UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA
MESTRADO EM BIOQUÍMICA E FISIOLOGIA

PURIFICAÇÃO, CARACTERIZAÇÃO DE UMA LECTINA E COMPOSTOS FENÓLICOS DA ENTRECASCA DE *Sebastiania jacobinensis* (MUILL. ARG.): EFEITO DE RADIAÇÃO GAMA SOBRE ESTRUTURA-ATIVIDADE PROTÉICA E PROTEÇÃO DE FLAVONÓIDES ISOLADOS

Antônio Fernando de Melo Vaz

Orientadora: Profa. Dra. Maria Tereza dos Santos Correia

Recife, 2009

ANTONIO FERNANDO DE MELO VAZ

"Purificação, caracterização de uma lectina e compostos fenólicos da entrecasca de *Sebastiania jacobinensis* (Muill. Arg.): efeito de radiação gama sobre estrutura-atividade protéica e proteção de flavonóides isolados"

Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

Aprovado por:

Profa. Dra. Maria Tereza dos Santos Correia, Presidente

Profa. Dra. Vera Lúcia de Menezes Lima – Examinador interno

Profa. Dra. Luana Cassandra Breitenbach B. Coelho – Examinador Interno

Profa. Dra. Maria Luiza Vilela Oliva – Examinador Externo

Vaz, Antônio Fernando de Melo

Purificação, caracterização de uma lectina e compostos fenólicos da entrecasca de Sebastiania jacobinensis (Muill. Arg.): efeito de radiação gama sobre estrutura-atividade protéica e proteção de flavonóides isolados / Antônio Fernando de Melo Vaz. – Recife: O Autor, 2009.

120 folhas: fig., tab.

Dissertação (mestrado) – Universidade Federal de Pernambuco.

CCB. Departamento de Bioquímica e Fisiologia, 2009.

Inclui bibliografia.

1. Lectina 2. Glicoproteína 3. Flavonóides isolados I Título.

"Uma rosa vermelha absorve todas as cores, menos a vermelha;
Vermelha, portanto, é a única cor que ela não é.
Essa Lei, Razão, Tempo e Espaço,
toda Limitação, cega-nos à Verdade
Tudo o que sabemos sobre o Homem, Natureza, Deus,
é apenas aquilo que eles não são;
é aquilo que rejeitam como repugnante."

(Aleister Crowley, Liber CCCXXXIII, Cap. 40)

SUMÁRIO

AGRADECIMENTOS

A Deus, por me dar forças para não me dobrar perante as dificuldades e por iluminar meus dias com saúde e serenidade.

Agradeço aos meus pais, Maria José e José Vaz, pelo incentivo, ensinamento, caráter e disciplina passados.

À minha irmã, Débora, por me suportar nas horas de estresse e por estar sempre ao meu lado.

À minha namorada, Marthyna, pelo apoio cedido nas horas difíceis e pelo carinho.

À professora e orientadora Maria Tereza dos Santos Correia, pela orientação científica, amizade, confiança e liberdade de busca nas colaborações durante a realização deste trabalho.

À professora Ana Mendonça de Melo e ao professor Levy dos Santos Guedes pelo apoio e ensinamentos.

À professora Maria Luiza pelo inestimável colaboração nos resultados, na eleboração dos artigos e ensinamentos.

À professora Rosemeire de Lucca pela colaboração nos resultados, na compreensão dos estudos de fluorescência.

Ao professor Haroldo Xavier pelo exemplo de pesquisador, pelo apoio e ensinamentos.

Ao professor Fernando Hallwass e a professora Daniela Navarro pelo apoio e ensinamentos nos ensaios de RMN.

Ao professor Toninho (UNIFESP) pelo apoio e ensinamentos de LC/MS.

À técnica e pesquisadora do Laboratório de Bioquímica da UNIFESP Lucimeire, pela paciência e ensinamentos que foram imprescindíveis na conclusão dos artigos.

À técnica e pesquisadora do Laboratório de Glicoproteínas Maria Barbosa Reis da Silva, pela amizade e ensinamentos que foram imprescindíveis na realização deste trabalho.

Ao técnico e pesquisador da Central Analítica Ricardo Oliveira da Silva, pelo apoio que foram imprescindíveis na finalização da caracterização estrutural dos flavonóides.

Aos meus colegas de mestrado, Guto, Filipe, Leo, Leucio, Ricardo, Romero, Douglas, Dewison, Thiago, Adenor, Caio, Gysele, Lidianne; e aos meus amigos, Seu joão, Michele, Regininha, Neila e Roberto pela amizade, compreensão e paciência, obrigado pessoal, gosto, admiro e torço por vocês.

Aos funcionários do INFAR da Universidade Federal de São Paulo, Magdonia, Patrícia, Magda, Isaurinha, obrigada pela ajuda e apoio.

A todos os que fazem parte do Laboratório de Bioquímica do INFAR da Universidade Federal de São Paulo, pela convivência, ajuda e apoio; em especial, Roberto, Nathalia, Cris, Flávio e Sheila.

Aos funcionários do Departamento de Bioquímica da Universidade Federal de Pernambuco, Flávio, Jorge, Albérico, Neide, Miron, Djalma, Helena, obrigada pela ajuda e amizade.

A todos os que fazem parte do Laboratório de Glicoproteínas do Departamento de Bioquímica da Universidade Federal de Pernambuco, pela convivência, ajuda, amizade e apoio; em especial, Rodrigo, Thiago, Lu, Cris, Déa, Chynthia, Vanessa, Francis, e Nathali.

À Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) pela concessão de bolsa e financiamento do projeto.

A todos que colaboraram de forma direta e indireta na concretização deste trabalho, com certeza, sem a ajuda de todos vocês não seria possível realizá-lo.

Meus sinceros agradecimentos.

Resumo

Uma lectina da entrecasca de *Sebastiania jacobinensis* (SejaBL) foi isolada por combinação de precipitação acetônica, fracionamento salino, cromatografia de troca iônica e gel filtração. A massa molecular de SejaBL foi determinada por SDS-PAGE e gel filtração, sendo de 52,0 kDa e 50,0 kDa, respectivamente. A lectina é uma glicoproteína com teor de carboidrato neutro de 6,94%, composta por duas subunidades com mesma massa molecular de 24 kDa. A lectina purificada hemaglutinou hemácias de coelho e humana. Glicoproteínas inibiram a hemaglutinação. SejaBL apresentou atividade máxima na faixa de pH 3,0-7,5, estabilidade térmica até 70 °C e é potencial inibidor da atividade da tripsina. Espectroscopia de fluorescência indicou a existência de superfícies hidrofóbicas. Lectina inibiu o crescimento micelial de *Fusarium moniliforme* e *Fusarium oxysporum* com um IC_{50} de 123±0,5µg e 303±0,9µg, respectivamente. Organismos não alvos, larvas *Artemia salina* e embriões de *Biomphalaria glabrata,* não foram afetados, indicando baixa toxicidade ambiental. Como modelo experimental, a lectina purificada de *S. jacobinensis* (SejaBL) foi utilizada como prova de que mudanças no centro hidrofóbico, causados pela irradiação gama, alteram estruturalmente proteínas. Para elucidar o efeito dos radicais livres nas propriedades moleculares de SejaBL atividade, estrutura terciária (intrínseca e bis-ANS fluorescência), massa molecular e perfil cromatográfico foram examinados após γ-irradiação em várias doses (0,020 a 35kGy). Atividade hemaglutinante específica (AHE), na lectina irradiada, mostrou perda significativa ($p<0,05$) acima de 12,5kGy e aumento significativo ($p<0,05$) em 0,1, 0,8 e 1kGy. Espectroscopia de fluorescência indicou supressão da emissão de fluorescência quando excitado a 280 e 295nm, com elevada ligação do bis-ANS, após altas doses. SDS-PAGE e HPLC-RP mostraram fragmentação polipeptídica. Mudança significativa na superfície hidrofóbica indicou captação de hidrogênio radical em aminoácidos, causando desorganização estrutural da lectina. Flavonóides são produtos secundários de plantas com capacidade para capturar radicais livres. A lectina da entrecasca de *S. jacobinensis* (SejaBL), proteína capaz de ligar a carboidrato, tem sua superfície hidrofóbica modificada por radicais livres produzidos pela água radiólise. No presente estudo, o efeito protetor sobre a proteína pela estabilidade antioxidante de flavonóides da entrecasca de *S. jacobinensis* (SejaBF), após irradiação gama, foi investigada. Dois flavonóides foram isolados com rápida recuperação da capacidade para capturar radical mensurado pelo 2,2-difenil-1-picrilhidracilo radical (DPPH) a 0,5 mg/mL, após alta dose de irradiação. Os danos causados por irradiação na AH foi minimizado em 55% na presença dos flavonóides. Os resultados mostraram radioestabilidade da capacidade antioxidante de SejaBF, protegendo a lectina de radicais livres após irradiação.

Palavras-chave: lectina; bis-ANS; fluorescência; atividade antifúngica; toxicidade ambiental; radiação gama; flavonóides; capacidade de capturar radical DPPH

Abstract

A lectin from *Sebastiania jacobinensis* bark (SejaBL) was isolated using a combination of acetone precipitation, fractionation by ammonium sulphate, ion exchange and gel filtration chromatographies. The molecular mass of SejaBL was determined as approximately 52.0kDa by SDS-PAGE and 50.0kDa by gel filtration. The lectin is a glycoprotein with a neutral carbohydrate content of 6.94%, composed of two subunits with the same molecular mass of 24kDa. The purified lectin hemagglutinated rabbit and human erythrocytes. Glycoproteins inhibited agglutination of rabbit erythrocytes. SejaBL showed maximum activity over the pH range 3.0-7.5, heat stability up to 70°C and a potential trypsin inhibitory activity. Fluorescence spectroscopy indicated the existence of hydrophobic surface. Lectin inhibited the mycelial growth of *Fusarium moniliforme* and *Fusarium oxysporum* with an IC_{50} value of 123±0.5µg and 303±0.9µg, respectively. Non-target organisms, *Artemia salina* Leach and embryo of *Biomphalaria glabrata,* were not affected, indicating low environmental toxicity. As an experimental model, the lectin purified from S. *jacobinensis* bark (SejaBL) was used as probe that changes on the hydrophobic center, by gamma radiation, structurally alter proteins. To elucidate the effect of free radicals in the molecular properties of SejaBL activity, tertiary structure (intrinsic and bis-ANS fluorescence), molecular weights and reversed phase chromatography were examined after γ-irradiation at various doses (0.020 to 35kGy). Specific Hemagglutinating Activity (SHA) in irradiated lectin showed significant loss (p<0.05), above 12.5kGy, and a significant increase (p<0.05) in 0.1, 0.8 and 1kGy. Fluorescence spectroscopy indicated suppression of emission intensity when excited at 280 and 295 nm, with high binding of bis-ANS, after high doses. SDS-PAGE and RP-HPLC showed polypeptide fragmentation. Significant change in the hydrophobic surface indicated hydrogen abstraction in amino acids, causing structural disorganization from the lectin. Flavonoids are ubiquitous plant secondary products with radical scavenger ability. The *S. jacobinensis* Bark Lectin (SejaBL), protein capable of binding to carbohydrates, has their hydrophobic surface modified by free radicals produced by water radiolysis. The present study shows the protective effect on protein and the antioxidant stability of *S. jacobinensis* bark flavonoids (SejaBF), after gamma irradiation. Two flavonoids isolated were present with fast scavenger ability measured by 2.2-diphenyl-1-picrylhydrazyl radical (DPPH) to 0.5mg/mL, after a high dose gamma radiation. The damage caused by irradiation on protein activity was reduced by 55% in the presence of the flavonoids. The results showed the radiostability of the antioxidant capacity of SejaBF, to protect the lectin from free radicals after irradiation.

Keywords: lectin; bis-ANS; fluorescence; antifungal activity; environmental toxicity, gamma radiation; flavonoids; DPPH radical scavenger capacity

LISTA DE ABREVIATURAS

AH: Atividade hemaglutinante

AHE: Atividade hemaglutinante específica

ALL: Lectina de musgo *Armillaria luteo-virens*

BSA: Albumina sérica bovina

Bis-ANS: 4,4'-Bis (1-anilinonaphthalene 8-sulfonate)

DTT: Dithiothreitol

Gal: Galactose

GlcNAc: N-acetilglicosamina

GalNAc: N-acetilgalactosamina

HA: Hemagglutinating activity

JCA: Lectina de *Artocarpus integrifólia* (Jacalina)

RIP: Proteína inativadora de ribossomos - tipo 2

HPLC-RP: Cromatografia líquida de fase reversa

SDS: Sulfato sódico de dodecila

SDS -PAGE: Eletroforese em gel de poliacrilamida contendo sulfato sódico de dodecila

SejaBL: Lectina da entrecasca de *Sebastiania jacobinensis*

SejaBF: Flavonóides da entrecasca de *Sebastiania jacobinensis*

SHA: Specific HA

TGL: Lectina da *tulipa gesneriana L*

LISTA DE FIGURAS

Dissertação

Artigo a ser submetido (primeiro)

Artigo a ser submetido (segundo)

Artigo a ser submetido (terceiro)

LISTA DE TABELAS

Dissertação

Artigo a ser submetido (primeiro)

Artigo a ser submetido (terceiro)

1. INTRODUÇÃO

A biodiversidade do Brasil abriga hoje entre 15% e 20% das espécies vegetais, animais e microrganismos do mundo. Apesar do crescente interesse internacional pela megabiodiversidade brasileira, sabemos que ela, por si só, não é garantia de crescimento econômico, tampouco de desenvolvimento sustentável. Mas espera-se que o País, além de importante exportador de matéria-prima, se converta no protagonista de uma nova economia mundial, baseado no uso sustentado da biodiversidade e dos seus recursos derivados, agregando valor para os diferentes setores produtivos (Ramalho, 1990).

Diferentes produtos utilizados pela sociedade possuem suas fontes na biodiversidade. São alimentos, fibras, itens farmacêuticos e químicos, óleos naturais e essenciais, entre outros. E sabemos que a geração de produtos de alto valor agregado a partir da biodiversidade está diretamente relacionada ao uso intensivo do conhecimento e do alto nível tecnológico. Este fato indica que a utilização de biotecnologia constitui uma opção viável e uma ferramenta fundamental para o uso sustentável e agregação de valor. O uso da biotecnologia implica na coleta de alguns poucos organismos ou parte deles, como fonte de matéria-prima para a prospecção e produção de novas biomoléculas, sendo assim uma estratégia adicional e efetiva de conservação da diversidade genética do meio ambiente (Wilson, 1988).

O controle de informações estratégicas, bem como das "tecno-ciências" que permitem agregar valor a essas informações – ao agregarem valor aos novos produtos e processos a partir daí gerados –, passa então a ocupar um dos centros de disputa e de conflito no jogo de forças políticas e econômicas internacionais. Tal controle pode ser exercido tanto com o domínio do acesso aos recursos da biodiversidade, quanto por intermédio de instrumentos de proteção de direitos à propriedade intelectual, seja sobre as modernas biotecnologias, seja sobre os conhecimentos tradicionais de populações locais (Sarita, 1998).

2. REVISÃO DA LITERATURA

2.1 Lectinas

No final do século XIX, começaram a acumular evidências sobre a existência na natureza de proteínas com habilidade para aglutinar eritrócitos. Tais proteínas foram chamadas fitoaglutininas, porque eram originalmente encontradas em extratos de plantas. A primeira citação a respeito se deu em 1888, quando Peter Hermann Stillmark estudando a toxicidade de extratos de *Ricinus communis* (mamona) observou a capacidade de aglutinar eritrócitos devido à presença de uma proteína extraída, a ricina. Pouco tempo depois, James B. Sumner isolou, da semente de *Canavalia ensiformis*, uma proteína cristalina denominada Concanavalina A (Sharon & Lis, 2004). No entanto, o estudo sobre essas proteínas só começou a ganhar foco em 1960, abrindo vasta área de aplicação (Gabor et al., 2004).

O termo lectina, originado do latim *legere* que significa escolhido, refere-se à habilidade dessas proteínas para reconhecerem e se ligarem reversivelmente a carboidratos ou glicoconjugados (Sharon & Lis, 1972). As lectinas diferem entre si quanto à seqüência de aminoácidos, requerimentos de metais, peso molecular e estrutura tridimensional e, ao contrário dos anticorpos, não são oriundas de resposta imune.

Lectinas estão amplamente distribuídas no meio ambiente, sendo encontradas em seres unicelulares (Sulagna et al., 2004; Lee et al., 2008) e animais pluricelulares (Dunphy et al., 2002; Haijie et al., 2006). Nos vegetais, os isolamentos são freqüentes, principalmente em sementes e entrecascas (Branco et al., 2004; Qiaojuan et al., 2005; Feng et al., 2006; Lan et al., 2008).

