenvolvimento das cepas o cultivo foi centrifugado em centrífuga Sorvall-Plus RC5b, a 35000G, a 4°C, durante 10 minutos. O material sobrenadante foi desprezado e o precipitado suspenso em tampão 20mM NaPB, 5mM β-mercaptoetanol, 1mM EDTA (tampão de extração).

- As células suspensas no tampão de extração foram lisadas, com a utilização de ultra-som (Ultrasonic Homogenizer 4710), com 10 pulsos de 30segundos, sob banho de gelo. Em seguida esse material foi novamente centrifugado em centrífuga Sorval Plus RC 5b, a 60000G, durante 20 minutos, o material precipitado foi descartado e o sobrenadante denominado como Extrato Bruto.

Medida do conteúdo protéico:

Dois métodos foram utilizados para determinação do conteúdo protéico nas amostras:

- Absorção no ultravioleta em 280nm, utilizando-se cubetas de quartzo com passo óptico de 1 cm.
- Método de Bradford: Para tal utilizou-se 2,5 mL do reagente de Bradford para um volume máximo de 50 μL de amostra. Após 30 minutos de reação à temperatura ambiente, foi feita a leitura de absorbância a 595 nm. Uma curva padrão com BSA na faixa de 5 e 50μg por tubo foi utilizada como referência (Bradford, 1976).

Ensaio para detecção de Atividade ureásica:

- Alíquotas de amostras de todas as etapas de purificação foram incubadas com 10mM de uréia, a 37°C, em tampão PBS 1X pH 7,0. A amônia liberada pela urease foi quantificada colorimetricamente pelo método de fenol-hipoclorito (Weatherb.MW, 1967). Utilizando-se uma curva padrão de sulfato de amônio na faixa de 1 a 40 μg. Uma unidade enzimática de urease foi definida como a quantidade de enzima capaz de liberar 1μmol de amônia por minuto, em pH 7,0 a 37°C.

Ensaio de Agregação plaquetária:

- Conforme Born & Cross, 1963. O plasma rico em plaquetas (PRP) foi preparado a partir de sangue de coelho coletado da artéria central auricular, na presença de citrato de sódio na concentração final de 0,313% (v/v) as amostras de sangue foram centrifugadas a 200 X G, por 20 minutos a temperatura ambiente para a obtenção de plasma rico em plaquetas. A agregação plaquetária e o *"shape change"* foram monitorados por turbidimetria usando Lummi-agregômetro (Chrono-Log Co. Havertown, Pa.) e registrada por 3 minutos. A agregação plaquetária também foi monitorada utilizando leitor de microplacas SpectraMax (Molecular Devices, USA). Resumidamente, as amostras de urease na presença ou não dos

potenciais inibidores foram adicionadas em placas de 96 poços com fundo plano e completadas para o volume finas de 50μL com solução salina. A agregação foi ativada com a adição de 100μL da suspensão de plaquetas. A placa foi incubada por 2 minutos a 37°C antes do início da agitação e leituras que foram acompanhadas a cada 11 segundos em comprimento de onda de 650nm, durante 20 minutos. Quando testados os potencias inibidores, as plaquetas e estes componentes forma incubados por 2 minutos a 37°C e a agregação foi ativada com a adição das amostras de urease ou do controle ADP. A mudança da turbidez foi medida por unidades de absorbância e os resultados expressos como a área sob a curva de agregação.

Ensaio de Edema de pata em camundongos:

- Grupos com 9 camundongos foram submetidos ao ensaio de edema de pata; onde na pata traseira direita foram aplicados com injeção sub-plantar 30 μL de amostra em diferentes concentrações, diluídas em soro fisiológico e como controle negativo interno, no mesmo animal, pata esquerda traseira 30 μL de soro fisiológico. A evolução do edema foi acompanhada durante 48 horas através da medida da pata com uso de micrômetro (Mitutoyo 0 – 25 mm x 0,002).

Análise Estatística:

- Os dados foram analisados por ANOVA seguido por Turkey-Kramer utilizando o programa Instat Graf Pad e os valores de $p < 0.05$ (*), $p < 0,01$ (**) e 0,001 (***) foram considerados estatisticamente significantes.

6. Capítulo I:

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

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

Resumo:

Neste trabalho comparamos duas ureases vegetais jackbean urease (JBU) e a urease embrião-específica de soja (SBU) e urease de *Bacillus pasteurii* (BPU), quanto a seus parâmetros cinéticos e outras propriedades biológicas recentemente descritas para as ureases e independentes de suas atividades ureolítica. O efeito inseticida foi investigado usando como modelo *Dysdercus peruvianus* (Hemíptera), praga do algodão. Em contraste com BPU, ambas as ureases vegetais apresentaram potente atividade inseticida, com valores de DL_{50} de 0,017% (m/m) e 0,052% (m/m) para JBU e SBU respectivamente. A atividade inseticida de JBU e SBU não foi afetada pelo tratamento com p-hidroximercuriobenzoato, um inibidor irreversível da atividade ureolítica de ambas proteínas. Em comparação à canatoxina uma isoforma de urease de sementes de *C. ensiformis* que apresenta toxicidade em camundongos (DL_{50} 2 mg/Kg), não foi detectada atividade tóxica com injeção intraperitoneal de JBU e SBU (20 mg/Kg). Semelhante a canatoxina, as três proteínas promoveram agregação plaquetária com EC_{50} (400 μg/mL, 22.2 μg/mL e 15,8 μg/mL) para BPU, SBU e JBU respectivamente. Essa ativação de plaquetas é independente da atividade ureolítica. A comparação dos parâmetros cinéticos indicou que SBU é cinco vezes menos suscetível a inibição por ácido acetohidroxâmico um agente quelante de níquel e zinco. As ureases também mostraram diferente suscetibilidade a agentes que modificam resíduos de cisteina, como p-hidroximercuriobenboato e p-benzoquinona. Em suma estes resultados indicam que as propriedades biológicas independentes da atividade ureolítica não se restringem apenas as ureases de *C. ensiformis* sugerindo que essas proteínas podem ter função na defesa da planta contra insetos predadores

Eur. J. Biochem. **271**, 1357–1363 (2004) © FEBS 2004 doi:10.1111/j.1432-1033.2004.04046.x

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).

(Received 10 December 2003, revised 24 January 2004, accepted 18 February 2004)

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).

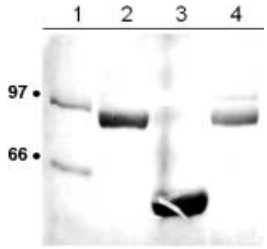

Fig. 1. SDS/PAGE patterns of JBU and SBU. SDS/PAGE analysis was performed using a 10% (w/v) polyacrylamide gel containing 0.1% SDS. After the run, the gel was stained with Coomassie Blue. Lane 1, molecular mass standards; lane 2, JBU (12 μg); lane 3, concanavalin A (12 μg, 27 kDa subunit); lane 4, SBU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

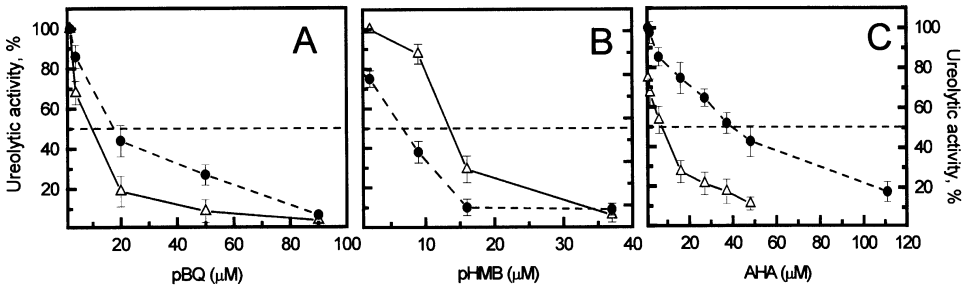

Fig. 2. Inhibition of ureolytic activity of JBU and SBU by *p*-benzoquinone (*p*-BQ), *p*-hydroxymercuribenzoate (*p*-HMB) and acetohydroxamic acid (AHA). Aliquots (0.1 mg·mL^{-1}) of JBU (△) or SBU (●) were incubated for 18–24 h at 4 °C with *p*-BQ (A), *p*-HMB (B) or AHA (C) and then assayed for residual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