2.1.1 Peculiaridades

As lectinas apresentam grande diversidade estrutural, e o aspecto comum entre elas é a presença de, ao menos, um sítio específico de ligação a carboidrato, denominado "domínio de reconhecimento de carboidrato". Em sua plenitude, liga-se a carboidratos ou glicoconjugados em solução ou que estejam conectadas ao envoltório celular (William & Drickamer, 1996). As lectinas podem ser monoméricas como a arcelina de *Phaseolus vulgaris* L (Lorís, 1998) e, quando apresentam duas subunidades polipeptídicas, são

denominadas diméricas, com cadeias semelhantes ou diferentes entre si, ganhando, respectivamente, a denominação de lectinas homodiméricas ou heterodiméricas. *Erythrina speciosa* Andr. é uma lectina homodimérica com 27,6kDa por subunidade (Konozy et al., 2003). A lectina presente em *Ricinus communis* L é heterodimérica, com uma subunidade de 32kDa e outra de 34kDa (Frigerio & Roberts, 1998). Ocorrem outros tipos estruturais, como as triméricas, pentaméricas, hexaméricas ou poliméricas, como a lectina de *Artocarpus heterophyllus*, também denominada jacalina, com 65kDa (Kabir, 1998).

A presença de lectina na amostra pode ser facilmente detectada a partir de ensaios de hemaglutinação, as quais interagem com carboidratos da superfície celular, através de seus sítios de ligação, formando diversas ligações reversíveis entre células opostas (Santos et al., 2005). Para assegurar que o agente aglutinante é uma lectina, são necessários ensaios subseqüentes de inibição da atividade hemaglutinante (AH), utilizando solução de distintos carboidratos (Kawagishi et al., 2001; Jayati et al., 2005).

Em relação às cadeias polipeptídicas, as lectinas são caracteristicamente ricas em aminoácidos ácidos e hidroxilados, associadas por interações hidrofóbicas, pontes de hidrogênio e, em alguns casos, pontes dissulfetos (Kennedy et al., 1995). As especificidades e afinidades dos sítios são alcançadas principalmente por pontes de hidrogênio, com o auxílio de forças de van der Waals e interações hidrofóbicas com resíduos de aminoácidos aromáticos que estão próximos às porções hidrofóbicas de monossacarídeos (Sharon & Lis, 2002), contribuindo para a estabilidade dos complexos formados.

A maioria das lectinas apresenta um complexo glicídico, ligado covalentemente à sua estrutura, perfazendo, em geral, 4-10% da massa total da molécula. A porção glicídica da lectina liga-se apenas à asparagina, serina ou treonina. Na asparagina pode ocorrer a ligação com GlcNAc em animais e vegetais (Helenius & Aebi, 2004).

Muitas lectinas requerem íons metálicos bivalentes em sítios específicos, que estimulam o reconhecimento do carboidrato pelo qual têm afinidade ao estabilizar a ligação por fixar as posições dos aminoácidos que interagem com o carboidrato. Ficou demonstrado, por exemplo, que a lectina de *Erythrina speciosa* Andr. necessita de Mg^{++} e Ca^{++} para capturar o carboidrato para o qual tem afinidade (Konozy et al., 2003).

Em 51 lectinas investigadas, há uma região conservada rica em aminoácidos hidrofóbicos, que forma uma cavidade na estrutura nativa. Esse sítio hidrofóbico possibilita interação entre as lectinas e grande número de compostos químicos distintos dos carboidratos como, por exemplo, a interação de compostos fenólicos, ligantes naturais dessa superfície hidrofóbica (Barre et al., 2002).

2.1.2 Classificação

2.1.2.1 Subdivisões das lectinas de acordo com a ocorrência e especificidade

De acordo com Sharon (Sharon & Lis, 2004), lectinas de plantas e animais demonstram características estruturais, composição da estrutura primária e nível de glicosilação distintos. Entretanto, ambas podem ser reunidas e classificadas com base na especificidade para carboidratos, estrutura molecular e ocorrência (Tabela 1).

Tabela 1 - Famílias de lectinas de plantas e animais

Superfamília	*Família*	*Especificidade*	*Lectina*	*Referência*
Lectina de vegetais	Lectina de leguminosas	Glicanos diversos	*Cratylia mollis* (Cramoll 1)	Correia e Coelho, 1995
	Lectina de floema curcubitaceae	(GlcNAc)n	*Urtica dioica*	Van Damme et al., 1998
	Jacalina relacionadas	Glicanos diversos	*Artocarpus integrifolia* (jacalina)	Van Damme et al., 1998
	Ligadoras de quitina (heveína, RIP tipo-2 e monocotiledônias)	$(GlcNAc)_n$ e Manose	Hevcína do látex de *Hevea brasiliensis* e RCA.	Van Damme et al., 1998
	Amarantáccas	GalNAc	ACA	Van Damme et al., 1998
Lectinas tipo C	Selectinas	Sialyl-Lewis^x	P-Selectina	Moore et al., 1992
	Colectinas	Ligante de Colageno	-------	Kawasaki et al., 1978
	Lectinas endocíticas	Asialoglicoproteína	-------	Sharon and Lis, 2004; Drickamer, 1988
	Pentraxinas			
Lectinas tipo P	Receptor manose-6P	Manose-6P	-------	Hoflack e Kornfeld, 1985
Lectinas tipo S	Galectina	β-galactosideo	Galectin-1	Barondes et al., 1994
Lectinas tipo N	-------	Glicanos complexos	IL-2	Sherblom et al., 1989
Ig	Siglectinas*	Glicanos complexos	Sialoadesina (siglec-4)	Crocker et al., 1994

GalNAc, N-acetilgalactosamina; GlcNAc, N-acetilglicosamina; ^{X} NeuAcα(2-3)Galβ(1-4)[Fucα(1-3)]GlcNAc;
* Esta família de lectina Ig conjugadas ligantes de ácido siálico, um membro da Superfamília Ig.

2.1.2.2 - Subdivisões de lectinas vegetais de acordo com a estrutura

Segundo Van Damme et al (1996), as lectinas vegetais podem ser classificadas de acordo com a característica estrutural em:

- merolectinas - são monovalentes e, por isso, não precipitam glicoconjugados ou aglutinam células. São representadas pela família da heveína;

- hololectinas - possuem dois ou mais sítios de ligação a carboidratos que são idênticos ou muito semelhantes; esse grupo compreende as lectinas que são capazes de aglutinar células e/ou precipitar glicoconjugados;

- quimerolectinas - são proteínas com um ou mais sítios de ligação a carboidratos e um domínio não relacionado. Esse domínio diferente pode ter atividade enzimática bem definida, ou outra atividade biológica, mas age independentemente do domínio ligante a carboidrato. Assim representadas pelos grupos RIPs tipo 2 e quitinase de plantas tipo I;

- superlectinas - são proteínas com dois domínios de ligação a carboidratos. Esse pode ser considerado um grupo especial de quimerolectinas, consistindo de dois domínios estrutural e funcionalmente diferentes de ligação a carboidratos, como a lectina TGL da *tulipa gesneriana L.,* que são formados por dois domínios de ligação a carboidrato, que reconhecem D-manose e L-fucose, respectivamente.

2.1.3 Lectinas de plantas (famílias)

2.1.3.1 Lectinas de leguminosa

Esta família é encontrada exclusivamente em leguminosa (Sharon & Lis, 2002). Todas são homólogas no nível molecular, mas mostram variação na especificidade ao carboidrato. Essa ampla especificidade certamente contribui para o sucesso dessa família de lectinas como ferramenta biotecnológica. Algumas lectinas estão envolvidas na simbiose entre legumes e a fixação de nitrogênio da bactéria do gênero Rhizobium (Van Damme et al., 1998).

2.1.3.2 Lectinas do floema de Cucurbitaceae

Muitas espécies de Cucurbitaceae contêm alta concentração de lectinas que ligam a oligômeros de GlcNAc. As lectinas do floema de Cucurbitaceae são proteínas diméricas, compostas por duas subunidades idênticas de 24kDa. Todas as lectinas conhecidas dos floemas de Cucurbitaceae mostram grau de similaridade seqüencial, mas não têm qualquer similaridade com outras famílias de lectinas de plantas (Peumans & Van Damme, 1998).

2.1.3.3 Lectinas relacionadas à jacalina

São lectinas específicas à galactose de sementes da fruta jaca (*Artocarpus integrifolia*). Lectinas similares têm sido encontrados em poucas espécies de Artocarpus e em *Maclura pomifera* (Young et al., 1989).

2.1.3.4 Lectinas ligadoras de quitina

A proteína ligante de quitina (polímero de N-acetil-glicosamina) contém o chamado domínio(s) heveína(s). O termo "heveína" refere-se à pequena proteína de 43 resíduos de aminoácidos, encontrados no látex de árvores de seringueira, *Hevea brasiliensis* (Waljuno et al., 1975). As quitinases classe I, por exemplo, são quimerolectinas compostas por um único domínio heveína, ligado a um domínio catalítico com atividade quitinásica (Sharon & Lis, 2004). Proteínas inativadoras de ribossomo (RIP), tipo 2, são quimerolectinas compostas de um polinucleotídeo constituído por um domínio adenosina glicosidase (cadeia A), ligado ao domínio ligante a carboidrato (cadeia B). A maioria das RIP, tipo 2, liga-se preferencialmente a Gal ou GalNAc. A RIP, tipo 2, tem sido encontrada em plantas das famílias Euphorbiaceae, Fabaceae, entre outras (Peumans & Van Damme, 1998).

2.1.3.5 Lectinas de Amaranthaceae

Clonagem molecular e análise de difração de raios X revelaram que amarantina, uma lectina de semente de *Amaranthus caudatus* não se assemelha a qualquer outra lectina

de planta com relação à seqüência de aminoácidos e à estrutura tridimensional (Transue et al., 1997). Com base nesses dados, a amarantina é agora considerada como um protótipo da família de lectina amarantina, uma lectina ligante específica de N-acetilgalactosamina (Van Damme et al., 1998).

2.1.4 Papel fisiológico e biotecnológico

Hipóteses sobre o papel fisiológico das lectinas foram formuladas devido à ocorrência dessa classe de proteínas em microrganismos, animais e plantas, mas admite-se que a função primária das lectinas seja a de reconhecimento de carboidratos. Lectinas vegetais desempenham importantes papéis tais como proteínas de reserva (Peumans & Van Damme, 1995), no mecanismo de defesa contra micro-organismos (Sá et al., 2008a), insetos, animais herbívoros (Sá et al., 2008b) e como mediadoras da simbiose planta-micro-organismo (Limpens & Bisseling, 2003).

Diferentemente, lectinas animais estão envolvidas com controle de qualidade, no processo de N-glicolização e na rota de secreção de glicoproteínas (Helenius & Aebi, 2004), como também nos processos específicos no citoplasma e/ou compartimentos nucleares (Levebvre, 2001). Lectinas de animais atuam como receptores e desempenham um papel no tráfego extracelular de glicoproteínas, adesão de celular, interações celulares e imunidade inata (Leffler, 2004; Hsu & Liu, 2004). Quanto aos micro-organismos, as lectinas desempenham papel de simbiose, sendo responsáveis pela associação específica entre bactérias fixadoras de nitrogênio e leguminosas (Bohlool & Schmidt, 1974), assim como entre bactérias da flora intestinal e mucosa (Sulagna et al., 2004).

É grande a versatilidade biotecnológica de lectinas. A lectina de *Arum maculatum* que previne eficientemente a infecção de culturas de fibroblastos por *Chlamydia pneumoniae* tem perspectivas de aplicação clínica (Mladenov et al., 2002); a propriedade de ligação a células tumorais torna as lectinas potenciais ferramentas bioadesivas na entrega de drogas (Gabor et al., 2004; Ponchel & Irache, 1998). A lectina de *Cratylia mollis* imobilizada em Sepharose CL 4B, foi capaz de isolar a enzima lecitina colesterol aciltransferase (Lima et al., 1997). Certas lectinas são capazes de atuar sobre linfócitos, fazendo com que essas células passem de um estado típico para um estado de crescimento e

proliferação. A lectina de semente de *Cratylia mollis* (Maciel et al., 2004) é exemplo de lectina com atividade mitogênica.

Em ensaios histoquímicos e imunoistoquímicos, as lectinas têm sido utilizadas para detecção de resíduos glicosilados em superfícies teciduais (Paessens et al., 2007). Essas proteínas têm sido aplicadas na diferenciação de tecidos normais, displásicos e neoplásicos, fornecendo contribuições importantes para o diagnóstico e distinção entre câncer de próstata e a hiperplasia benigna (Basu et al., 2003). Atuações larvicida e inseticida têm sido citadas. A lectina do Cerne de *Myracrodruon urundeuva* mostrou-se excelente na atuação sobre estágios larvais de *Aedes aegypti* (Sá et al., 2008c) e a lectina da folha de *Bauhinia monandra* delimitou ampla ação inseticida (Macedo et al., 2006).

2.2 Radioatividade

Foi descoberta casualmente por Antoine Henri Becquerel em 1896, quando o mesmo realizava experiências com um mineral do urânio. Becquerel observou que esse mineral [K_2U0_2 $(S0_4)_2$. $2H_20$] era capaz de impressionar filmes fotográficos. Esses raios foram inicialmente denominados "raios do urânio". Em 1897, Marie Sklodowska Curie concluiu que era a quantidade de urânio que determinava a intensidade da radiação e, conseqüentemente, as mesmas (radiações) dependeriam do elemento (urânio). Em seguida, foi evidenciado que esse fenômeno se tratava de fenômeno atômico, ou, na realidade, fenômeno nuclear (Knool, 1979).

2.2.1 Radiação

O termo radiação vem do latim *radiare*, que indica um fenômeno básico em que a energia se propaga através do espaço, ainda que interceptada pela matéria. O termo irradiação vem do latim *in* e *radiare*, empregado para indicar o tratamento da matéria pela energia radiante. As radiações são produzidas por processos de ajustes que ocorrem no núcleo ou nas camadas eletrônicas, ou pela interação de outras radiações ou partículas com o núcleo ou com o átomo (Tauhata et al., 2003). Distinguem-se, assim, dois tipos de radiação: as chamadas corpusculares, feitas por intermédio de elétrons (raios beta), núcleos

de hélio (raios alfa), núcleos de hidrogênio (prótons; p. ou H1) ou nêutrons (n ou n1); e as eletromagnéticas, constituídas pelos raios de comprimento de onda muito curto, os raios X e os raios gama (Pitorri, 1993). Enquanto os raios X são produzidos por geradores especiais, os raios gama emanam espontaneamente de substâncias radioativas como rádio, tório e cobalto (Magalhães & Tauhata, 1994).

2.2.2 Medidas de intensidade das radiações ionizantes

A intensidade das radiações ionizantes é medida na base do número de íons que elas produzem num certo volume padrão. A unidade padrão é denominada Roentgem (R). No organismo humano, isso corresponde a cerca de duas ionizações por mícron cúbico (Knool, 1979). Em linhas gerais, as várias unidades de medida das radiações podem ser assim definidas:

- Roentgen (1 R), aplicado em tecidos moles, causa a absorção, por partes desses, de quantidade de energia igual a 93 ergs por grama. Um erg representa a energia desenvolvida pela massa de 1 grama, movendo-se com a velocidade de 1 centímetro por segundo;

- Rad, unidade empregada para raios alfa e beta, em tecidos moles, 1 R pode ser aceito como equivalente a 1 Rad;

- Gray (Gy), unidade empregada a qualquer radiação ionizante, quando submetida a qualquer material, representa a dose absorvida por um material no ponto P. 1 Gy equivale a 100 Rad.

2.2.3 Dose e taxa de dose

Dose é a quantidade total de radiação emitida; taxa de dose é a maneira como essa dose é distribuída ao longo do tempo. Assim, uma mesma dose (digamos 100Gy), podendo ser aplicada durante diferentes períodos de tempo (1 minuto, 10 minutos, 100 minutos etc.) se apresentará com diferentes taxas (de 100Gy/min, de 10Gy/min, de 1Gy/min etc.), apesar de, em todos os casos, a dose final de radiação emitida seja a mesma (100Gy). Quanto maior a taxa e a dose, maior o dano (MS/S.N.V.S, 1992).

2.2.4 Efeitos biológicos gerais da radiação

É fácil compreender que a radiação ionizante pode agir sobre a célula e modificar a concentração de íons hidrogênio e o potencial de oxirredução de diferentes biomoléculas, alterando, profundamente, a funcionalidade molecular. As alterações químicas, decorrentes da radiação ionizante, são realizadas por dois mecanismos: a) por ação direta - na qual a molécula sofre alterações, tornando-se ionizada ou excitada pela passagem de elétrons ou ondas eletromagnéticas; b) por ação indireta - na qual a molécula não absorve energia, mas recebe, por transferência, energia de outra molécula (Kempner, 2001).

Os mecanismos pelos quais a radiação age sobre a célula são vários e, em parte, desconhecidos, no entanto os efeitos agudos possivelmente se devem à ionização da água. A água se decompõe e, como conseqüência, verifica-se a formação de compostos químicos ativos, que influenciam várias classes de biomoléculas (Tauhata et al., 2003). Os compostos que se formam são instáveis, de curta duração, mas seus efeitos podem ser profundos. Entre eles são citados ânions superóxidos, radicais peróxidos, radicais hidroxil e peroxinitritos. Essa oxidação afeta facilmente grupos protéicos, em especial o grupo sulfidril e sítios hidrofóbicos (Riley, 1994).

Em sistemas biológicos, os efeitos das radiações ionizantes diferem qualitativamente segundo a dose da radiação. Pequenas doses agem por ação indireta e produzem principalmente oxidações. Grandes doses agem por ação direta e indireta ao mesmo tempo. Vários fatores influenciam os efeitos radiobiológicos. Sendo especialmente importante: a) a intensidade da radiação (efeito reversível e irreversível); b) a temperatura de exposição c) a maneira da exposição, isto é, se simples, continuada ou fracionada; d) o tempo de exposição; e) presença de oxigênio (MS/S.N.V.S, 1992).

2.2.5 Aplicação biotecnológica da radiação gama

A aplicação de radiação gama para inativação de patógenos é evento crucial para iniciar a comercialização de produtos derivados de plasma humano em grande escala, sem o potencial risco de contaminação para receptores. Um método de inativação viral, usando irradiação gama em alta dose (45kGy), provoca a inviabilidade de todo vírus presente no

plasma e essa efetividade antiviral está diretamente relacionada ao dano no genoma viral (Miekka *et* al., 1998; Reid, 1998; Hiemstra et al., 1991). No entanto, a aplicação de radiação gama pode afetar a integridade estrutural e a atividade protéica. A irradiação foi inicialmente descartada como ferramenta para a inativação de patógenos no plasma e derivados, por causa da pobre recuperação protéica. Pesquisas, envolvendo a proteção de proteínas contra os efeitos das radiações ionizantes, têm sido desenvolvidas com uso de antioxidantes (Zbikowska et al., 2006) e estratégicos modelos de exposição (Terryn et al., 2007).

2.3 Flavonóides

Sydler, em 1915, propôs o termo "farmacognosia" para o estudo de drogas e medicamentos de origem natural, a maioria deles de origem vegetal. Essa área da Farmacologia, como ciência, passou a ser subdividida em áreas mais específicas como, por exemplo, a farmacoquímica (fitoquímica) que estuda a origem, a síntese e as formas de extração dos compostos (Di Stasi, 1995).

Os compostos produzidos pelos vegetais são divididos em dois grupos: os metabólitos primários, tais como carboidratos, aminoácidos e lipídeos e os metabólitos secundários: compostos elaborados a partir dos metabólitos primários, tais como compostos fenólicos, terpenóides e alcalóides. São esses compostos os responsáveis pelos efeitos medicinais, ou tóxicos, das plantas, com grande importância ecológica, uma vez que podem atuar na atração de polinizadores ou representar defesa química contra estresse ambiental (Baladrin et al., 1985).

Os flavonóides seguem uma estrutura básica de C_6-C_3-C_6 (dois aneis fenil - A e B - ligados através de um anel pirano – C) (**Figura 1**). São bastante comuns na natureza, atuando na atração de polinizadores, e como copigmentos das antocianinas (Bruneton, 1995). Apresentam atividades anti-inflamatórias, antimicrobianas e antitumorais (Barnes et al., 2001), exercendo esses benefícios pelo seu poder antioxidante (Wollgast & Anklan, 2000).

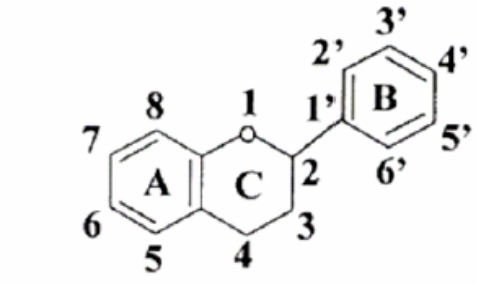


Figura 1 – Estrutura básica dos flavonóides (Bruneton, 1995)

Esses compostos têm origem biossintética mista, com parte da molécula proveniente da rota do ácido chiquímico e parte do ácido mevalônico, com estrutura química baseada no esqueleto 2-fenilcromano (Bruneton, 1995). Podem ser encontrados na forma livre ou conjugados a açúcares e, dependendo da substituição e do nível de oxidação no C_4, os flavonóides podem ser divididos em 14 classes (**Figura 2a**), tais como auronas, antocianidinas, flavonas, flavonóis, isoflavonóides e proantocianidinas (Harborne, 1984).

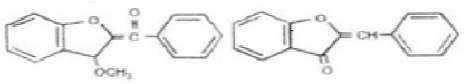
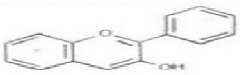
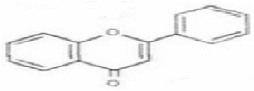
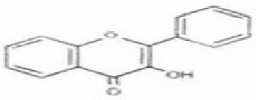
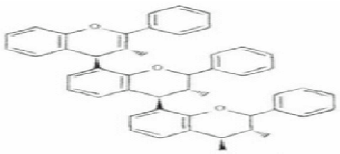
Flavonóides	Estrutura Básica
Auronas	
Antocianidinas	
Flavonas	
Flavonois	
Isoflavonóides	
Proantocianidinas	

Figura 2a - Diferentes classes de flavonóides segundo Bravo (1998)

Na biossíntese dos flavonóides, a primeira substância formada é uma chalcona, que está em equilíbrio enzimático com sua flavanona correspondente (**Figura 2b**). O par chalcona/flavanona é precursor das subclasses dos flavonóides. Diretamente deles, derivam as flavonas, os isoflavonóides e os dihidroflavonóides. Esses últimos são intermediários da síntese de catequinas, antocianidinas e flavonóis. As auronas derivam diretamente das chalconas (Winkel-Shirley, 2002). Os últimos passos na biossíntese dos flavonóides estão associados aos processos de O- e C- glicosilações, acilação dos açúcares e alquilações (Saleh, 1979). Estudos têm demonstrado a influência de alguns fatores ambientais na regulação gênica de flavonóides (Dooner, 1983).

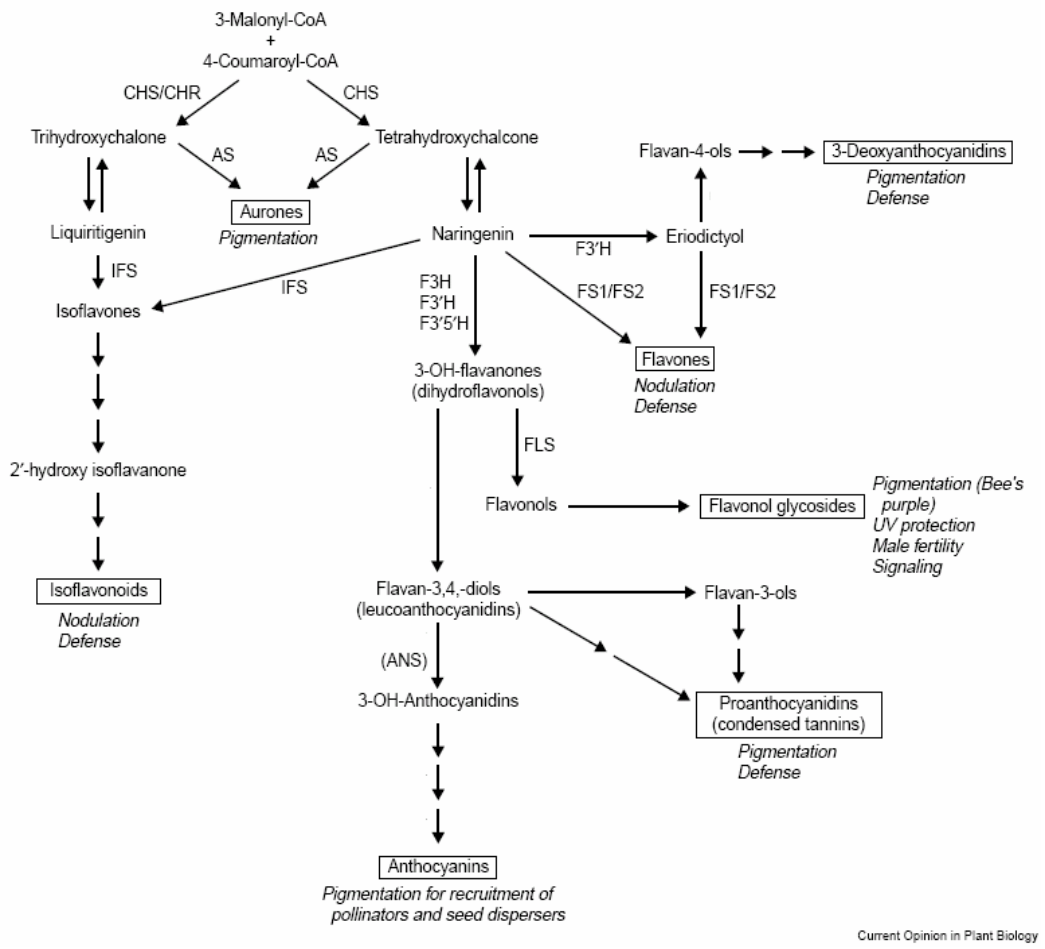


Figura 2b Esquema da via biosintética de flavonóides (adaptado de Winkel-Shirley, 2002)

2.4 *Sebastiania jacobinensis*

Segundo descrito em Flora Brasiliensis (2001), *Sebastiania jacobinensis* é uma árvore nativa, inerme, latescente, possui copa pequena, irregular, semipersistente, de coloração verde-escura (**Figura 3a**). O tronco é geralmente curto, com casca externa rugosa e cor marrom-acinzentada (**Figura 3b**). Suas folhas são simples, alternas, glabras, de consistência coriácea, medem de 2 a 7cm de comprimento por 1 a 3cm de largura (**Figura 3c**). Apresentam geralmente ápice agudo, base cuneada, margem levemente serreada a dentado-crenada e pecíolo de até 7mm de comprimento. As flores são pequenas, amarelas, dispostas em espigas terminais de 3 a 6cm de comprimento (**Figura 3d**). Os frutos são cápsulas globosas com seis ângulos, de coloração parda, medem de 8 a 10mm de diâmetro e possuem 3 a 6 sementes em seu interior (**Figura 3e**). No levantamento bibliográfico não foi encontrado nenhum relato de investigação científica sobre essa espécie, deixando, desde já, aqui registrado, apenas aplicações fitoterápicas contra infecções e processos de hipersensibilidade.

Figura 3 ***Sebastiania jacobinensis***

3. OBJETIVOS

3.1 Objetivo Geral

Purificar, caracterizar uma lectina e compostos fenólicos da entrecasca de *sebastiania jacobinensis* (Muill. Arg.) e avaliar o efeito da radiação gama sobre a estrutura-atividade protéica sobre a proteção dos flavonóides isolados.

3.2 Objetivos Específicos

- Isolar a lectina, com homogeneizados da entrecasca de *S. Jacobinensis,* da melhor fração obtida pelo fracionamento utilizando cromatografia de troca iônica e filtração em gel;

- Caracterizar a lectina parcialmente purificada quanto à especificidade a carboidratos ou glicoproteínas, estabilidade térmica, especificidade para eritrócitos, estabilidade frente a variações de pH, influência de íons bivalentes e resistência a proteinases;

- Avaliar através de SDS-PAGE e Gel filtração a massa molecular, o padrão de pureza e o caráter glicoprotéico da lectina;

- Avaliar a atividade antifúngica e a seletividade ambiental "in vitro" da lectina purificada;

- Avaliar o efeito de radiação gama de alta taxa de dose na atividade e estrutura da lectina purificada;

- Isolar e caracterizar polifénois da entrecasca de *S. jacobinensis* baseado na sua atividade antioxidante, no tempo de retenção cromatográfico e massa molecular;

- Avaliar a proteção da lectina purificada e a radioestabilidade contra radiação ionizante do antioxidante (i.e. flavonóides isolados).

4. REFERENCIAS BIBLIOGRAFICAS

BALADRIN, M.F. KLOKE, J.A. WURTELE, E.S. BOLINGE, W.H. Natural plant chemicals. Source of industrial and medicinal materials. **Science**, v. 228, p. 1054-1060, 1985.

BARNES, J. ANDERSON, L.A. PHILLIPSON, J.D. St. John's worth (*Hypericum perforatum*): A review of chemistry, pharmacology and chemical properties. **Journal of pharmacy and pharmacology**, v. 53, p. 583-600, 2001.

BARONDES, S.H. COOPER, D.N.W. GITT, M.A. LEFFLER, H. Structure and function of a large family of animal lectins. **J. Biol. Chem.,** v. 269, p. 20807-20810, 1994.

BARRE, A. HERVÉ, C. LESCURE, B. ROUGÉ, P. Lectin receptor kinases in plants. **Plant science**, v. 21, p. 379-399, 2002.

BASU, P.S. MAHJAB, R. BATABYL, S.K. Lectin and serum-PSA interaction as a screening test for prostate cancer. **Clinical Biochemistry**, v. 36, p. 373-376, 2003.

BOHLOOL, B.B. SCHMIDT, E.L. Lectins: a possible basis for specificity in the Rhizobium-legume root module symbiosis. **Science**, v. 188, p. 269-271, 1974.

BRANCO, A.T. BERNABÉ, R.B. FERREIRA, B.S. OLIVEIRA, M.V.V. GARCIA, A.B. SOUZA-FILHO, G.A. Expression and purification recombinant lectin from rice (*Oryza sativa* L.). **Protein expression & Purification**, v. 33, p. 34-38, 2004.

BRAVO, L. Polyphenols: chemistry, dietary, sources, metabolism, and nutrition significance. **Nutr. Ver**, v.56, p.317-333, 1998.

BRUNETON, J. Phenols and Phenolic acids. In Bruneton, J. Pharmacognosy, phytochemistry and medical plants. **Lavoisier Press**., p. 211-227, 1995.

CORREIA, M.T.S. COELHO, L.C.B.B. Purification of a glucose/manose specific Lectin, isoforma 1, from seeds of *Cratylia mollis* Mart. (Camaratu bean). **Applied Biochemistry and biotechnology**, v. 55, p 261-273, 1995.

CROCKER, P.R. MUCKLOW, S. BOUCKSON, V. MCWILLIAM, A. WILLIS, A.C. GORDON, S. MILON, G. KELM, S. BRADFIELD, P. Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. **EMBO J.**, v. 13, p. 4490–4503, 1994.

DI STASI, L.C. Plantas Medicinais: Arte e Ciência. **UNESP**., Brasil. p. 230 1995.

DOONER, H. Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. **Mol Gen Genet,** v. 189, p. 136-141, 1983.

DRICKAMER, K. Two distinct classes of carbohydrate-recognition domains in animal lectins, **J. Biol. Chem.,** v. 263, p. 9557–9560, 1988.

DUNPHY, J.L. BARCHAM, G.J. BISCHOF, R.J. YOUNG, A.R. NASH, A. MEEUSEN, E. N. T. Isolation and characterization of a novel eosinophil-specific galectin released into the lungs in response to allergen challenge. **The Journal of Biological Chemistry**, v. 277, p. 14916-14924, 2002.

FENG, K. LIU, Q.H. NG, T.B. LIU, H.Z. LI, J.Q. CHEN, G. SHENG, H.Y. XIE, Z.L. WANG, H.X. Isolation and characterization of a novel lectin from the mushroom *Armillaria luteo-virens*. **Biochemical and Biophysical Research Communications**, v. 345, p. 1573-1578, 2006.

FLORA BRASILIENSIS a obra, v. 11, Part 2, p. 589, 2001.

FRIGERIO, L. ROBERTS, L.M. The enemy within: ricin and plant cells. **Journal of experimental botany**, v. 49, p. 1473-1480, 1998.

GABOR, F. BOGNER, E. WEISSENBOECK, A. WIRTH, M. The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. **Advanced Drug Delivery Reviews,** v. 3, p. 459-480, 2004.

HAIJEI, Y. LUO, H. LI, F. LI, S. XU, X. Purification and characterisation of a calcium-independent lectin (PjLec) from the haemolymph of the shrimp *Penaeus japonicus*. **Fish & Shellfish Immunology**, v. 22, p. 88-97, 2006.

HARBORNE, J.B. Phytochemical methods. **A guide to modern techniques of plant analysis**, Londres. Chapman and Hall. p. 288, 1984.

HELENIUS, A. AEBI, M. Roles of N-linked glycans in the endoplasmic reticulum. **Annu. Rev. Biochem.,** v. 73, p. 1019-1049, 2004.