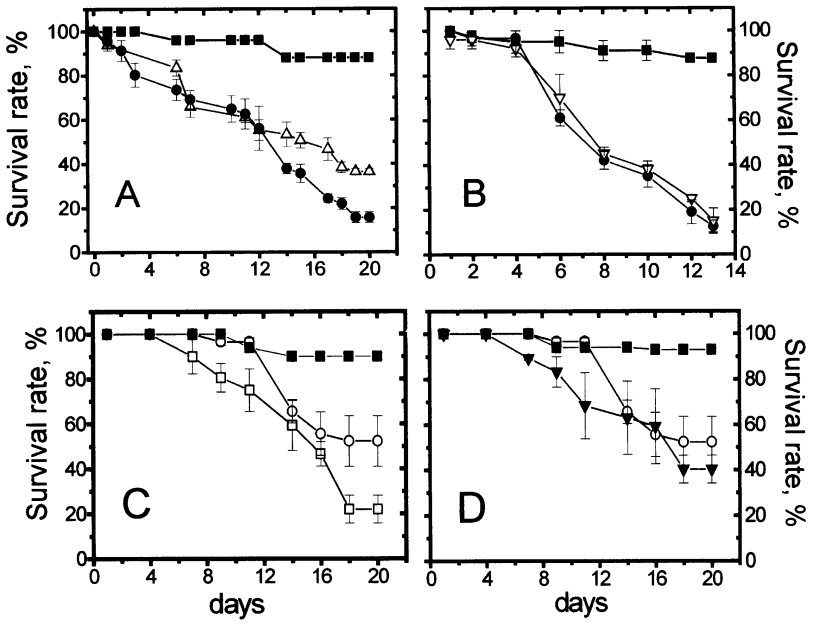

Fig. 3. Insecticidal effect of JBU and SBU in *Dysdercus peruvianus*. The toxic activity of ureases was assayed in feeding trials with *Dysdercus peruvianus* (second instar) using different concentrations of the freeze-dried proteins added to cotton meal. (A) Insecticidal effect of JBU: 0.02% (w/w) (△), 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.

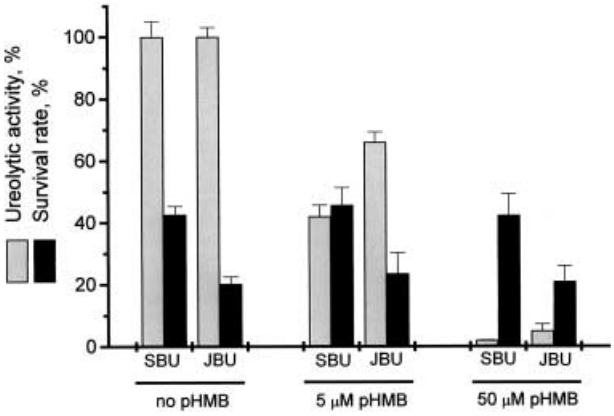

Fig. 4. Effect of *p*-hydroxymercuribenzoate treatment on the insecticidal and ureolytic activities of JBU and SBU. Ureases were incubated for 18–24 h at 4 °C with different concentrations of *p*-HMB (5 μM and 50 μM), dialysed against buffer A and then assayed for ureolytic activity and toxicity for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

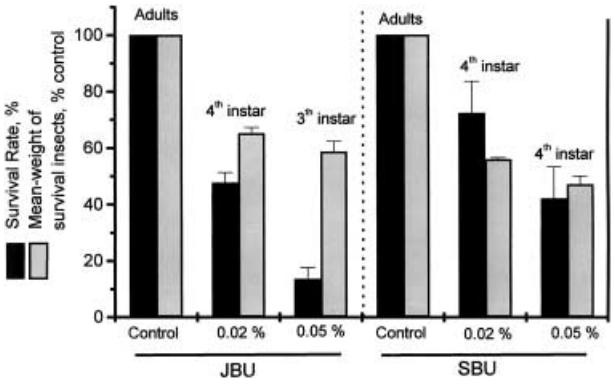

Fig. 5. Detrimental effects of JBU and SBU on the cotton stainer bug, *Dysdercus peruvianus*. The entomotoxic effects of JBU and SBU fed to *Dysdercus peruvianus* were evaluated as survival rate, mean body weight and stage of development of the surviving insects after two weeks. The freeze-dried proteins 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

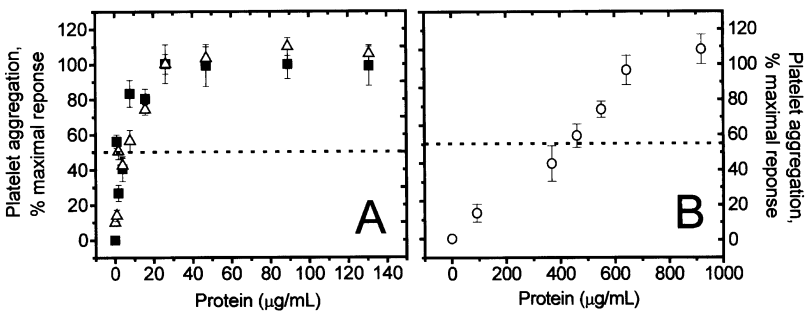

Fig. 6. Platelet aggregation induced by ureases. Platelet suspensions were challenged with (A) JBU (■), SBU (△) or (B) BPU (○), and aggregation of platelets was measured turbidimetrically. Data are means ± SEM of at least four independent experiments ($P < 0.001$).

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.

Acknowledgements

This work was supported by Conselho Nacional Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (Procad-CAPES-MEC), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Programa de Apoio a Núcleos de Excelência (MCT-FINEP-CNPq).

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7. Capítulo II

Urease display biological effects independent of enzymatic activity. Is there connection to diseases causes by urease-producing bacteria?

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Brazilian Journal of Medical and Biological Research (2006) 39 (x): Review

Resumo:

Ureases são enzimas encontradas plantas, fungos e bactérias que catalisam a hidrólise da uréia para a formação de amônia e dióxido de carbono. Enquanto as ureases fungicas e vegetais são homo-oligômeros com subunidades de aproximadamente 90-kDa, as ureases bacterianas são formadas por hetero-oligômeros compostos por duas ou três cadeias polipeptídicas. Trabalhos anteriores de nosso grupo mostraram que isoformas de urease encontradas em *Canavalia ensiformis*, a canatoxina e a urease clássica ligam-se a glicoconjugados e induzem agregação plaquetária. A canatoxina também induz a liberação de histamina em mastócitos; insulina em células pancreáticas e neurotransmissores em sinaptossomas. *In vivo* induz edema de pata e quimiotaxia de neutrófilos em ratos. Estes efeitos são independentes de sua atividade ureolítica e requerem a ativação do metabolismo de eicosanóides e canais de cálcio. *Helicobacter pylori* uma bactéria gram-negativa que coloniza a mucosa gástrica humana, causa úlceras gástricas e câncer por um mecanismo que não é, todavia, elucidado. *H. pylori* produz fatores que causam danos às células do epitélio gástrico, como a citotoxina de vacuolização VacA, a citotoxina CagA e a urease (até 10% de proteína bacteriana) a qual neutraliza o meio ácido em torno da bactéria e permite sua sobrevivência no estômago. Células inteiras de *H. pylori* ou extratos aquosos de proteínas de superfície promovem inflamação, ativam neutrófilos e liberação de citocinas. Neste trabalho revisamos dados da literatura que sugerem que a urease de *H.pylori* apresenta muitas das atividades biológicas observadas em *C. ensiformis* e mostra que ureases bacterianas tem um efeito secretagogo modulado por metabólitos de eicosanóides via rota da lipoxigenase. Estes achados. Estes achados podem ser importantes à elucidação da função da urease na patogênese das doenças gastrointestinais causadas por *H. pylori*.