HIEMSTRA, H. TERSMETTE, M. VOS, A.H. OVER, J. VAN BERKEL, M.P. DE BREE, H. Inactivation of human immunodeficiency virus by gamma radiation and its effect on plasma and coagulation factors. **Transfusion,** v. 31, p. 32-39, 1991.

HOFLACK, B. AND KORNFELD, S. Lysosomal enzyme binding to mouse P388D1 macrophage membranes lacking the 215-kDa mannose 6-phosphate receptor: evidence for the existence of a second mannose 6-phosphate receptor. **Proc. Natl Acad. Sci,** USA. v. 82, p. 4428-4432, 1985.

HSU, D.K. LIU, F.T. Regulation of cellular homeostasis by galectins. **Glycoconj. J**., v. 19, p. 507-515, 2004.

JAYATI, B. ARUN, K.G. BISHNU, P.C. Purication and molecular characterization of a sialic acid specfic lectin from the phytopathogenic fungus *Macrophomina phaseolina*. **Carbohydrate Research**., v. 340, p. 1973-1982, 2005.

KABIR, S. Jacalin: a jackfruit (*Artocarpus heterophyllus*) seed-devived lectin of versatile applications in immunobiological research. **Journal of immunological methods**., v. 212, p. 193-211, 1998.

KAWAGISHI, H. TAKAGI, J. TAIRA, T. MURATA, T. USUI, T. Purification and characterization of a lectin from the mushroom *Mycoleptodonoides aitchisonii*. **Phytochemistry,** v. 56, p. 53-58, 2001.

KAWASAKI, T. ETOH, R. YAMASHINA, I. Isolation and characterization of a mannan-binding protein from rabbit liver. **Biochem Biophys. Res. Commun**., v. 81, p. 1018–1024, 1978.

KEMPNER, E.S. Effects of high-energy electrons and gamma rays directly on protein molecules. **J. Pharm. Sci.**, v. 90, p.1637-1646, 2001.

KENNEDY, J.F. PAIVA, P.M.G CORREIA, M.T.S. CAVALCANTI, M.S.M. COELHO, L.C.B.B. Lectins, versatile proteins of recognition: a review. **Carbohydrate Polymers**, v. 26, p. 219-230, 1995.

KNOOL, G.F. **Radiation detection and measurement**, J. wiley and Sons, New York. 1979.

KONOZY, E.H.E. BERNARDES, E.S. ROSA, C. FACA, V. GREENE, L.J. WARD, R.J. Isolation, purification, and physicochemical characterization of a D-galactose-binding lectin from seeds of *Erythrina speciosa*. **Arch. Biochem. Biophys.,** v. 410, p. 222–229, 2003.

LAM, S.K. HAN, Q.F. NG, T.B. Isolation and characterization of a lectin with potentially exploitable activities from caper (*Capparis spinosa*) seeds. **Biosci Rep.,** 2008.

LEE, S. NALINI, M. KIM, Y. A viral lectin encoded in *Cotesia plutellae* bracovirus and its immunosuppressive effect on host hemocytes. **Comp. Biochem. Physiol. Mol. Integr. Physiol.,** v. 149, p. 351-61, 2008.

LEFEBVRE, T. Identification of N-acetyl-D-glucosaminespecific lectins from rat liver cytosolic and nuclear compartments as heat-shock proteins. **Biochem. J.**, v. 360, p. 179-188, 2001.

LEFFLER, H. Introduction to galectins. **Glycoconj. J.,** v. 19, p. 433–440, 2004.

LIMA, V.L.M. CORREIA, M.T.S. CECHINEL, Y.M.N. SAMPAIO, C.A. OWEN, J.S. COELHO, L.C.B.B. Immobilized *Cratylia mollis* lectin as potential matrix to isolate plasma glycoproteins Incluinding lecithin cholesterol acyltransferase. **Carbohydrate Polymers.**, v. 31, p, 27-32, 1997.

LIMPENS, E. BISSELING, T. Signaling in symbiosis. **Current Opinion in Plant Biology,** v. 6, p. 343-350, 2003.

LORIS, R. Principles of structures of animal and plant lectins. **Biochimica et biophysica acta,** v. 1572, p. 198-208, 1998.

MACEDO, M.L.R. FREIRE, M.G.M. DA SILVA, M.B.R. COELHO, L.C.B.B. Insecticidal action of *Bauhinia monandra* leaf lectin (BmoLL) against *Anagasta kuehniella* (Lepidoptera: Pyralidae), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). **Comparative Biochemistry and Physiology,** v. 146, p. 486-498, 2007.

MACIEL, E.V.M. ARAÚJO-FILHO V.S. NAKAZAWA, M. GOMES, Y.M. COELHO, L.C.B.B. CORREIA, M.T.S. Mitogenic activity of *Cratylia mollis* lectin on human lymphocytes. **Biologicals,** v. 32, p. 57-60, 2004.

MAGALHÃES, E.S. TAUHATA, L. **Energia nuclear e suas aplicações**, 1994.

MIEKKA, S. I. BUSBY, T. F. REID, B. POLLOCK, R. RALSTON, A. DROHAN, W. N. New methods for inactivation of lipid-enveloped and non-enveloped viruses. **Haemophilia,** v. 4, p. 402- 408; 1998.

MLADENOV, I.V. IANA H. HARALAMBIEVA, I.D.I. IVAN, G.M. Characterisation of 20-kDa lectin-spermagglutinin from *Arum maculatum* that prevents *Chlamydia pneumoniae* infection of L-929 fibroblast cells. **FEMS Immunology and Medical Microbiology,** v. 32, p. 249-254, 2002.

MOORE, K.L. STULTS, N.L. DIAZ, S. SMITH, D.F. CUMMINGS, R.D. VARKI, A. AND MCEVER, R.P. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. **J. Cell Biol.,** v. 118, p. 445-456, 2004.

MS/S.N.V.S – M.S./CNEN/IRD; **Apostila do curso de introdução ao controle de radiações ionizantes**, 1992.

PAESSENS, L.C. GARCIA-VALLEJO, J.J. FERNANDES, R.J. VAN KOOYK, Y. The glycosylation of thymic microenvironments. A microscopic study using plant lectins. **Immunology Letters,** v. 110, p. 65-73, 2007.

PEUMANS, W.J. VAN DAMME, E.J.M. Lectins as plant defense proteins. **Plant physiol,** v. 109, p. 347-352, 1995.

PEUMANS, W.J. VAN DAMME, E.J.M. Plant lectins versateli proteins with important perspectives in biotechnology. **Biotechnology and Geneteic Engineering Rewiews,** v. 15, p. 199-228, 1998.

PITORRI, C.R. **Apostila do curso de físicas de radiações e qualidade de imagem radiográficas**, 1993.

PONCHEL, G. IRACHE, J. M. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. **Advanced Drug Delivery Reviews**, v. 34, p. 191-219, 1998.

QIAOJUAN, Y. ZHENGQIANG, J. SHAOQING, Y. WEI, D. LUJIA, H. A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity. **Archives of Biochemistry and Biophysics**, v. 442, p. 72-81, 2005.

RAMALHO, Y.M.M. **Biotecnologia: cenário internacional e perspectivas para o Brasil**, Rio de Janeiro, BNDES/DEEST/AP, 1990.

REID, B. D. The Sterways process: a new approach to inactivating viruses using gamma radiation. **Biologicals,** v. 26, p. 125-129, 1998.

RILEY, P.A. Free radicals in biology: oxidative stress and the effects of ionizing radiation, **Int. J. Radiat. Biol.,** v. 65, p. 27-33, 1994.

SÁ, R. A. ; GOMES, F. S. ; NAPOLEÃO, T. H. ; SANTOS, N. D. L. ; MELO, C.M.L. ; GUSMÃO, N.B. ; COELHO, L.C.B.B. ; PAIVA, P.M.G. ; BIEBER, L.W. . Antibacterial and antifungal activities of *Myracrodruon urundeuva* heartwood. **Wood Science and Technology**, p. 1-11, 2008a.

SÁ, R. A. ; NAPOLEÃO, T. H. ; SANTOS, N. D. L. ; GOMES, F. S. ; ALBUQUERQUE, A.C. ; XAVIER, H.S. ; COELHO, L.C.B.B. ; BIEBER, L.W. ; PAIVA, P.M.G. . Induction of mortality on *Nasutitermes corniger* (Isoptera, Termitidae) by *Myracrodruon urundeuva* heartwood lectin. **International Biodeterioration and Biodegradation**, v. 62, p. 460-464, 2008b.

SÁ, R. A. ; SANTOS, N. D. L. ; SILVA, C.S.B. ; NAPOLEÃO, T. H. ; GOMES, F. S. ; CAVADA, B. S. ; COELHO, L.C.B.B. ; NAVARRO, D.M.A.F. ; BIEBER, L.W. ; PAIVA, P.M.G. . Larvicidal activity of lectins from *Myracrodruon urundeuva* on *Aedes aegypti*. **Comparative Biochemistry and Physiology. C, Toxicology & Pharmacology**, 2008c.

SALEH, N.A.M. The biosynthesis of flavonoid glycosides and their importance in chemosystemactic. **Biochemical Systematics and Ecology**, v. 7, p. 37-45, 1979.

SANTOS, A.F.S. ARGOLO, A.C.C. COELHO, L.C.B.B. PAIVA, P.M.G. Detection of water soluble lectin and antioxidant component from *Moringa oleifera* seeds. **Water Research,** v.39, p. 975-980, 2005.

SARITA A. **Da biodiversidade à biotecnologia: a nova fronteira da Informação**, Ci. Inf., Brasília, v. 27, n. 1, p. 7-10, jan./abr. 1998.

SHARON, N. LIS, H. History of lectins: from hemagglutinins to biological recognition molecules. **Glycobiology,** v. 14, p. 53-62, 2004.

SHARON, N. LIS, H. How proteins bind carbohydrates: lessons from legume lectins. **Journal of Agricultural and Food Chemistry,** v. 50, p. 6586-6591, 2002.

SHARON, N. LIS, H. Lectins: cell-agglutinating and sugarspecific proteins. **Science,** v. 177, p. 949–959.1972.

SHERBLOM, A.P. SATHYAMOORTHY, N. DECKER, J.M. MUCHMORE, A.V. IL-2, a lectin with specificity for high mannose glycopeptides, **J. Immunol.**, v. 143, p. 939–944, 1989

SULAGNA, B. SUJATA, N.K. GANGULY, G. MAJUMDAR, S. A biologically active lectin of enteroaggregative *Escherichia coli*. **Biochimie,** v. 86, p. 657-666, 2004.

TAUHATA, L., SALATI, I.P.A. PRINZIO, R.DI. PRINZIO, M.A.R.R. **Radioproteção e dosimetria: Fundamentos** – 5ª Revisão, Rio de Janeiro IRD/CNEN, 2003.

TERRYN, H. MAQUILLE, A. LEVIN, C.H. TILQUIN, B. Irradiation of human insulin in aqueous solution, first step towards radiosterilization. **International Journal of Pharmaceutics,** v.343, p. 4-11, 2007.

TRANSUE, T.R., SMITH, A.K., MO, H., GOLDSTEIN, I.J. AND SAPER, M.A. Structure of benzyl T-antigen disaccharide bound to *Amaranthus caudatus* lectin. **Nature Structural Biology,** v. 10, p. 779-783, 1997.

VAN DAMME, E.J.M. BARRE, A. ROUGÉ, P. LEUVEN, F.V. PEUMANS, W.J. The NeuAc (a-2,6)-GalNAc-binding lectin from elderberry (*Sambucus nigra*) bark type-2 ribosome-inativatining protein with an usual specificity and structure. **European Journal of Biochemistry,** v. 235, p. 128-137, 1996.

VAN DAMME, E.J.M. PEUMANS, W.J. BARRE, A.J. ROUGÉ, P.J. Plant Lectins: A Composite of Several Distinct Families of Structurally and Evolutionary Related Proteins with Diverse Biological Roles. **Critical Reviews in Plant Sciences,** v. 17, p. 575-692, 1998.

WALJUNO, K., SCHOLMA, R.A., BEINTEMA, J., MARIONO, A., HAHN, A.N. Amino acid sequence of hevein. **Proc. Int. Rubber Conf. Kuala Lumpur,** v. 2, p. 518-531, 1995.

WANG, R. KONG, C. KOLATKAR, P. CHUNG, M.C.M. A novel dimer of a C-type lectin-like heterodimer from the venom of *Calloselasma roddostoma* (Malayan pit viper). **FEBS Letters,** v. 508, p. 447-453, 2001.

WILLIAM, I.W. DRICKAMER, K. Structural basis of recognition lectin-carbohydrate. **Anna Rev. Biochen,** v. 65, p. 441-473, 1996.

WILSON, E.O. Biodiversity. Washigton: **National Academy Press,** 1988.

WINKEL-SHIRLEY, B. Biosynthesis of flavonoids and effects of stress, **Curr. Opin. Plant Biol.,** v. 5, p. 218-223, 2002.

WOLLGAST, J. ANKLAN, E. Polyphenols in chocolate: is there a contribution to human health? **Food Research International,** v. 33, p.449-459, 2000.

YOUNG, M.N., JOHNSTON, R.A.Z., SZBO,A.G. WATSON, D.C. Homology of the D-galactose-specific lectin from *Artocarpus integrifolia* and *Maclura pomifera* and the role of an unusual small polypeptide subunit. **Biochemica et Biophysica Acta,** v. 270, p. 596-603, 1989.

ZBIKOWSKA, H.M. NOWAK, P. WACHOWICZ, B. Protein modification caused by a high dose of gamma irradiation in cryo-sterilized plasma: Protective effects of ascorbate. **Free Radical Biology & Medicine,** v. 40, p. 536-542, 2006.

Capítulo 1

Artigo a ser submetido à publicação

Biochemical and Biophysical Research Communications

Impacto: 2.749 (2007)

http://www.sciencedirect.com/science/journal/0006291X

Manuscript

A nontoxic lectin from *Sebastiania jacobinensis* with antifungal activity

Antônio F. M. Vaz [a,*], Ana M. M. A. Melo [b], Maria L. V. Oliva [c], Lucimeire A. Santana [c], Rosemeire A. S. Lucca [c], Luana C. B. B. Coelho [a], Maria T. S. Correia [a]

[a]Department of Biochemistry, Federal University of Pernambuco, Recife, Brazil

[b]Department of Biophysic and Radiobiology, Federal University of Pernambuco, Recife, Brazil

[c]Department of Biochemistry, Federal University of São Paulo, São Paulo, Brazil

[*]**Corresponding author**: Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE - CEP: 50670-901, Recife, PE, Brazil. (Maria Tereza dos Santos Correia)

Tel: 55 (81) 2126.8574; Fax: 55 (81) 21268576; E-mail address: melo_vaz@ig.com.br

Abstract

A lectin from *Sebastiania jacobinensis* bark (SejaBL) was isolated using a combination of precipitated acetone, ammonium sulphate fraction, ion exchange and gel filtration chromatographies. The molecular mass of SejaBL was 52.0 and 50.0 kDa by SDS-PAGE and gel filtration, respectively. The lectin was a glycoprotein with a neutral carbohydrate content of 6.94% composed of two identical subunits of 24 kDa. The purified lectin hemagglutinated both rabbit and human erythrocytes and this activity was not by monosaccharides. However, glycoproteins inhibited agglutination of rabbit erythrocytes. SejaBL showed maximum activity over the pH range 3.0-7.5, heat stability up to 70 °C and a potential trypsin inhibitor. Fluorescence spectroscopy indicated the existence of hydrophobic surface. Lectin inhibited the mycelial growth of *Fusarium moniliforme* and *Fusarium oxysporum* with an IC_{50} value of 123 ± 0.5 and 303 ± 0.9 µg, respectively. Nontarget organisms, *Artemia salina* Leach and embryo of *Biomphalaria glabrata* were not affected indicating low environmental toxicity. Alternative viewpoints are presented that can hopefully help in future efforts to develop safer and more effective microbial control agents.

***Keywords*:** Lectin; Bis-ANS; Fluorescence; Antifungal activity; Environmental toxicity

Introduction

Plant lectins are a heterogeneous group of proteins or glycoproteins that share the capacity to identify a specific carbohydrate. Their widespread distributions in the plant kingdom suggest a physiologically important function [1]. They have attracted great interest because of their various biological activities, as antiproliferative, antitumor, antifungal and antiviral properties [2]. Seeds, especially of leguminous species, are common sources of lectins, but they are also present in rice vegetative tissues, latex and bark of different species [3-6]. Intrinsic fluorescence and Bis-ANS have been used in folding, stability studies and as evidence of conformational change in proteins, by assessing hydrophobic regions [7,8].

The *Artemia salina* Leach is a salt-water crustacean used as food for fish. *A. Saline* is sensitive to the effect of active substances and its mortality is used to monitor toxicity because it is well sensitive to many chemical substances [9]. The *Biomphalaria glabrata* (Say, 1818) is the clam of the Planorbidae family with the widest distribution in Brazil. The antibiotic effect of drugs on the embryo development of *B. glabrata* and mortality of *A. Saline* have urged their use as a way to monitor the environmental impact and selectivity of microbiological control agents [10,11]. Antifungal proteins have been isolated from a large number of plants separated into many types comprising thaumatin-like proteins [12] chitinases and β-1.3-glucanases [13], thionins [14], plant defensins [15], ribosome-inactivating proteins [16], protease inhibitor-like proteins [17] and lectins [18].

Sebastiania jacobinensis Müll. Arg. (Euphorbiaceae family) is a common tree found in the tropical regions of Brazil. The bark of this plant is popularly used against infections and hypersensitivity processes. In view of the benefits that microbiological control agents with

low environmental toxicity provide to vegetable biotechnology, we describe the isolation and partial characterization of an antifungal lectin from the bark of *S. jacobinensis,* with ecotoxicological profile.

Material and methods

Purification lectin. S. jacobinensis bark was homogenized overnight at 4 °C in 10 mM Tris–HCl buffer (pH 8.5) with 0.2% (v/v) Triton X-100 detergent. The homogenate was centrifuged 5,000 g for 20 min (crude extract) followed by lyophilization. In brief, two volumes of cold acetone were added, mixed well and kept on ice for 10 min. The solution was centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was concentrated under vacuum until total evaporation of the acetone. The residual aqueous solution obtained was precipitated with 60% $(NH_4)_2SO_4$. The precipitate was dialyzed against the Tris–HCl buffer. This fraction was applied to a CM-Cellulose column (2 x 20 cm) previously equilibrated with the Tris–HCl buffer into 150 mM of NaCl. Following removal of the unadsorbed proteins, the column was eluted with 1 M acetic acid. The largest fractions of absorbance eluted with acetic acid were pooled, dialyzed and concentrated by ultrafiltration (Amicon ultra-15, Mr 10.000 cut-off) and chromatographed on a Sephadex G-100 column (2 x 70 cm) equilibrated with 150 mM NaCl. The column was eluted with the same solution at a flow rate of 0.5 mL min^{-1}. The primary unadsorbed fraction (SejaBL), after concentration by ultrafiltration in 10 mM phosphate buffer (pH 7.0), was applied to a Superdex 75 HR 10/30 column coupled to an ÄKTA purifier system (Amersham Pharmacia Biotech). The column was preequilibrated and eluted with 300 mM NaCl, at a flow rate of 0.5 mL min^{-1}, monitored by absorbance at 215 nm, collected in 1-ml fractions. The lectin was submitted

to reverse-phase chromatography on a C-18 column performed on a HPLC system (Shimadzu) and monitored at 215 nm, as described below for chain separations. The total protein content from the crude extract and the purified lectin were determined by the Lowry *et al.* method [19], using BSA standard curve.

Hemagglutination activity and specificity sugar. Hemagglutinating activity (HA) was evaluated as described by Correia and Coelho [20] defined as the lowest sample dilution showing hemagglutination. Specific HA (SHA) corresponded to the relation between HA and protein concentration. The carbohydrate binding specificity of the lectin was determined by HA inhibition using several sugars (D-glucose, N-acetil-D-glucosamine, D-arabinose, D-mannose, L-fucose, L-raphinose, D-galactose, L-threalose, D-xylose, D-sacarose, L-rhamnose, L-celobiose, D-lactose, methyl-α-D-mannopyranoside and methyl-α-D-glucopyranoside) and glycoproteins (bovine serum albumin, casein, tyroglobulin, ovoalbumin, fetuin and asialofetuin) as described by Coelho and Silva [21].

Effect of pH, temperature and metal ions. The effect of pH on SejaBL HA was evaluated by incubating the lectin (0.5 mg mL^{-1}) at different pH values for 1 h at room temperature in selected buffers (10 mM citrate phosphate buffer, pH 2 – 7 and 10 mM tris-hydrochloric acid buffer, pH 8 - 12). The heat stability was determined by incubation of lectin solution at different temperatures (30-90 °C for 30 min and 100 °C, 30 to 90 min) and remaining SHA was determined. The effects of Mg^{2+}, Zn^{2+} and Ca^{2+} were determined by incubation at same volume of any metal ion (5, 10 and 20 mM) in 150 mM NaCl and lectin. An aliquot (50 μL) of the mixture was distributed in microtitre plate wells and the HA was proceeded as described for carbohydrate inhibition assay.

Molecular weight determination. Chain analyses were performed after disulfid bridges split by reduction and alkylation. Previously lyophilized SejaBL samples were reduced by the Friedman reaction [22] with some modifications as follows: lectin (1.5 mg) was dissolved in 250 µL 50 mM Tris–HCl pH 8.6, 6 M urea, 10 mM EDTA, 179 mM DTT and incubated for 3 h at 37 °C, in the dark, before N_2 purging. The free sulphydryl groups were exposed to 100 µL iodoacetate and the reaction was continued for another 2 h in the same initial conditions. The iodoacetate derivative chains were desalted and separated on a reverse-phase C-18 column (Vydac-protein peptide ultrasphere) performed on a HPLC system (Shimadzu LC-10AD-kyto, Japan) and monitored at 215 nm. The column was equilibrated with solvent A (0.1% TFA in H_2O) and eluted using solvent B (90% acetonitrile: 10% H_2O: 0.1% TFA) non-linear gradient in A - B = 5% at t = 5 min; B = 70% at t = 27 min; B = 80% at t = 60 min and B = 100% at t = 69 min. SDS–PAGE was carried out according to Laemmli [23] and acid gel electrophoresis for native proteins was performed with Davis' system [24]. SDS–PAGE on reducing conditions was made after Friedman reaction. The broad range standard marker proteins were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glycoproteins were detected by the periodic acid-schiffs (PAS) [25] and estimation of carbohydrate content from the protein samples was done by the phenol sulphuric acid method [26], with a curve of D-mannose as standard.

Fluorescence spectroscopy. Intrinsic fluorescence emission of lectin solution (0.2 mg ml^{-1} in 10 mM phosphate buffer, pH 7.0) was measured at 25 °C using the spectrofluorimeter (JASCO FP-6300, Tokyo, Japan) in a cuvette (1-cm pathlength rectangular quartz). The excitation wavelength was 295 and 280 nm. The emission spectra were recorded at a range of 305 to 450 nm. The band passes were 5 nm. The hydrophobic surface was determined using the same condition as intrinsic fluorescence. The samples were transferred to the

cuvette and then mixed with 5 µM bis-ANS (Molecular Probes Inc., USA) and the fluorescence measured. The fluorescence emission obtained was of 400 to 600 nm with excitation at 360 nm [27].

Resistance proteases assay and trypsin inhibitory activity. Sensitivity of SejaBL to protease was developed according to method of Rios et al. [28]. 5.0 µg of α-chymotrypsin and trypsin at 0.1 M Tris–HCl buffer (pH 8.2) were preincubated for 3 h with 50 µL of SejaBL (0.5 mg ml^{-1}). After, the reaction was stopped with phenylmethyl sulfonyl fluoride (Sigma). The sensitivity to protease was confirmed by lectin HA. The determination of trypsin inhibitory activity was carried out according to the method of Lee and Lin [29] by inhibition of hydrolysis of N-Benzoyl-L-arginine-4-nitroanilide (Sigma) at 0.1 M Tris-HCl buffer (pH 8.2) catalyzed by trypsin. Four determinations were averaged for trypsin inhibitory activity and expressed as µg trypsin inhibited.

Antifungal activity. Assay of antifungal activity and quantitative assay to determine the IC_{50} were performed as described by Wang and Ng [30]. The selected fungi, i.e.: *Trichoderma viride* (URM-3344), *Fusarium oxysporum* (URM-2489), *Colletotrichum gloeosporioides* (URM-4911), *Fusarium moniliforme* (URM-3226), *Aspergillus niger* (URM-5238) and *Candida albicans* (URM-4388), stock obtained from the Federal University of Pernambuco fungal cultures, were carried out in 60 mm – 15 mm petri plates containing 7 mL of potato dextrose agar (PDA). After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the border of the mycelial colony. A SejaBL aliquot (20 µL with 300 µg) was added to the disk. Positive control was Cercobim (2 µg) and negative control (150 mM of NaCl). The plates were incubated at 23 °C for 72 h until mycelial growth had involved the disks containing the

negative control and had formed crescents of inhibition around disks containing samples with antifungal activity.

To determine the IC_{50} value, three concentrations of SejaBL (75, 150, and 300 µg) were added, separately, to three aliquots each containing PDA (4 mL) at 45 °C, mixed quickly and poured into three separate small petri dishes. After the agar had cooled down, a small amount of mycelia (same amount to each plate) was added. Buffer only without SejaBL served as control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth determined.

Environmental Toxicity. A. salina (Brine Shrimp) encysted eggs (25 mg) were hatched in the becker filled with seawater under artificial light at 30 °C, pH 8–9 in constant aeration. After 28 h the nauplii were collected with a pasteur pipette macroscopically and counted in the stem of the pipette against a lighted background. Nauplii were transferred to test tubes containing the samples. The lectin concentration ranged from 10 to 1000 µg ml^{-1} in vials containing 5 mL of seawater. Fifteen shrimp nauplii were added to each vial (45 shrimps per concentration). The plates were maintained under illumination. Survivors were counted after 24 h of incubation and the percentage of deaths at each dose and control was determined. The bioassay was performed as described previously by Meyer et al. [31] and developed with minor modifications. *B. glabrata* embryos (0 to 15 h after spawning) were exposed for 48 h to SejaBL (10 to 200 µg ml^{-1}) and the embryo development was followed for an additional 10 days in absence of exposure. The assay was carried out according to Oliveira-Filho and Paumgartten [32].

Statistical analysis. Data are represented as mean ± S.E.M. The antifungal activity IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GraphPrism® (GraphPad Software Inc., San Diego, CA, USA). LC_{50} on brine

shrimp and *B. glabrata* embryos was obtained from 24 and 48 h respectively; the percentage of deaths were estimated using the method described by Finney [33].

Results and Discussion

The lectin was best extracted with 0.2% Triton X-100 present in the solution. Only one peak bioactivity was detected per acetic acid elution at the CM-Cellulose column (Fig. 1A) and into Sephadex G-100 column (Fig. 1B) with SHA of 8192 (Table 1). Superdex-75 gel filtration (Fig. 1C) showed apparent molecular weight of 50 kDa. On the C-18 column (Fig. 1D) the lectin was eluted about 70% of acetonitrile. SejaBL is a single band of 52 kDa and under reducing conditions, analyzed by SDS-PAGE (Fig. 2A;B), showed dimeric protein composed of two subunits with the same molecular mass of 24 kDa and another fragment of less than 6 kDa. After desalting on the C-18 column, the two chains showed different mobility (Fig. 2C). This suggests that the chains are covalently connected by disulfide bonds and have no homology. In general, molecular mass of subunits back lectin is around 30 kDa, with the fully active lectin forming either dimers or tetramers. Molecular mass ranging from several kilodaltons to about 67 kDa has been reported for antifungal proteins [34-36].

The lectin is an acidic protein (Fig. 2D) with pH stability ranges of 5-8 (Fig. 3A). The lectin band was weakly stained by PAS, and positive phenol–sulphuric acid showed a glycoprotein with 6.94% covalently linked carbohydrate. The native active form was heat-stable up to 70 °C. A soft decrease in its activity was observed when temperature was raised from 70 to 100 °C (Fig. 3B). The loss of hemagglutinating activity with increasing temperature is evidently due to heat-induced denaturation of the lectin. The relative ease

with which SejaBL can be destroyed by heat enables its use as microbial control agents in cereal. SejaBL agglutinated erythrocytes from rabbits and humans of all blood groups except B. Fetuin, asialofetuin, bovine serum albumin and casein inhibited SejaBL induced rabbit erythrocyte agglutination, whereas monosaccharides did not inhibit its activity. Ions did not affect SejaBL HA and the lectin did not suffer apparent hydrolysis front proteases keeping HA. However, SejaBL had a dose-dependent inhibition against trypsin from the porcine pancreas (Table 2). Inhibitor-like proteins are generally found in vegetative tissues, believed to play a role of defence against predators [17].

The intrinsic protein fluorescence spectra of SejaBL (Fig. 4) revealed one major peak at 330 nm, indicating the presence of tryptophan in a highly hydrophobic region. Interestingly, bis-ANS fluorescence observed in SejaBL when compared only with buffer (i.e., the control) indicates hydrophobic surface turned to the solvent (Fig. 4). Prasad *et al*. [37] suggested that the immediate increase in the bis-ANS fluorescence intensity observed in proteins is due to the binding at primary hydrophobic site(s). This demonstrates the hydrophobic character of the protein confirmed by tryptophan and tyrosine intrinsic fluorescence. Steadman *et al.* [38] noted that a hydrophobic surface produced decreasing stability and increasing structural alteration in protein after chemical or physical stress. The lower agglutination activity, at the basic pH values and high temperature may be due to the hydrophobic character of SejaBL.

Among the fungi tested, SejaBL exerted antifungal action against *F. moniliforme* and *F. oxysporum*. The IC_{50} values of its antifungal activity towards *F. moniliforme* and *F. oxysporum* was, respectively, 123 ± 0.5 and 303 ± 0.9 µg (Table 3), similar to those of previously reported for antifungal proteins [39,40], and some are linked to protease inhibitory activities [41,42]. The species of Fusarium sp are pathogens to banana, corn and

rice. Damage produced by these fungi cause loss of cereals with harmful effects on human health and agriculture.

The lectin showed percentage of *A. salina* deaths with LC_{50} of at 715.89 ± 1 μg ml^{-1} (Table 3). Lectin did not present toxicity in blastulae and embryo development of *B. glabrata*. Lethality was not observed until 200 μg ml^{-1} (Table 3). This data suggests that the antifungal activity is unrelated to the lytic action or membrane instability. The inhibitory effect on embryo development was not observed since the first cleavage, and the cells presented a nuclear organization and homogeneous cytoplasm, with no DNA or protein synthesis in injury [43]. SejaBL, compared with the reference chemical fungicides, presents a higher degree of selectivity, does not affect the conservation and access to biological diversity and does not involve environmental contamination.

Conclusion

Some *Fusarium sp* species produce mycotoxins in cereal crops that can affect human and animal health [44,45]. The isolation of a plant lectin with inhibitory activity toward fungi and without environmental toxicity has important application in vegetable biotechnology as a promising biological control agent to genetically modified plants.

Acknowledgements

This work was financially supported by CAPES. The authors are deeply grateful for the technical assistance of Maria Barbosa Reis da Silva and João Antônio Virgilio.

References

[1] N. Sharon, Lectins: Carbohydrate-specific Reagents and Biological Recognition Molecules, J. Biol. Chem. 282 (2007) 2753-2764.

[2] W.J. Peumans, E.J.M. Van Damme, Plant lectins versateli proteins with important perspectives in biotechnology, Biotec. Gen. Engine. Rew. 15 (1998) 199-228.

[3] A.T. Branco, R.B. Bernabé, B.S. Ferreira, M.V.V. Oliveira, A.B. Garcia, G.A. Souza-Filho, Expression and purification recombinant lectin from rice (*Oryza sativa* L.), Prot. Express. Purif. 33 (2004) 34-38.

[4] R. Wititsuwannakul, K. Rukseree, K. Kanokwiroon, D. Wititsuwannakul, A rubber particle protein specific for *Hevea latex* lectin binding involved in latex coagulation, Phytochem. 69 (2008) 1111-1118.

[5] W.F. Broekaert, M. Nsimba-Lubaki, B. Peeters, W. J. Peumans, A lectin from elder (*Sambucus nigra* L.) bark, J. Biochem. 221 (1984) 163-169.

[6] V. Horejsi, C. Hashkovec, J. Kocourek, Isolation and characterization of the lectin from black locust bark (*Robinia pseudacacia* L.), Biochim. Biophys. Acta. 532 (1978) 98-104.

[7] W.C. Johnson, Analyzing protein circular dichroism spectra for accurate secondary structures, Proteins. 35 (1999) 307-312.

[8] A. Hawe, M. Sutter, W. Jiskoot, Extrinsic Fluorescent Dyes as Tools for Protein Characterization, Pharm. Res. 25 (2008)1487-1499.

[9] P.A. Almeida, T.M.S. Silva, A. Echevarria, Mesoionic 5-alkyl-1.3-dithiolium-4-thiolates: Synthesis and brine shrimp toxicity, Heterocycl. Comm. 8 (2002) 593-600.

[10] J.L. Mclaughlin, L.L. Rogers, The use of biological assays to evaluate botanicals, Drug Inform. J. 32 (1998) 513-524.

[11] A. Münzinger, *Biomphalaria glabrata* (Say), a suitable organism for a biotest, Environ. Tech. Lett. 8 (1987) 141-148.

[12] J. Grenier, C. Potvin, J. Trudel, A. Asselin, Some thaumatinlike proteins hydrolyse polymeric beta-1.3-glucans, Plant J. 19 (1999) 473-480.