Brazilian Journal of Medical and Biological Research (2006) 39: 851-861
ISSN 0100-879X Review

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*.

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Research supported by CNPq, CAPES and FAPERGS. D. Olivera-Severo and G.E. Wassermann were recipients of fellowships from CAPES.

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Received October 27, 2005
Accepted March 30, 2006

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 mirabili*s 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.

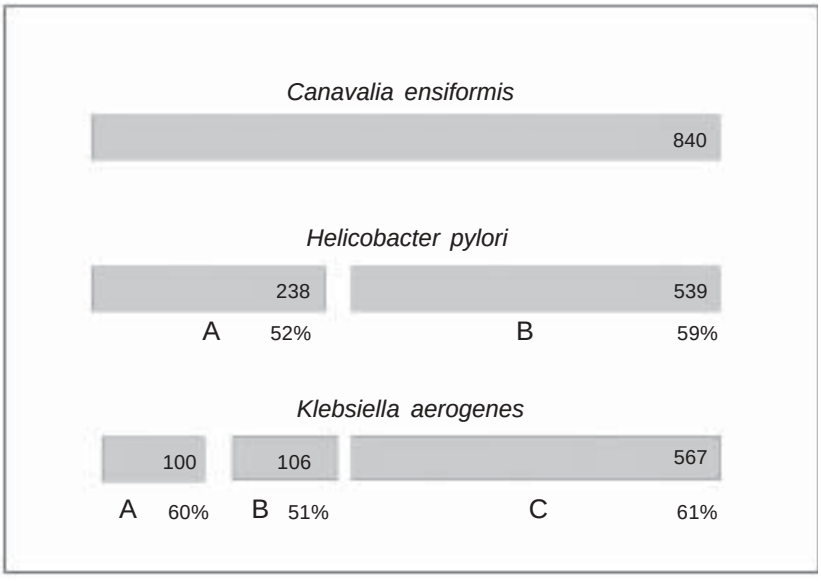

Figure 1. Schematic structure of ureases. Plant ureases such as the jack bean (*Canavalia ensiformis*) enzyme have a single subunit while bacterial ureases have two (*Helicobacter pylori*) or three (*Klebsiella aerogenes*) subunits. The number of amino acid residues of each subunit is indicated. Note 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-

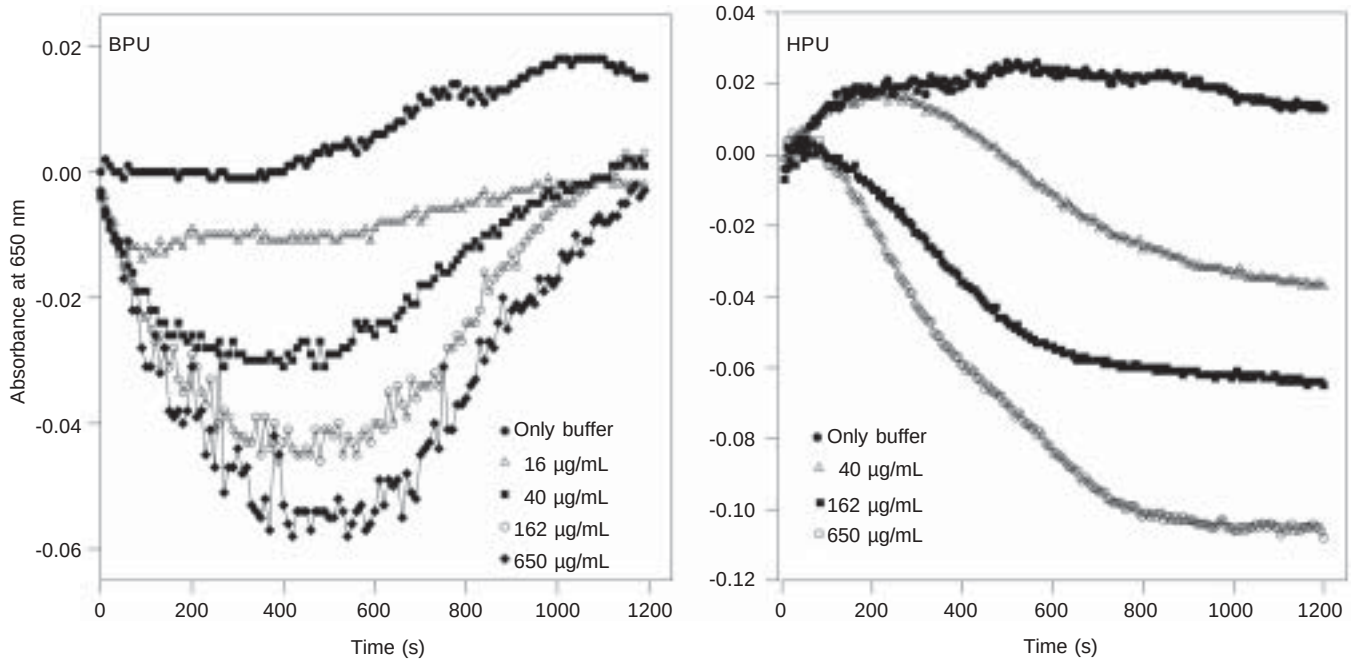

Figure 2. Dose-response curve of platelet aggregation induced by purified *Bacillus pasteurii* (BPU, left panel) and *Helicobacter pylori* ureases (HPU, right panel). Rabbit platelet-rich plasma suspensions were challenged with different concentrations of purified *B. pasteurii* (Sigma, St. Louis, MO, 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.

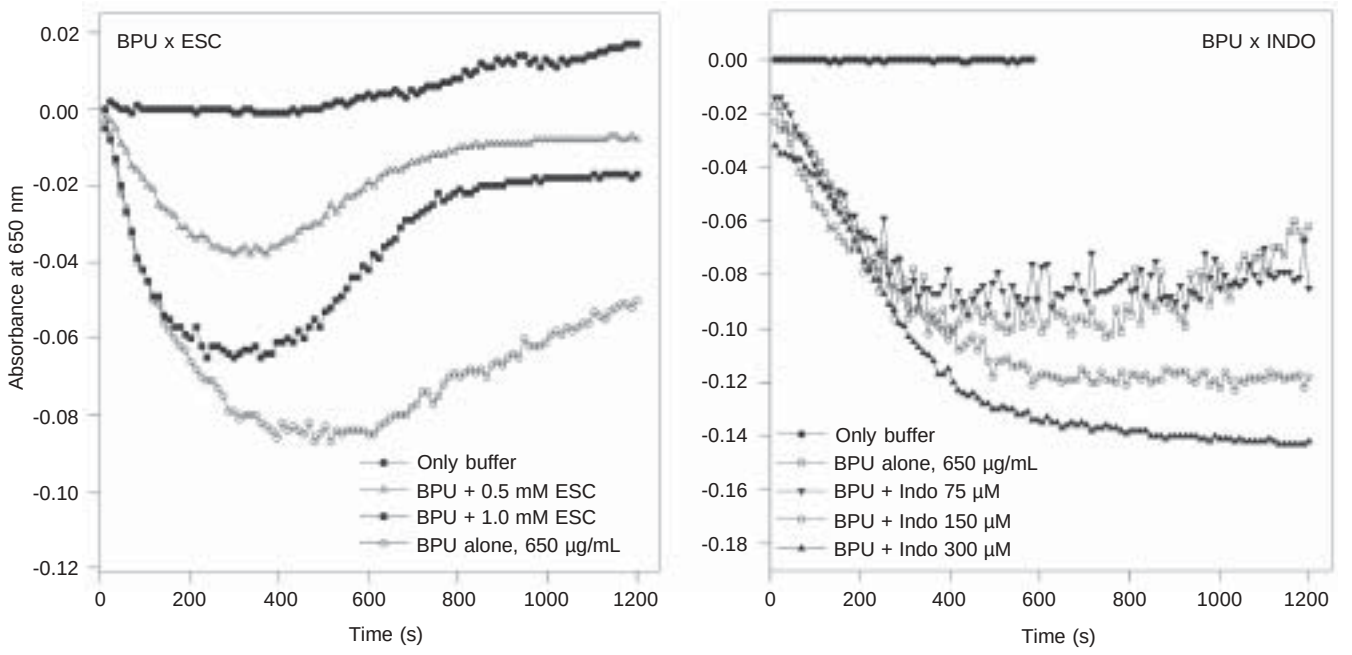

Figure 3. Effect of inhibitors of arachidonate metabolism on platelet aggregation induced by *Bacillus pasteurii* urease. Rabbit platelet-rich plasma suspensions were pretreated for 2 min with the indicated final concentrations of esculetin (ESC, left panel) and indomethacin (INDO, right panel) and then 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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8. Capítulo III

Baccilus pasteurii urease shares whith plant ureases the ability to induce aggregation of blood platelets.

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Archives of Biochemistry and Biophysics

Resumo:

Ureases (EC 3.5.1.5) são enzimas altamente homólogas presentes em plantas, bactérias e fungos. A Canatoxina, uma isoforma de urease em sementes de *Canavalia ensiformis*, tem várias propriedades biológicas independentes de sua atividade ureolítica, tais como agregação de plaquetas e efeitos pró-inflamatórios. Neste trabalho descrevemos a propriedade da urease de *Bacillus pasteurii* de também induzir agregação plaquetária em plaquetas de efeito semelhante ao apresentado pela canatoxina (ED_{50} 0,4 e 0,015 mg/mL, respectivamente). A agregação plaquetária induzida por BPU foi bloqueada utilizando plaquetas pretratadas com dexametasona e esculetina; inibidores de fosfolipase A2 e lipoxigenase respectivamente. Por outro lado, plaquetas tratadas com indometacina, um inibidor de cicloxigenase apresentou um incremento na resposta de agregação por BPU. Metoxiverapamil (bloqueador de canais de cálcio) e AMP (antagonista de ADP) reduziram a agregação plaquetária induzida por BPU enquanto WEB 2170, 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 das plaquetas através de um mecanismo cálcio dependente. Estes achados têm implicação na elucidação das interações bactéria-planta e na discussão da 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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Received 5 April 2006, and in revised form 1 June 2006
Available online 19 June 2006

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.

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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doi:10.1016/j.abb.2006.06.001

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

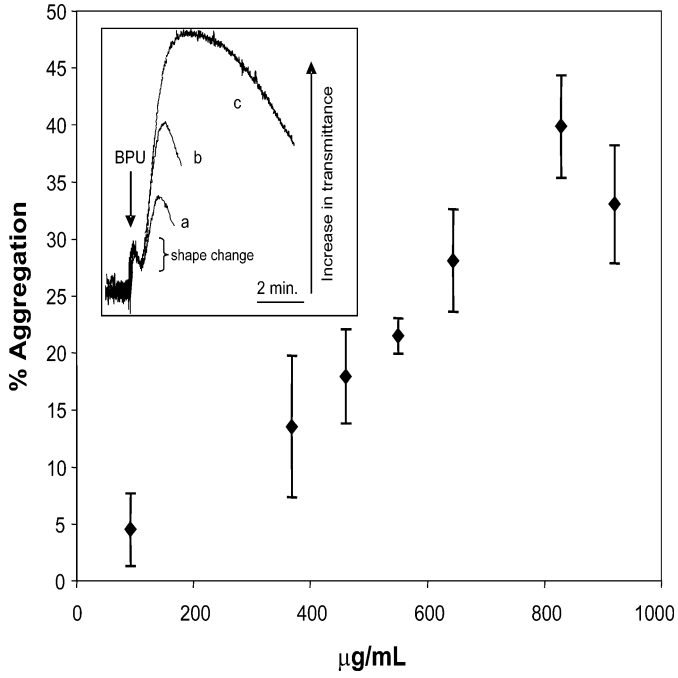

Fig. 1. Dose–effect curve of aggregation of rabbit platelet-rich plasma suspensions induced by *B. pasteurii* urease. Rabbit platelet-rich plasma suspension in microwell plates were exposed to increasing concentrations of BPU or 5 μM ADP (100% aggregation). Aggregation of platelets was monitored ever 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

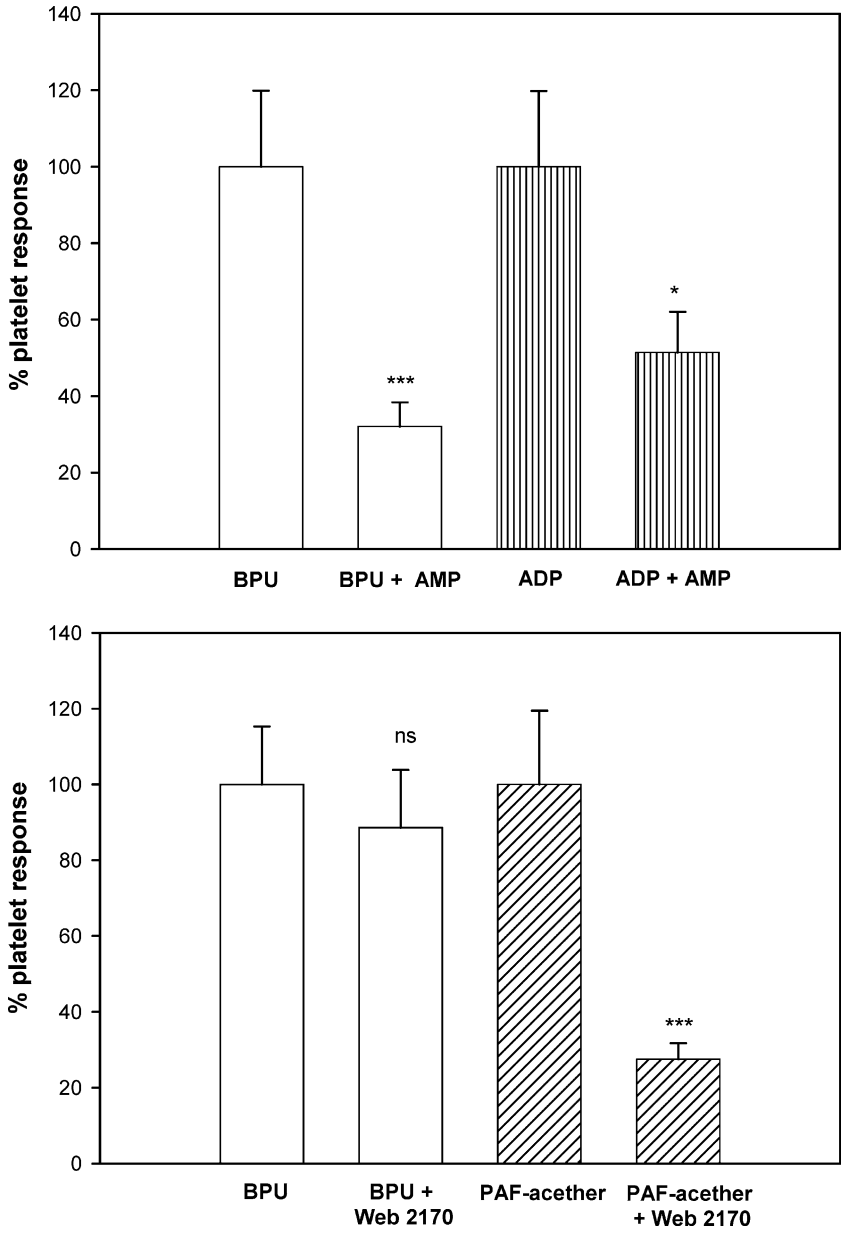

Fig. 2. Effect of adenosine monophosphate (AMP) (upper panel) or Web 2170 (a PAF antagonist) (lower panel) on the BPU-induced platelet aggregation. Rabbit platelet-rich plasma suspension in microwell plates were exposed to BPU (0.7 mg/mL), 5 μM ADP or 50 nM PAF (100% aggregation), in the presence or 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.