[13] S. Krishnaveni, G.H. Liang, S. Muthukrishnan, A. Manickam, Purification and partial characterization of chitinases from sorghum seeds, Plant Sci. 144 (1999) 1-7.

[14] P. Epple, K. Apel, H. Bohlmann, Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum*, Plant Cell. 9 (1997) 509-520.

[15] B. Thomma, B.P.A. Cammue, K. Thevissen, Plant defensins, Planta 216 (2002) 193-202.

[16] L. Barbieri, M.G. Battelli, F. Stirpe, Ribosome-inactivating proteins from plants, Biochim Biophys Acta. 1154 (1993) 237-282.

[17] Z.Y. Chen, R.L. Brown, J.S. Russin, A.R. Lax, T.E. Cleveland, A corn trypsin inhibitor with antifungal act inhibits *Aspergillus flavus* alpha-amylase, Phytopathol. 89 (1999) 902-907.

[18] J.H. Wong, H.Y. Edwin Chan, T.B. Ng, A mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*), Biochim. Biophys. Acta 1780 (2008) 1017-1022.

[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. (1951) 193-265.

[20] M.T.S. Correia, L.C.B.B. Coelho, Purification of a glucose/manose specific Lectin, isoforma 1, from seeds of *Cratylia mollis* Mart. (Camaratu bean), App. Biochem. Biotech. 55 (1995) 261-273.

[21] L.C.B.B. Coelho, M.B.R. Da Silva, Simple method to purify milligram quantities of the galactose – specific lectin from the leaves of *Bauhinia monandra*, Phytoch. Analysis. 11 (2000) 295-300.

[22] M. Friedman, L.H. Krull, J.F. Cavins, The chromatographic determination of cystine and cysteine residues in proteins as S-h-(4-Pyridylethyl) cysteine, J. Biol. Chem. 245 (1970) 3868– 3871.

[23] U.K. Laemmli, Cleavage of structural protein during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.

[24] B.J. Davis, Ann. N. Y. Acad. Sci., 121 (1964) 404-427.

[25] R.M. Zacharius, T.E. Zell, J.H. Morrison, J.J. Woodlock, Glycoprotein staining following electrophoresis on acrylamide gels, Anal. Biochem. 30 (1969) 148-152.

[26] T. Masuko, A. Minami, N. Iwasaki, T. Majima, S. Nishimura, W.C. Lee, Carbohydrate analysis by a phenol–sulfuric acid method in microplate format, Anal. Biochem. 339 (2005) 69-72.

[27] A. Bhattacharyya, A.K. Mandal, R. Banerjee, S. Roy, Dynamics of compact denatured states of glutaminyl-tRNA synthetase probed by bis-ANS binding kinetics, Biophy. Chem. 87 (2000) 201-212.

[28] F.J.B. Rios, B.S. Cavada, D.A. Meeiros, R.A. Moreira, L.M. Vasconcelos, J.T.A. Oliveira, Digestibility of plant lectins from canavalia, cratylia, Dioclea and *Artocarpus genera*, Lect. Biol. Biochem. Clin. Biochem. 11 (1996) 277-284.

[29] T.M. Lee, Y.H. Lin, Trypsin inhibitor and trypsin-like protease activity in air-or submergence-grown rice (*Oryza sativa* L.) coleoptiles, Plant Sci. 106 (1995) 43-54.

[30] H.X. Wang, T.B. Ng, An antifungal peptide from red lentil seeds, Peptides. 28 (2007) 547-552.

[31] B.N. Meyer, Ferrigni, N.R. Putnam, J.E. Jacobsen, L.B. Nichols, D.E. McLaughlin, J.L. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45 (1982) 31-34.

[32] E.C. Oliveira-Filho, F.J.R. Paumgartten, Toxicity of *Euphorbia milii* latex and niclosamide to snails and nontarget aquatic species, Ecotoxicol. Environ. Saf. 46 (2000) 342-350.

[33] D.J. Finney, Statistical aspects of monitoring for dangers in drug therapy, Meth. Inform. Med. 10 (1971) 1-8.

[34] K.T. Chu, K.H. Liu, T.B. Ng, Cicerarin, a novel antifungal peptide from the green chickpea, Peptides 24 (2003) 659-663.

[35] Y. Qiaojuan, J. Zhengqiang, Y. Shaoqing, D. Wei, H. Lujia, A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity, Arch. Biochem. Biophys. 442 (2005) 72-81.

[36] T.B. Ng, Antifungal proteins and peptides of leguminous and non-leguminous origins, Peptides 25 (2004) 1215-1222.

[37] A.R.S. Prasad, R.F. Luduena, P.M. Horowitz, Bis(8-anilinonaphthalene-1-sulfonate) as a probe for tubulin decay, Biochemistry 25 (1986) 739-742.

[38] B.L. Steadman, K.C. Thompson, C.R. Middaugh, K. Matsuno, S. Vrona, E.Q. Lawson, R. V. Lewis, The effects of surface adsorption on the thermal stability of proteins, Biotech. Bioengin. 40 (2004) 8–15.

[39] X.Y. Ye, T.B. Ng, P.W.K. Tsang, J. Wang, Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*) activities, J. Protein Chem. 20 (2001) 367–375.

[40] M. Sitohy, M. Doheim, H. Badr, Isolation and characterization of a lectin with antifungal activity from *Egyptian Pisum* sativum seeds, Food Chem. 104 (2007) 971-979.

[41] G. Chilosi, C. Caruso, C. Caporale, L. Leonardi, L. Bertini, A. Buzi, M. Nobile, Antifungal activity of a Bowman-Birk type trypsin inhibitor from wheat kernel, J. Phytopathol. 148 (2000) 77-481.

[42] J.Y. Kim, S.C. Park, M.H. Kim, H.T. Lim, Y. Park, K.S. Hahm, Antimicrobial activity studies on a trypsin–chymotrypsin protease inhibitor obtained from potato, Biochem. Biophys. Res. Commun. 330 (1999) 921-927.

[43] N. Fusetani, Marine metabolities which inhibit development of echinoderm embryos, In: P. Scheuer, (Ed.), Bioorg. Mar. Chem. Springer-Verlag, Berlin, (1987) 61-91.

[44] M. Jiménez, T. Huerta, R. Mateo, Mycotoxin Production by *Fusarium* Species Isolated from Bananas, Appl. Environ. Microbiol. 63 (1997) 364-369.

[45] C. W. Hesseltine, Natural occurrence of mycotoxins in cereals, Mycopathol. 53 (1974) 141-153.

Table 1

Table 1

Yields and hemagglutinating activities of various fractions (from 100 g meal of *S. jacobinensis* bark)

Sample	Yield (mg)	Specific hemagglutinating activity (units/mg)	Total hemagglutinating activity (units)	Recovery of hemagglutinating activity (%)	Folds of purification
Crude extract	14400	284.4	4.09×10^6	100	1
$(NH_4)_2SO_4$ fraction	1323.2	3951	5.22×10^6	127.67	13.9
CM-Cellulose	96	6827	6.55×10^5	16.01	24
Sephadex G-100	20	8192	1.63×10^5	4	28.8

Table 2

Table 2 Inhibition of purified SejaBL against trypsin

µg of SejaBL	0.5	1.25	2.5	5	7.5	12.5
% of inhibition	8.5	10	17	28	39	56.5

The assay for the inhibition of trypsin was carried out using 5µg porcine pancreas trypsin.

Table 3

Table 3 Linearity value for the antifungal activity and acute environmental toxicity

	Antifungal activity	IC_{50} ± S.D.	Regression equation	Coefficient of determination $(r^2)^2$
A	*F. moniliforme*	123 ± 0.5 µg	y = 0.1955x + 4.386	0.992
	F. oxysporum	303 ± 0.9 µg	y = 0.0741x	0.99
	Toxicity environmental	**LC_{50} ± S.D.**	**Regression equation**	**Coefficient of determination $(r^2)^2$**
B	Embryo of *B. glabrata*	nontoxic	—	—-
	A. salina leach	715.89 ± 1 µg/mL	y = -0.1799x + 178.79	0.983

Figure

Figure captions

Fig. 1. (A) Purification by ion-exchange (40 mg of protein) was applied at a flow rate of 0.3 mL min^{-1} and 2 mL fractions were collected. Arrows indicated elution with Tris–HCl buffer (1), followed by 1 M of acetic acid (2). Absorbance at 280 nm (- -); log of HA (-●-). (B) Gel filtration on Sephadex G-100 equilibrated and eluted with NaCl 150 mM at 0.5 mL min^{-1} collected on 1-mL fractions. Absorbance at 280 nm (- -); log of HA (-●-). (C) Superdex 75 HR 10/30 column coupled to an ÄKTA purifier system equilibrated and eluted with NaCl 300 mM at 0.5 mL min^{-1} collected on 1-mL fractions. (D) Reverse phase chromatography in C-18 column HPLC system. The column was equilibrated with solvent A (0.1% TFA in H_2O) and used a non-linear gradient elution (5-100%) with solvent B (90% acetonitrile: 10% H_2O: 0.1% TFA).

Fig. 2. (A) Molecular weight of SejaBL on a 10% SDS-PAGE. MW, molecular weight. (B) On reducing conditions. (C) Acid native protein. (D) Chains separations after desalting on the C-18 column.

Fig. 3. Effects of temperature and pH on SHA of SejaBL. SHA in NaCl 150 mM with rabbit erythrocytes was 3.01.

Fig. 4. Intrinsic fluorescence emission of SejaBL, excitation at 280 nm ( ) and 295 nm (•).Bis-ANS fluorescence emission, excitation at 360 nm (■).

Figure

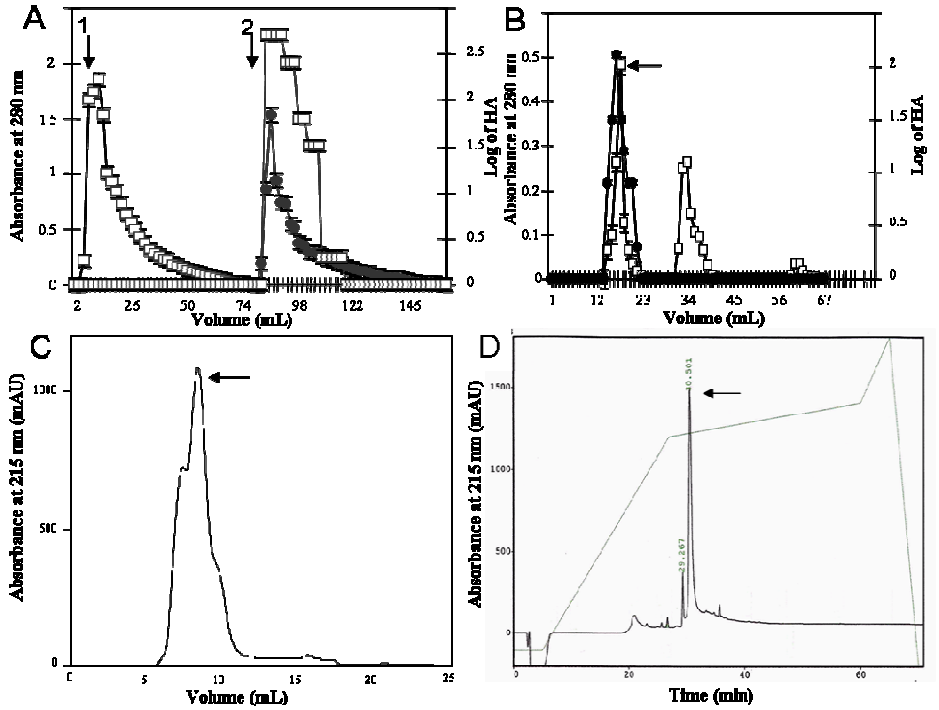


Figure 1

Figure

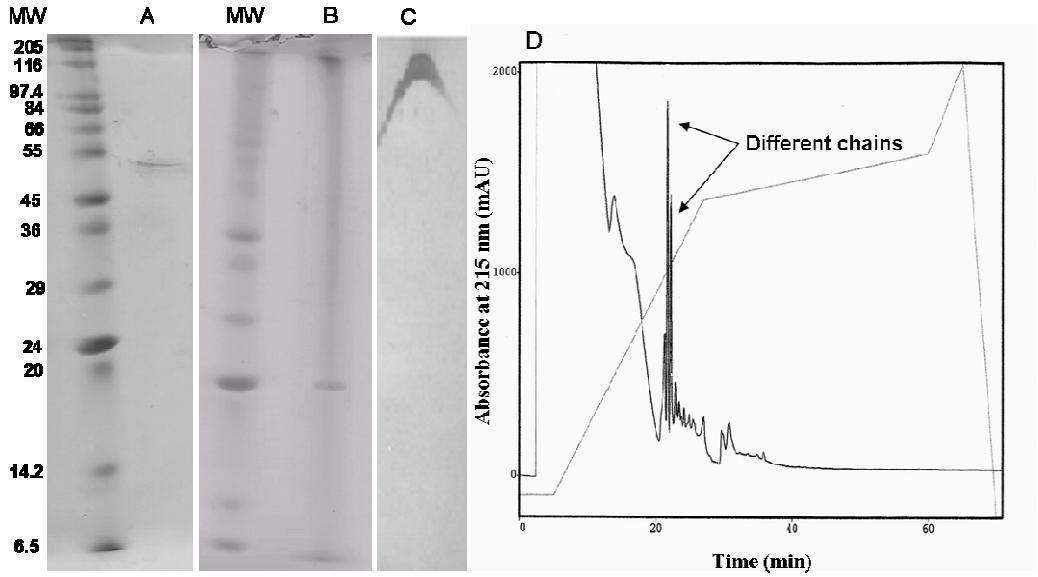


Figure 2

Figure

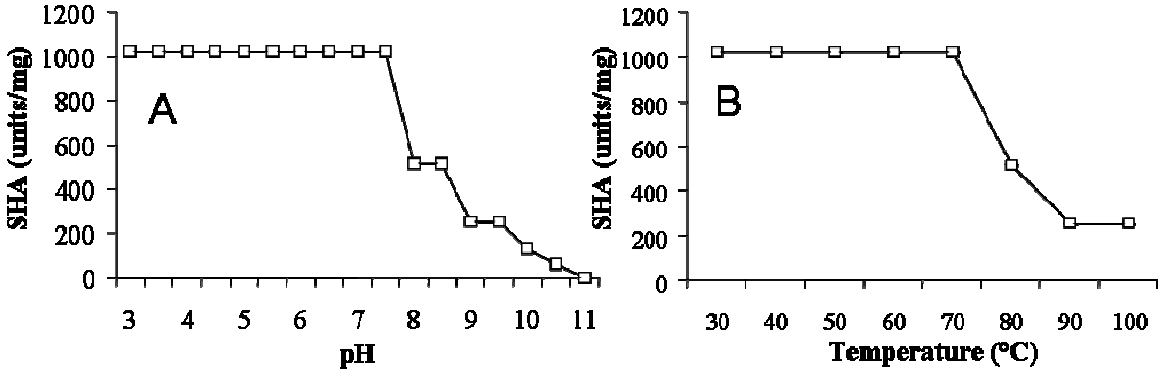


Figure 3

Figure

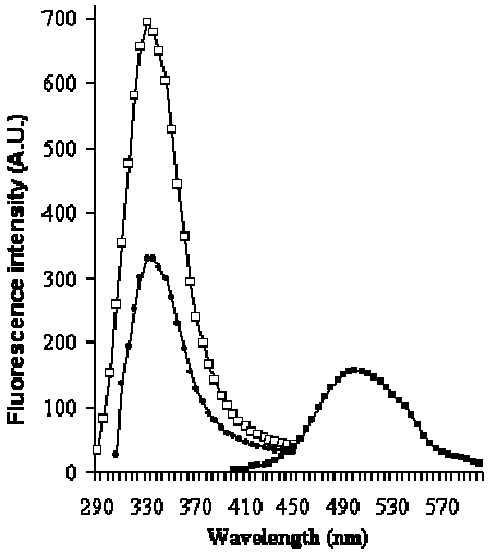


Figure 4

Capítulo 2

Artigo a ser submetido à publicação

Archives of Biochemistry and Biophysics

Impacto: 2.578 (2007)

http://www.elsevier.com/wps/find/journaldescription.cws_home/622787/description

Manuscript

Effect of gamma radiation on the molecular properties of S*ebastiania jacobinensis* bark lectin

Antônio Fernando de Melo Vaz [a,*], Ana Maria Mendonça de Melo [b], Maria Luiza Vilela Oliva [c], Lucimeire Aparecida de Santana [c], Luana Cassandra Breitenbach Barroso Coelho [a], Maria Tereza dos Santos Correia[a]

[a]Department of Biochemistry, Federal University of Pernambuco, Recife, Brazil

[b]Department of Biophysic and Radiobiology, Federal University of Pernambuco, Recife, Brazil

[c]Department of Biochemistry, Federal University of São Paulo, São Paulo, Brazil

[*]**Corresponding author**: Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE - CEP: 50670-901, Recife, PE, Brazil. (Maria Tereza dos Santos Correia)

Tel: 55 (81) 2126.8574; Fax: 55 (81) 21268576; E-mail address: melo_vaz@ig.com.br

Abbreviations used: CM-cellulose, Carboxymethyl cellulose; SDS–PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SejaBL, *Sebastiania jacobinensis* bark Lectin; %SHA, percentage relative Specific Hemagglutinating Activity.