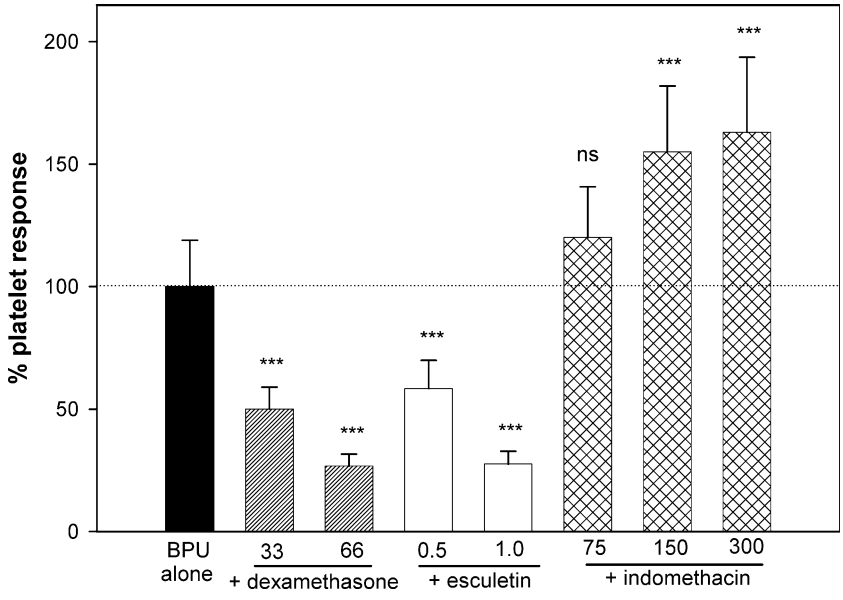

Fig. 3. Effect of inhibitors of platelet phospholipase A_2 or arachidonic acid metabolism on the BPU-induced platelet aggregation. Rabbit platelet-rich plasma suspensions in microwell plates were incubated for 2 min at r.t. in the presence or absence of the indicated concentrations of the drugs and aggregation 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.

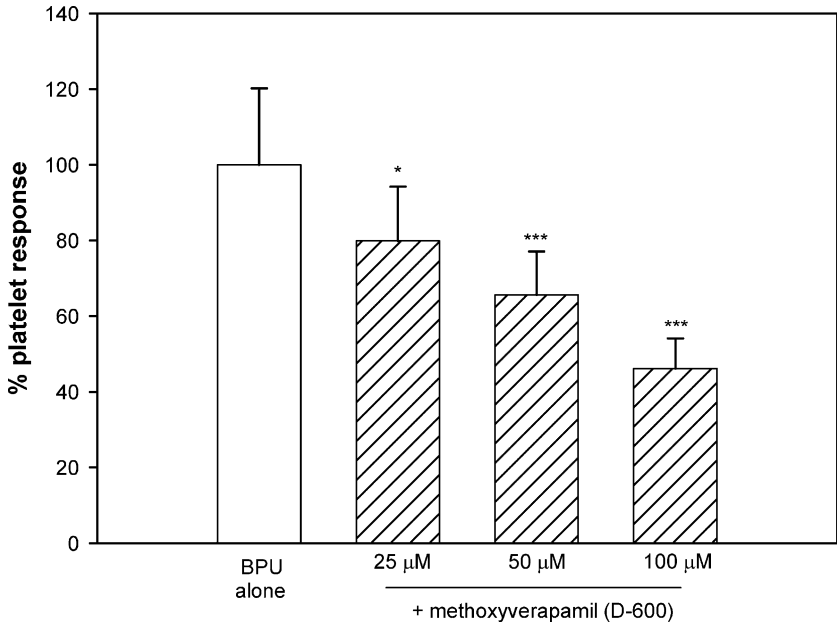

Fig. 4. Effect of methoxyverapamil (D-600), a blocker of voltage-gated Ca^{2+} channels, on the BPU-induced platelet aggregation. Rabbit platelet-rich plasma suspensions in microwell plates were incubated for 2 min at r.t. in the presence or absence of D-600 at the indicated concentrations and aggregation 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.

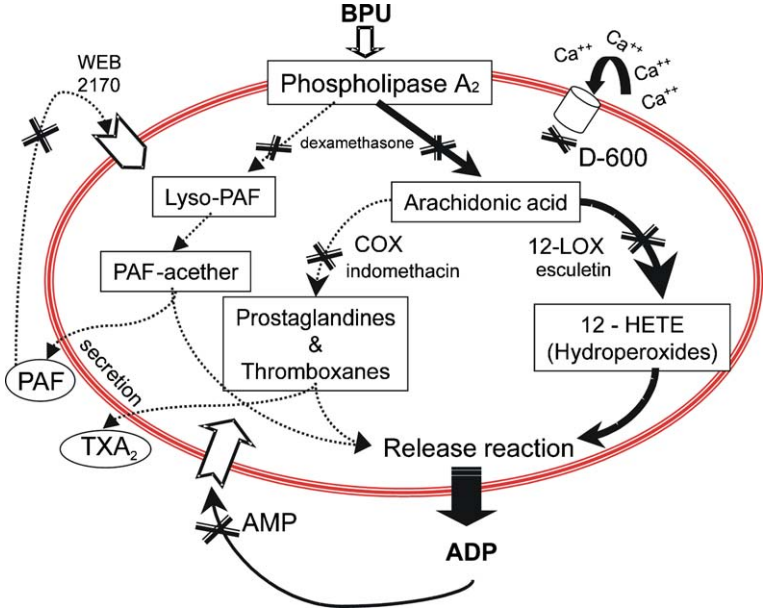

Fig. 5. Proposed mechanism of platelet aggregation induced by *Bacillus pasteurii* urease. The biochemical pathways that underlie the platelet-aggregating activity of *Bacillus pasteurii* urease (BPU) are indicated as continuous lines. BPU activates platelets through a phospholipase A_2 and calcium-dependent 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.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq; Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior—CAPES and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul—FAPERGS. D.O-S. and G.E.W. recipients of fellowships from CAPES. The authors are grateful to Dr. Arthur Fett Neto and Dr. Janette Palma Fett, Dept. Botany, Federal University of Rio Grande do Sul, Brazil, for critical reading and suggestions that help improving the manuscript.

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9. Capítulo IV:

Dados Não Publicados:

- Produção e purificação de HPU

- Agregação plaquetária induzida por HPU

- Edema de pata induzido por HPU

Transformação de colônias de *E.coli* cepa SE 5000.

Para verificar o estado de transformação das colônias utilizou-se placas de cultura contendo meio de cultura *urea segregation Agar* onde se pode identificar as colônias produtoras de urease pelo aumento de pH o qual modifica a coloração do meio para vermelha pela presença do marcador vermelho de cresol, figura 1R.

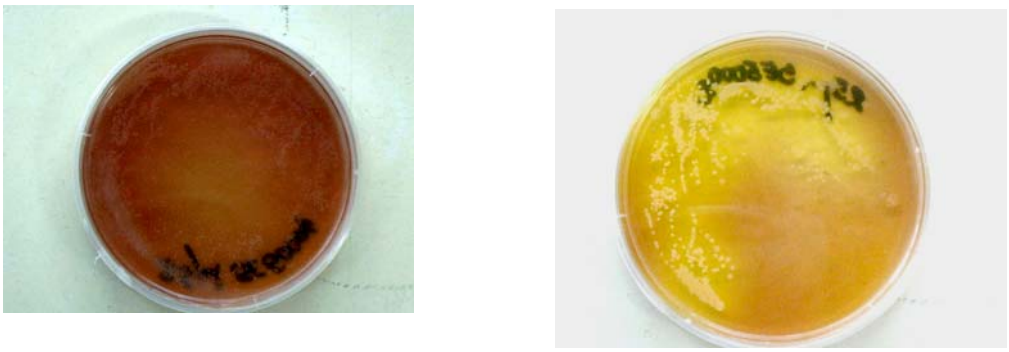

Figura 1R: (A) Placa com colônias transformadas com plasmídio php 8080; (B) placa com colônias não transformadas com plamídio php 8080.

Purificação das urease de *Helicobacter pylori*:

Um método de purificação da urease recombinante de ***H. pylori*** foi desenvolvido neste trabalho, com o objetivo de aumentar o rendimento da metodologia da metodologia descrita por (Hu *et al*., 1992). A FIGURA 1R ilustra a marcha de purificação aqui desenvolvida.

Fracionamento por precipitação com Sulfato de Amônio:

O extrato bruto, obtido a partir da cultura de células de *E.coli* SE5000, dialisado contra o tampão de extração, submetido à dosagem de proteínas, e ensaiado para atividade ureásica, foi em seguida fracionado por precipitação com

sulfato de amônio, obtendo-se três frações: Fração I - de 0 a 30%; Fração II – de 30 a 70% e Fração III - o sobrenadante de 70% de saturação.

As frações obtidas a partir do fracionamento com sulfato de amônio foram dialisadas para retirada do sal, submetidas ao ensaio para detecção de atividade ureásica determinando-se que fração rica em urease é a Fração II.

Cromatografia de troca iônica Q-Sepharose:

A fração rica em atividade ureásica (FraçãoII) foi submetida a cromatografia de troca iônica Q-Sepharose, na forma de "batch", na proporção de 1mL de resina para cada 10mg de proteína; a resina foi equilibrada em tampão 20mM NaPB, 5mM β-mercaptoetanol, 1mM de EDTA, pH 7,8. Após a adsorção da amostra à resina, esta foi lavada com o tampão de equilíbrio (L) e então eluida com gradiente descontínuo: Os tampões de eluição foram: 1ª eluição - 20mM NaPB, 5mM β-mercaptoetanol, 1mM de EDTA, 100 mM NaCl pH 7,8; 2ª eluição - 20 mM NaPB, 5mM β-mercaptoetanol, 1mM de EDTA, 200mM NaCl pH 7,8; 3ª eluição - 20mM NaPB, 5mM β-mercaptoetanol, 1mM de EDTA,300mM NaCl pH 7,8; 4ª eluição (D) - 20 mM NaPB, 5mM β-mercaptoetanol, 1mM de EDTA, 500mM NaCl pH 7,8. A atividade ureásica concentrou-se na fração oriunda da 2ª eluição (B).

Cromatografia de exclusão molecular:

A fração rica em urease, oriunda da 2ª eluição, na cromatografia de troca iônica foi submetida à cromatografia de gel filtração em coluna Superose 6, equilibrada com tampão de extração, em FPLC-System, obtendo-se o perfil cromatográfico apresentado na Figura.3R.

Fluxograma de purificação da urease de *Helicobacter pylori*

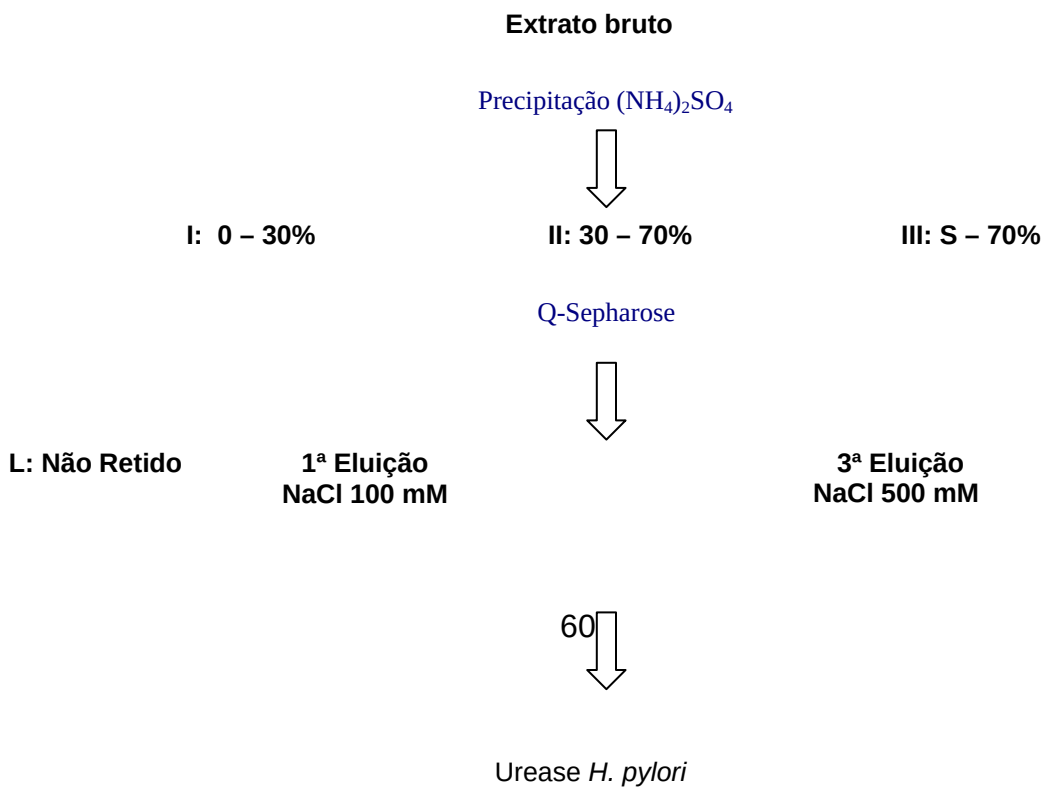

FIGURA.2R: Fluxograma de purificação da urease de *H. pylori*.

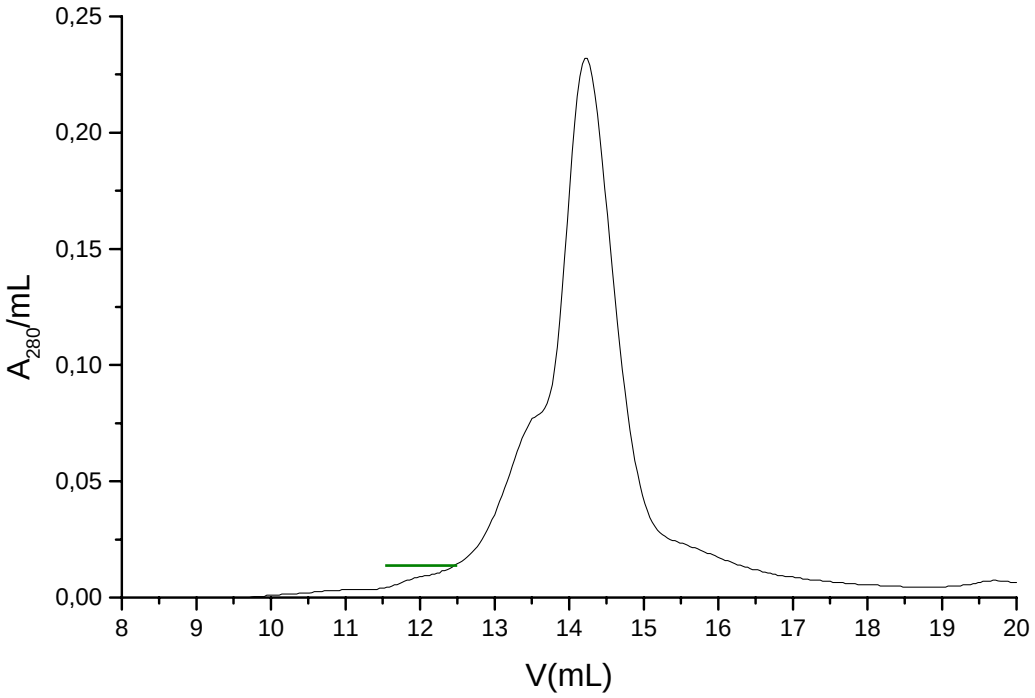

FIGURA.3R: Perfil cromatográfico da urease de ***H.pylori*** em cromatografia de exclusão molecular em coluna Superose-6. ——— Representa a fração com atividade ureolítica, o volume de eluição, conforme curva de calibração da coluna é de 540 KDa, equivalente ao hexâmero da urease de *H.pylori*.