Abstract

Purified lectin from S*ebastiania jacobinensis* bark (SejaBL) is an effective antifungal agent of low environmental toxicity with hydrophobic surface that is displayed to free radicals produced by water radiolysis. In an experimental model, SejaBL was used as probe that changes on the hydrophobic center by irradiation, structurally altered proteins. To elucidate the effect of free radicals in molecular properties of SejaBL hemagglutinating activity (HA), tertiary structure (intrinsic and bis-ANS fluorescence), molecular weights and reversed phase chromatography were examined after γ-irradiation at various doses (0.020 to 35 kGy). Specific Hemagglutinating Activity (SHA) in irradiated lectin showed significant loss ($p < 0.05$), above 12.5 kGy, and a significant increase ($p < 0.05$) in 0.1, 0.8 and 1 kGy. Fluorescence spectroscopy indicated suppression of emission intensity when excited at 280 and 295 nm with high binding of bis-ANS after high doses. SDS-PAGE and reversed phase chromatography showed polypeptide fragmentation. Significant change in the hydrophobic surface indicated hydrogen abstraction in the amino acids causing structural disorganization from the lectin. Irradiation is a promising new safety technology that can eliminate pathogens in biomolecules, and to ratify their application it is important to clarify changes in various biological materials to start the application in large scale.

***Keywords*:** Gamma radiation; Lectin; Intrinsic fluorescence; Bis-ANS

Introduction

Radiation treatments of biological materials have been applied in various processes as the sterilization of medical supplies and food. The applicable high dose of gamma radiation may affect the functional integrity of biopolymers. In proteins, structural damage is extensive abolishing biological activity [1-3]. Irradiation can inactivate proteins through two different mechanisms. First, it can split covalent bonds in protein target as a direct result of depositing photon energy [4]. Second, it can act indirectly, via water molecules, producing reactive oxygen species (ROS) responsible for the majority of protein damage [5]. These products interact with biological molecules, forming secondary organic radicals [6]. The primary damages are hydrogen abstraction in amino acid side chain, rings of aromatic residues, and reaction with sulfur [7,8].

Exposure of proteins to radiation has altered their physical and chemical structures with fragmentation, cross-linking, aggregation, unfolding and formation of new reactive groups, resulting in distortions of secondary and tertiary structures [9-11]. This amendment depends on several factors, such as protein concentration, presence of oxygen and quaternary structure [12]. Many conformation studies involved intrinsic and extrinsic fluorescence measurements of proteins. Denaturant proteins often exhibit changes in solvent accessibility to hydrophobic regions. One of the most important probes for study protein denaturation is bis-ANS [13]. Bis-ANS is non-fluorescent in aqueous mediums but shows fluorescence when bound to proteins. It has been used to probe the structure-function relationship in several proteins, such as tubulin [14] and myosin [15].

Lectins are proteins or glycoproteins of ubiquitous distribution in nature, which have one or several carbohydrate binding sites without catalytic function or immunological

characteristic [16]. They present a variety of chemical and biological strategies as insecticidal, fungicidal, antibacterial actives [17,18]. SejaBL is a purified lectin from S*ebastiania jacobinensis* bark with antifungal activity of low environmental toxicity which has a hydrophobic surface sensitive to the effect of free radicals. In this paper we describe the effect of gamma radiation on the activity and molecular properties of SejaBL, with focus on the changes in hydrophobic regions suffered by free radicals generated by the water radiolysis.

Material and methods

Purified lectin S. jacobinensis

S. jacobinensis bark was homogenized overnight at 4 °C in 10 mM Tris–HCl buffer (pH 8.5) with 0.2% (v/v) Triton X-100 detergent. The homogenate was centrifuged 5,000 g for 20 min (crude extract) followed by lyophilization. In brief, two volumes of cold acetone were added, mixed well and kept on ice for 10 min. The solution was centrifuged at 10,000 g for 20 min, at 4 °C. The supernatant was concentrated under vacuum until total evaporation of the acetone. The residual aqueous solution obtained was precipitated with 60% $(NH_4)_2SO_4$. The precipitate was dialyzed against the Tris–HCl buffer. This fraction was applied to a CM-Cellulose column (2 x 20 cm) previously equilibrated with the Tris–HCl buffer into 150 mM of NaCl. Following removal of the unadsorbed proteins, the column was eluted with 1 M acetic acid. The largest fractions of absorbance eluted with acetic acid were pooled, dialyzed and concentrated by ultrafiltration (Amicon ultra-15, Mr 10.000 cut-off) and chromatographed on a Sephadex G-100 column (2 x 70 cm) equilibrated with 150 mM NaCl. The column was eluted with the same solution at a flow

rate of 0.5 mL min^{-1}. The primary unadsorbed fraction (SejaBL) was concentrated by ultrafiltration in 10 mM phosphate buffer (pH 7.0) and stored. All other chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA). All solutions were made with water purified by the Milli-Q system.

Sample irradiation

The lectin 0.5 mg mL^{-1} in phosphate buffer (pH 7.0) added in borosilicate glass vials (16-125 mm) were frozen and irradiated into atmospheric O_2, using the ^{60}Co gamma ray irradiator Gammacell 220 Excel (Ontario, Canada); in doses of 0.020; 0.050; 0.075; 0.1; 0.2; 0.4; 0.6; 0.8; 1.0; 3.0; 6.0; 12.5; 25; 35 kGy. The dose rate was 12.5 kGy / h. Each sample was analyzed 1 h, 24 h and 7 days after exposition by the methods below.

Hemagglutination activity and Protein concentration

Hemagglutinating activity (HA) was evaluated as described by Correia and Coelho [19] defined as the lowest sample dilution showing hemagglutination. Specific HA (SHA) corresponded to the relation between HA and protein concentration measured according Lowry *et al.* [20], using the BSA standard curve. The percentage of remaining SHA ($\%SHA_{REM}$) was calculated according to the equation: $\%SHA_{REM} = [SHA]_{GM} / [SHA]_{GO} \times 100$, where G_M is the lectin SHA of each dose irradiated (0.02-35 kGy) and G_0 is the not irradiated lectin SHA (control).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed to Laemmli [21]. Protein samples were mixed with sample

buffer (60 mM Tris–HCl, 2% SDS, 25% glycerol, 0.1% bromophenol blue, pH 6.8), resolved on a 10% separation gel and stained with silver kit (Bio-Rad). The broad range standard marker proteins were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Reverse phase chromatography analysis

The fraction irradiated lectins (0.1, 3 and 35 kGy) were submitted to reverse phase chromatography on the C-18 column (Vydac-protein peptide ultrasphere), performed on the HPLC system (Shimadzu LC-10AD-kyto, Japan) and monitored at 215 nm. The column was equilibrated with solvent A (0.1% TFA in H_2O) at a flow rate of 0.7 mL min^{-1} and a non-linear gradient elution was used with solvent B (90% acetonitrile: 10% H_2O: 0.1% TFA) in A, with B = 5% at t = 5 min; B = 70% at t = 27 min; B = 80% at t = 60 min and B = 100% at t = 69 min.

Fluorescence spectroscopy

Fluorescence emission intensity of the irradiated lectin solution was measured at 25 °C using the spectrofluorimeter (JASCO FP-6300, Tokyo, Japan), at 0.2 mg mL^{-1} of SejaBL (phosphate buffer, pH 7.0) in cuvette (1-cm pathlength rectangular quartz). The excitation wavelength was 295 and 280 nm. The emission spectra were recorded at a range of 305 to 450 nm. The band passes were 5 nm. Light scattering was measured at 90° for aggregation assays, in lectin previously irradiated, and light scattering values at 320 nm were monitored at 300 to 340 nm. The spectra reported averages of 3 scans. The spectrum of the solution was subtracted from each spectrum.

Hydrophobic surface

The lectin hydrophobic surface was determined using the same condition to intrinsic fluorescence. The samples were transferred to the quartz cuvette and then mixed with 5 µM bis-ANS (Molecular Probes Inc., USA) and the fluorescence measured in the JASCO spectrofluorimeter. The fluorescence emission was obtained at 400 to 600 nm with excitation at 360 nm [22].

Statistical analysis

Statistical analysis for paired data was carried out by Student's t test using the GraphPrism® (GraphPad Software Inc., San Diego, CA, USA). The significance accepted difference between the mean values of parameter was analyzed to the level of $p < 0.05$.

Results and Discussion

Effects of irradiation on SHA

Lectin showed significant loss ($p < 0.05$) with 12.5, 25 and 35 kGy due to direct action of radiation on structure in 1 h after exposure. In doses (0.02 to 6 kGy) no significant change was observed. The doses 0.1, 0.8 and 1 kGy showed a significant increase ($p < 0.05$) with 1 h and 24 h after radiation target analysis (Fig. 1). The amplification is associated with reduced dosage of protein, except for dose of 0.1 kGy, where there was an increase in HA. This reduction has been observed [23-25] and is a consequence of chemistry oxidation suffered by aromatical amino acids (mainly tyrosine and tryptophan residues) responsible for the formation of the measurable colorful complex based on the

Lowry et al. method [20]. These data are confirmed by intrinsic fluorescence emission reduction of tyrosine and tryptophan amino acids (Fig. 3). In relation to the increase of HA with 0.1 kGy, according to Macklis [26] and Shuzheng [27], low doses of radiation yield a rise of active molecules exerted into closed systems. This peculiar behavior, confirmed by increased intrinsic fluorescence, reveals changes in native tertiary structure with a possible modification of hydrogen bonding, van der Waals and hydrophobic interactions. Although there was a significant loss of HA, SejaBL showed high resistance compared to other previously analyzed proteins [3]. The concentration of proteins influenced the degree of structural change of the irradiated proteins. Thus, the damage is greater to low protein concentrations [12].

Structural analysis

None of these doses (0.02 to 0.075 kGy) changed the molecular weight pattern of SejaBL. The initial degradation of lectin (MW 52 kDa) was observed in doses (0.2 to 6 kGy) with aggregation tracked by light scattering (data not shown). Above 3 kGy, only a degradation of the main band was viewed. The reverse phase chromatography analysis revealed loss of the peak area with structural collapse (Fig. 2). Puchala & Schuessler [28] suggested that break points, caused by radiation on protein, are fragile bonds in the polypeptide chain. Proteins can be converted to higher molecular weight aggregates, due generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, as well as the formation of disulfide bonds [5,24,29].

Intrinsic fluorescence

The intrinsic fluorescence emission showed increase (0.02 to 0.1 kGy) and decreased (0.2 to 35 kGy) without change in λ_{max} at around 330 nm for tryptophan and tyrosine residues (Fig. 3). The hydroxyl radicals are generated by water radiolysis [30] and all aliphatic amino acids are potential targets of modification [11,31]. Aromatic amino acids residues become particularly sensitive, producing hydroxylated products. The formation of formylkynurenine from tryptophan, 3.4-dihydroxyphenylalanine (DOPA) from tyrosine, and o-tyrosine from phenylalanine as major oxidation products was recognized [32]. The partial aromatic amino acid substitution by hydroxylated products results in a lesser emission of fluorescence [33]. Such oxidative damage distorts the polarity of these residues modifying the hydrophobic surface and tertiary structure favoring the denaturation and aggregation.

Hydrophobicity

Bis-ANS fluorescence increased at all doses with maximum blue shifts to 490 nm above 0.8 kGy (Fig. 4). Bis-ANS fluorescence is observed in non-irradiated SejaBL (i.e., the control) and when compared only with the buffer it indicates that the hydrophobic surface turned to the solvent. In protein, hydrophobic surfaces are easily attacked by hydroxyl radicals and, in this case, the change in protein hydrophobicity is considered the preponderant factor for the structural collapse. Hydrogen abstraction of aromatic amino acids promotes greater exposure of the hydrophobic surface to the solvent, due to decrease of apolarity surface, leading to greater binding bis-ANS (maximum blue shift emission at 490 nm).

A survey of the literature showed that lectins bind their ligands most commonly through hydrogen bonds (some mediated by water), hydrophobic interactions, and that in rare cases electrostatic interactions (ion pairing) and coordination with metal ions also play a role [16]. The hydrophobic face of sugar rings on aromatic side chains of amino acids is a common feature of the complexes with carbohydrates of other lectins, for instance, of the galectins, and certain bacterial toxins, such as *Escherichia coli* lytic toxin, and also of non-lectin proteins [34]. Several independent properties of irradiated SejaBL revealed different responses. They indicate that the tertiary structure plays a critical role in HA, where the various modifications of hydrophobic amino acid residues via free radicals promote the collapse of hydrophobic regions, changing several kinetic properties in lectin. Irradiation at appropriate doses is a promising approach for producing safe and pathogen-free biomolecules. Therefore, it is important to clarify changes in various biological materials in order to ratify the application of gamma radiation, a crucial event to begin the production of bioderivatives in large scale.

Acknowledgements

This study was financially supported by the CAPES. We are grateful to the Department of Nuclear Energy (UFPE) for the access and help.

References

[1] M.E. Lowe, E.S. Kempner, Radiation Inactivation of the Glycoprotein, Invertase, J. Biol. Chem. 257 (1982) 12478-12480.

[2] M.D. Davis, M.A. Parniak, S. Kaufman, E. Kempner, Structure–Function Relationships of Phenylalanine Hydroxylase Revealed by Radiation Target Analysis, Arch. Biochem. Biophys. 94 (1997) 491-495.

[3] H.M. Zbikowska, P. Nowak, B. Wachowicz, Protein modification caused by a high dose of gamma irradiation in cryo-sterilized plasma: Protective effects of ascorbate, Free Radic. Biol. Med. 40 (2006) 536-542.

[4] E.S. Kempner, Effects of high-energy electrons and gamma rays directly on protein molecules, J. Pharm. Sci. 90 (2001) 1637-1646.

[5] P.A. Riley, Free radicals in biology: oxidative stress and the effects of ionizing radiation, Int. J. Radiat. Biol. 65 (1994) 27-33.

[6] M.H. Klapper, M. Faraggi, Applications of pulse radiolysis to protein chemistry, Quart. Rev. Biophys. 12 (1979) 465-519.

[7] M. Charlier, S. Eon, E. Sèche, S. Bouffard, F. Culard, M. Spotheim-Maurizot, Radiolysis of lac Repressor by γ-Rays and Heavy Ions: A Two-Hit Model for Protein Inactivation, Biophys. J. 82 (2002) 2373-2382.

[8] G. Xu, M.R. Chance, Radiolytic Modification of Sulfur-Containing Amino Acid Residues in Model Peptides: Fundamental Studies for Protein Footprinting, Anal. Chem. 77 (2005) 2437-2449.

[9] A. Filali-Mouhim, M. Audette, M. St-Louis, L. Thauvette, L. Denoroy, F. Penin, X. Chen, N. Rouleau, J.P. Le Caer, J. Rossier, M. Potier, M. Le Maire, Lysozyme fragmentation induced by radiolysis, Int. J. Radiat. Biol. 72 (1997) 63-70.

[10] Y.C.J.S. Yang, K.B. Song, Effect of ascorbic acid and protein concentration on the molecular weight profile of bovine serum albumin and β-lactoglobulin by γ-irradiation, Food Res. Int. 32 (1999) 515-519.

[11] W.M. Garrison, Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins, Chem. Rev. 87 (1987) 381-398.

[12] E.S. Kempner, Advances in Radiation Target Analysis, Anal. Biochem. 276 (1999) 113-123.

[13] F.J. Farris, G. Weber, C.C. Chiang, I.C. Paul, Preparation, crystalline structure, and spectral properties of the fluorescent probe 4.4-bis-1-phenylamino-8-naph-thalenesulfonate, J. Am. Chem. Soc. 100 (1978) 4469-4475.

[14] P. Horowitz, V. Prasad, R.F. Luduena, Bis(1.8-anilinonaphthalenesulfonate): a novel and potent inhibitor of microtubule assembly, J. Biol. Chem., 259 (1984) 14647-14650.

[15] R. Takashi, Y. Tonomura, M.F. Morales, 4.4'-Bis (1-anilinonaphthalene-8-sulfonate): a new probe of the active site of myosin, Proc. Natl. Acad. Sci. 74 (1977) 2334-2338.

[16] N. Sharon, H. Lis, History of lectins: from hemagglutinins to biological recognition molecules, Glycob. 14 (2004) 53-62.