SDS-PAGE e Western-Blot:

A fração com atividade ureolítica oriunda da cromatgrafia de exclusão molecular foi submetida a eletroforese em gel de ploacrilamida 10% obendo-se duas bandas majoritárias com massas relativas em torno de 60 e 30 kDA, o equivalente a as subunidades da urease de *H. pylori*. O ensaio western-blot utilizando anticorpos anticanatoxina revelaram imunorreatividade cruzada entre estes anticorpos e a subunidade UreB de 60 kDa da urease de *H. pylori*. (dados não mostrados).

Agregação plaquetária:

A figura 4R apresenta uma curva dose resposta para a agregação plaquetária induzida por HPU em plasma rico em plaquetas de coelho com DE_{50} de aproximadamente 150 μg/mL, com "*shape change*" e agregação semelhante a agregação induzida por colágeno e canatoxina.

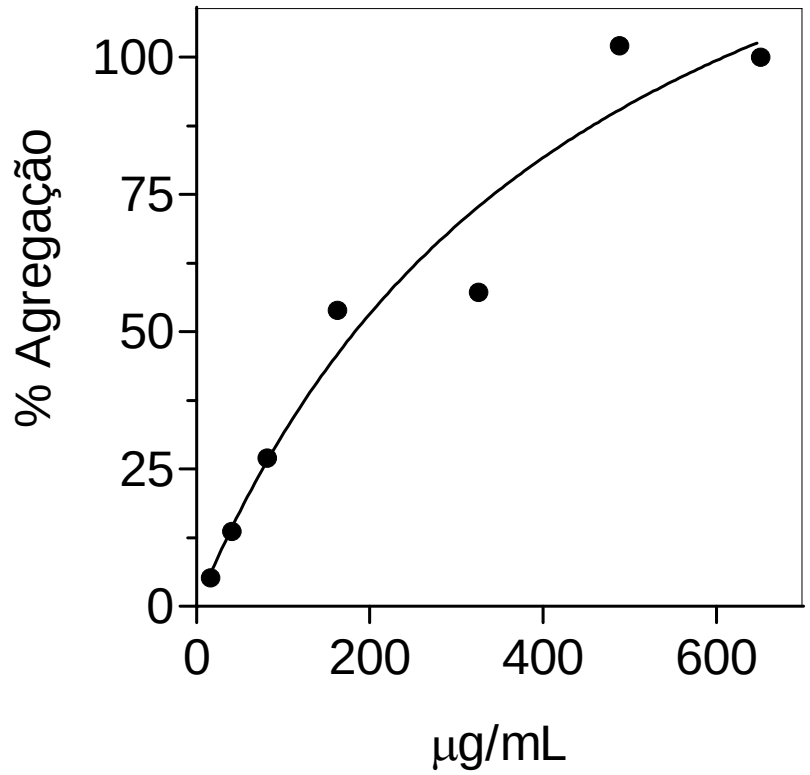

Figura 4R: Curva dose resposta de agregação plaquetária induzida por urease recombinante de *H. pylori.* Experimento representativo da média de quadriplicatas.

O envolvimento dos metabólitos do ácido araquidônico na agregação plaquetária induzida por HPU foi investigado em plaquetas pré-tratadas com dexametasona (um inibidor de fosfolipase A_2), indometacina (um inibidor de cocloxigenase) e esculetina (um inibidor de 12-lipoxigenase). A Figura 5R mostra que a dexametasona bloqueia a agregação plaquetária induzida por HPU, o que indica o envolvimento de ácido araquidônico livre para a ocorrência do fenômeno.

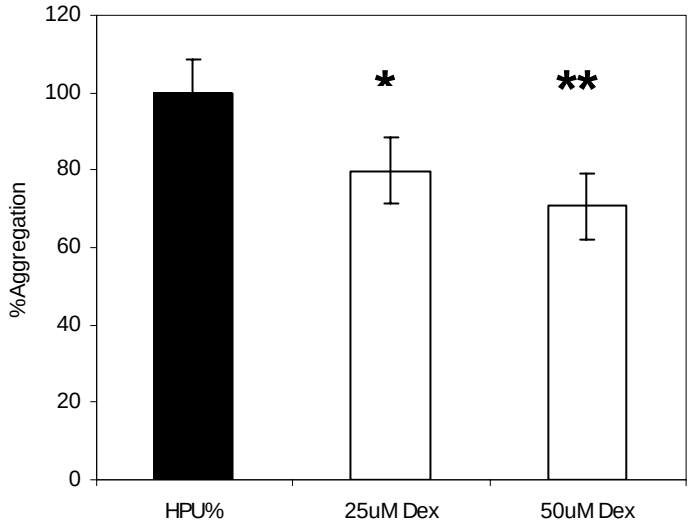

Figura 5R: Efeito da dexametasona na agregação plaquetária induzida por 650 ug/mL de HPU. Ensaios realizados em quadriplicata. * P<0.05; ** p < 0,01.

Em plaquetas tratadas com indometacina, a agregação plaquetária induzida por HPU foi aumentada em até 3 vezes, o que exclui a participação de tromboxana A_2, um produto indireto da ação da cicloxigenase na agregação como apresentado na Figura 6R.

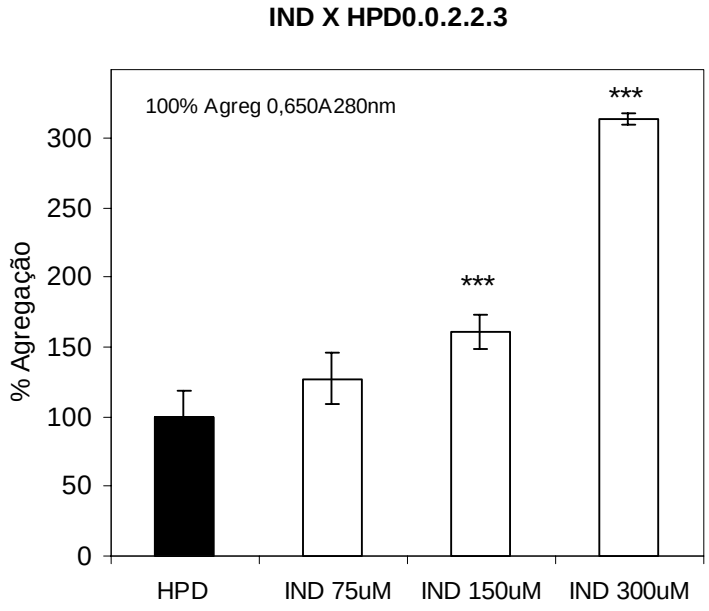

Figura 6R: Efeito da indometacina na agregação plaquetária induzida por 650 ug/mL de HPU. Ensaios realizados em quadriplicata. *** p < 0,001.

Os experimentos com esculetina apresentaram uma redução na resposta de agregação induzida por HPU, indicando a participação de produtos de lipoxigenase, na mediação da resposta de agregação induzida pela amostra, Figura 7R.

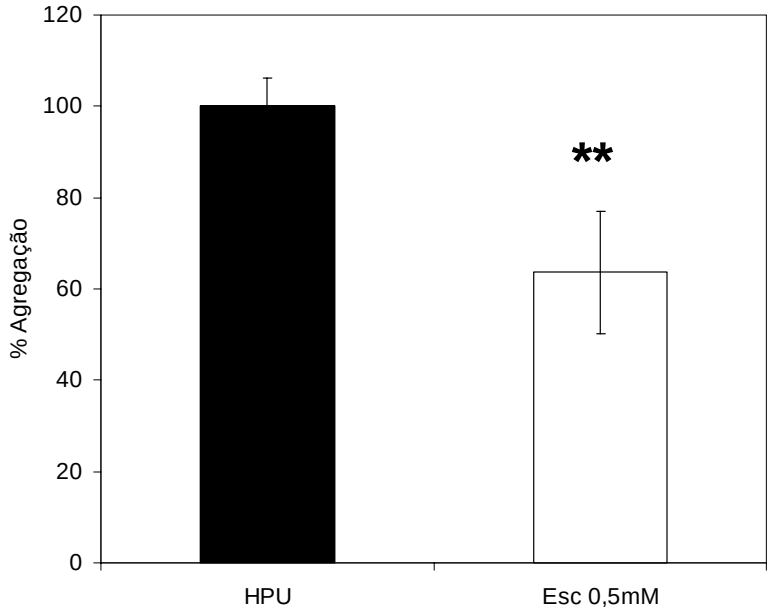

Figura 7R: Efeito da esculetina na agregação plaquetária induzida por 650 ug/mL de HPU. Ensaios realizados em quadriplicata. ** p < 0,01.

Para investigar a o potencial pró-inflamatório de HPU foi realizado o ensaio usando o modelo de edema de pata em camundongos, com a injeção sub-plantar da amostra (0,5; 5 e 45 μg). Como resultado obteve-se uma resposta com a formação de edema dependente da dose administrada, com pico entre 4 e 6 horas e redução completa em 24 horas (Figura 8R), o que sugere que esta molécula tem potencial pró-inflamatório Este resultado é semelhante ao edema induzido por canatoxina em ratos.

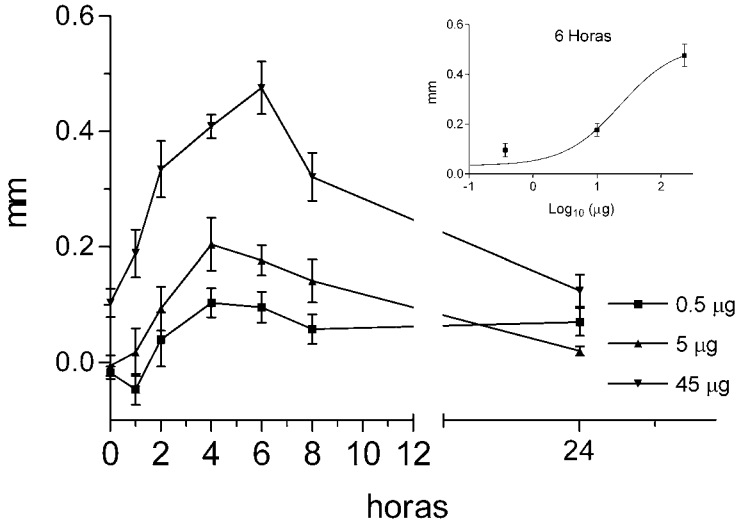

Figura 8R: Edema de pata em camundongo induzido por diferentes doses de HPU recombinante, acompanhado ao longo de 48 horas. Cada grupo foi composto por um n=9 indivíduos. *In set* curva dose resposta do experimento.

10. Discussão Geral:

As ureases são uma família de moléculas amplamente conservadas, presentes em uma vasta gama de organismos, como plantas fungos e bactérias. Essas proteínas apesar de suas estruturas quaternárias diversas mantiveram uma alta conservação evolutiva tendo em vista a homologia de 50 – 60% dessas moléculas em espécies muito distintas. Essa conservação ao longo da evolução sugere fortemente que estas moléculas exerçam um papel fisiológico relevante para os organismos que as produzem.

Em plantas, a presença de ureases tem sido abordada por alguns autores como importantes no metabolismo da arginina e disponibilidade de nitrogênio para o indivíduo, tendo em vista sua atividade enzimática intensa; contudo permanece a pergunta: *Se a uréia não é um metabólito majoritário em plantas será que sua função fisiológica nestes organismos está somente relacionada à hidrólise desse substrato?*

Contudo vale a pena ressaltar a atividade inseticida apresentada por essa molécula e por outras ureases vegetais. Pode residir ai uma importante função biológica para essas moléculas em vegetais *"defesa"*.

Mesmo tendo sido a primeira enzima a ser cristalizada, por Sumner em 1926 comprovando assim o caráter protéico das enzimas; até hoje permanecem muitas dúvidas sobre estrutura e função das ureases. Em *C. ensiformis*, por exemplo, a estrutura tridimensional da urease até a data não foi resolvida; em microrganismos temos apenas três estruturas 3D à disposição nos bancos de dados. Todavia, não é indício que as ureases foram pouco estudadas ao longo dos anos: há uma vasta literatura a respeito de suas funções para os organismos que as produzem, e suas relações com outros organismos.

Pelo fato de não haver ainda um consenso de suas funções fisiológicas, é pertinente uma abordagem que extrapole os limites de sua atividade como enzima, ampliando assim a chance de obter informações que permitam entender a presença e a manutenção de tal molécula em organismos distintos.

Com este enfoque chegamos aos trabalhos desenvolvidos pelo nosso grupo de pesquisas. A canatoxina, uma isoforma de urease presente em sementes de *C. ensiformis* exerce um leque de atividades biológicas independentes de sua atividade ureolítica, já descritas em várias publicações e apresentadas neste trabalho como arcabouço teórico para as hipóteses construídas para as ureases bacterianas estudadas.

Em se tratando de ureases bacterianas. *Que outras atividades estariam envolvidas na fisiologia desses microrganismos independentes de sua atividade enzimática?* Em *Bacillus pasteurii*, por exemplo, uma bactéria de solo não patogênica, as interações entre este microrganismo e outros que convivem no mesmo ambiente são importantes, em particular as interações com as plantas. E em bactérias patogênicas como *H pylori: Que funções essa proteína pode exercer na virulência e patogenicidade desse organismo?*

Este trabalho apresenta resultados que enfatizam a grande conservação não apenas estrutural, mas também funcional das ureases em diferentes organismos. Os achados aqui apresentados para as duas ureases bacterianas estudadas corroboram os resultados conhecidos para canatoxina. A atividade secretagoga mediada por eicosanóides derivados do ácido araquidônico via rota das lipoxigenases, e o potencial pró-inflamatório dessas enzimas podem estar intimamente ligados à fisiopatologia desses microrganismos.

Em *B. pasteurii*, o potencial secretagogo dessa proteína pode estar envolvido com a indução de exudados de raízes vegetais e nas interações intertróficas com outros organismos de solo.

Em *H. pylori* os dados apresentados representam uma nova abordagem na temática urease como fator de virulência desse organismo. Alem da evidente importância da atividade ureolítica em criar um microclima adequado para a colonização gástrica pelo *H. pylori*, a ativação de plaquetas, o potencial secretagogo e pró-inflamatório dessa molécula e o envolvimento de eicosanóides via lipoxigenases,

podem contribuir de maneira significativa como fatores de patogenicidade de *H.pylori,* como também abrir uma nova luz aos estudos de doenças cardiovasculares associados a esta bactéria.

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