[17] W.J. Peumans, E.J.M. Van Damme, Plant lectins versateli proteins with important perspectives in biotechnology, Biotech. Gen. Eng. Rev. 15 (1998) 199-228.

[18] N. Sharon, Lectins: Carbohydrate-specific Reagents and Biological Recognition Molecules, J. Biol. Chem. 282 (2007) 2753-2764.

[19] M.T.S. Correia, L.C.B.B. Coelho, Purification of a glucose/manose specific lectin, isoforma 1, from seeds of *Cratylia mollis* Mart. (Camaratu bean), App. Biochem. Biotech. 55 (1995) 261-273.

[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. (1951) 193-265.

[21] U.K. Laemmli, Cleavage of structural protein during the assembly of the head of bacteriophage T4, Nature. 227 (1970) 680-685.

[22] A. Bhattacharyya, A.K. Mandal, R. Banerjee, S. Roy, Dynamics of compact denatured states of glutaminyl-tRNA synthetase probed by bis-ANS binding kinetics, Biophys. Chem. 87 (2000) 201-212.

[23] J.H. Miller, D.A. Fedoronko, B.D. Hass, M. Myint, E.S. Kempner, Radiation Effects on the Native Structure of Proteins: Fragmentation without Dissociation, Arch. Biochem. Biophys. 352 (1998) 281-287.

[24] S. Moon, K.B. Song, Effect of gamma-irradiation on the molecular properties of ovalbumin and ovomucoid and protection by ascorbic acid, Food Chem. 74 (2001) 479-483.

[25] J.H. Miller, G. Bolger, E.S. Kempner, Radiation target analysis of enzymes with stable free radicals, Rad. Phys. Chem. 62 (2001) 33-38.

[26] R.M. Macklis, B. Beresford, Radiation hormesis, J. Nuclear Med. 32 (1991) 350-359.

[27] S. Kondo, Health effects of low-level radiation, Kinki University, Medical Physics, Osaka, 1993.

[28] M. Puchala, H. Schuessler, Oxygen effect in the radiolysis of proteins. IV. Myoglobin, Int. J. Pept. Protein Res. 46 (1995) 326-332.

[29] C.L.P. Oliveira, L. Hoz, J.C. Silva, I.L. Torriani, F.M. Netto, Effects of gamma radiation on β-lactoglobulin: Oligomerization and aggregation, Biopolym. 85 (2006) 284-294.

[30] E. Franzini, H. Sellak, J. Hakim, C. Pasquier, Oxidative damage to lysozyme by the hydroxyl radical: comparative effects of scavengers, Biochim. Biophys. Acta 1203 (1993) 11-17.

[31] G. Xu, K. Takamoto, M.R. Chance, Radiolytic Modification of Basic Amino Acid Residues in Peptides: Probes for Examining Protein-Protein Interactions, Anal. Chem. 75 (2003) 6995-7007.

[32] W.Q. Shi, J.C. Dong, Y.F. Zhao, W.Y. Li, Hydroxylation of 3-Nitrotyrosine and Its Derivatives by Gamma Irradiation, Rad. Res. 166 (2006) 639-645.

[33] H. Steinhart, Stability of tryptophan in peptides against oxidation and irradiation, Adv. Exp. Med. Biol. 294 (1991) 29-40.

[34] J. Rini, Lectin structure, Annu. Rev. Biophys. Biomol. Struct. 24 (1995) 551-577.

Figure

Figure captions

Fig. 1. γ-radiation effect on lectin activity. %SHA_{REM} (▲) 01 h; (O) 24 h; ( ) 7 days after irradiation. The error in the determination of %SHA_{REM} for different doses is approximately ± 1%, smaller than the size of the symbols. *Significant difference (p < 0.05) in relation to those non-irradiated lectin (i.e., the control).

Fig. 2. Chromatography profile of SejaBL under gamma irradiation. (▬) control non-irradiated; (▬) 0.1 kGy; (▬) 3 kGy; (▬) 35 kGy. Reverse phase chromatography in C-18 column HPLC system (Shimadzu). The column was equilibrated with solvent A (0.1% TFA in H_2O) and a non-linear gradient elution was used with solvent B (90% acetonitrile: 10% H_2O: 0.1% TFA) in A, with B = 5% at t = 5 min; B = 70% at t = 27 min; B = 80% at t = 60 min and B = 100% at t = 69 min.

Fig. 3. SejaBL intrinsic fluorescence. (**A**) Total intrinsic fluorescence; relative intensity (%) with (▲) 01 h; (O) 24 h; ( ) 7 days after irradiation lectin. (**B**) Tryptophan fluorescence. The error in the determination of fluorescence from the relative peak areas of integral intensities to different doses is approximately ± 0.5%, smaller than the size of the symbols. (**C**), (**D**) and (**E**) fluorescence with 1 h, 24 h and 7 days after irradiation, respectively. Excitation 295 nm. Emission 305-450 nm. (▬) control non-irradiated; (---) 0.02 to 0.075 kGy; (–) 0.02 to 6 kGy; (…) 12.5 to 35 kGy.

Fig. 4. SejaBL fluorescence treated with bis-ANS. (**A**) bis-ANS Fluorescence; relative intensity (%) with (▲) 01 h; (O) 24 h; ( ) 7 days after irradiation. (**B**) Center mass. The

error in the determination of the fluorescence from the relative peak areas of the integral intensities to different doses is approximately ± 1%, smaller than the size of the symbols. (**C**), (**D**) and (**E**) fluorescence with 1 h, 24 h and 7 days after irradiation, respectively. Excitation 360 nm. Emission 400–600 nm. (▬) control non-irradiated; (---) 0.02 to 0.075 kGy; (–) 0.02 to 6 kGy; (…) 12.5 to 35 kGy.

Figure

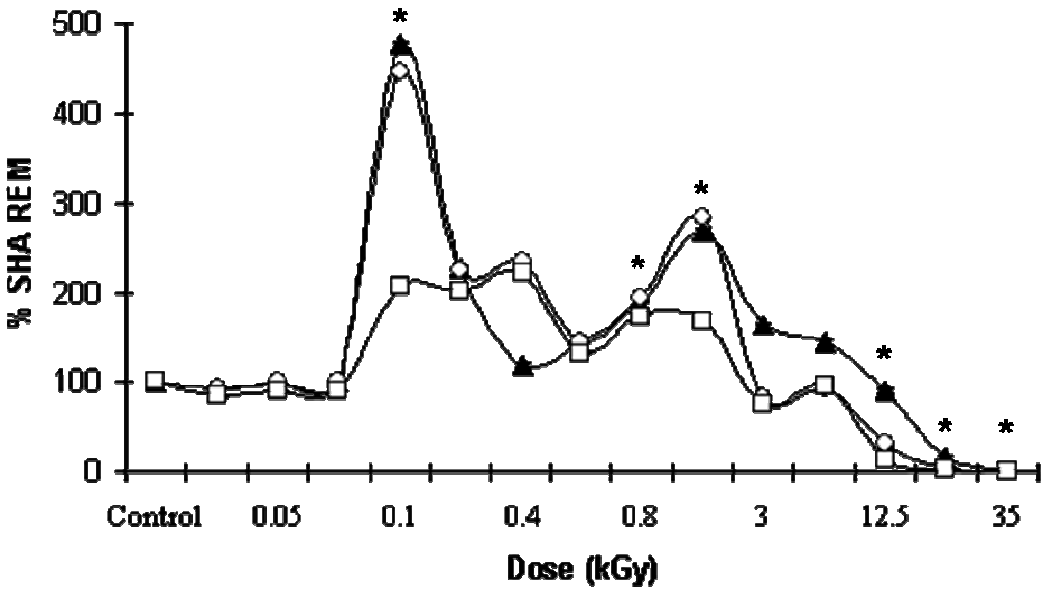


Figure 1

Figure

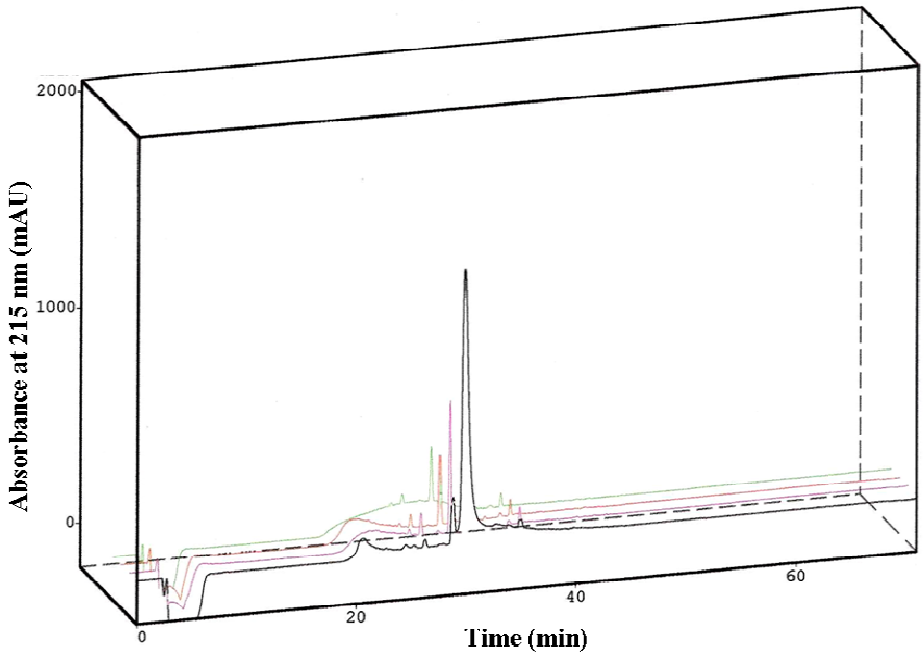


Figure 2

Figure

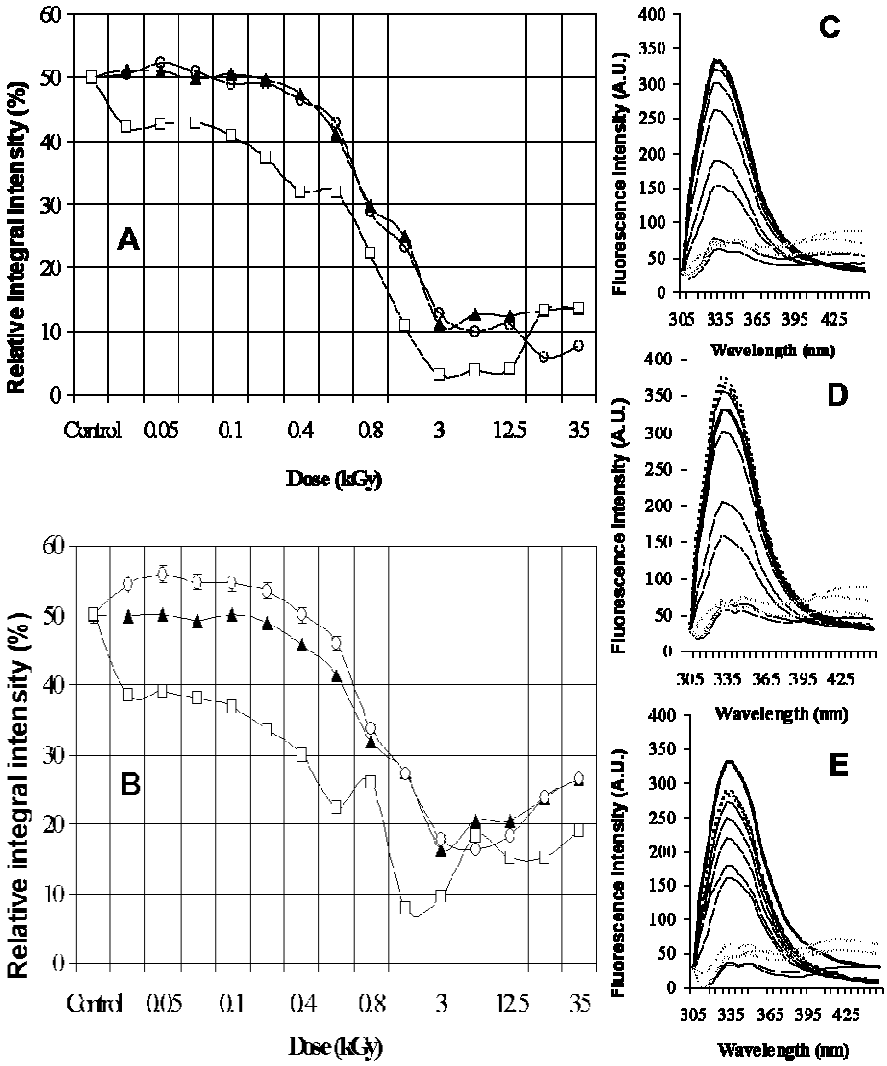


Figure 3

Figure

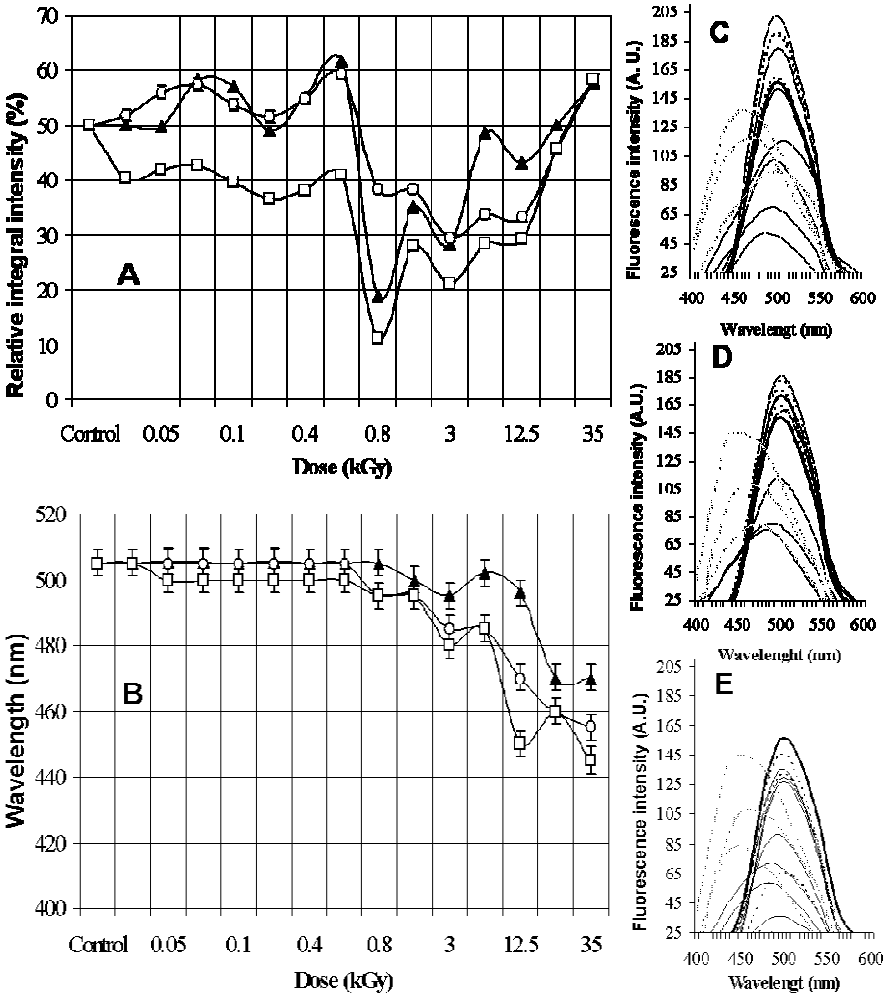


Figure 4

Capítulo 3

Artigo a ser submetido à publicação

Radiation Physics and Chemistry

Impacto: 0.934 (2007)

http://www.elsevier.com/wps/find/journaldescription.cws_home/331/

Manuscript

Original Paper

Radical scavenger ability of flavonoids isolated from *Sebastiania jacobinensis* with protective action on protein oxidation after gamma irradiation

A. F. M. Vaz [a,*], A. C. C. M. Argolo [a], A. M. M. A. Melo [b], M. L. V. Oliva [c], H. S. Xavier [d], L. C. B. B. Coelho [a] and M. T. S. Correia [a]

[a] Department of Biochemistry, Federal University of Pernambuco, Recife, PE, Brazil. E-Mail: melo_vaz@hotmail.com

[b] Department of Biophysic and Radiobiology, Federal University of Pernambuco, Recife, PE, Brazil.

[c] Department of Biochemistry, Federal University of São Paulo, São Paulo, SP, Brazil.

[d] Department of pharmaceutical science, Federal University of Pernambuco, Recife, PE, Brazil.

* Author to whom correspondence should be addressed; E-Mail: melo_vaz@ig.com.br; Tel. +5581-2126-8540; Fax: +5581-2126-8576

Abbreviations used: CM-cellulose, Carboxymethyl cellulose; DEAE-Cellulose, diethylaminoethyl cellulose; DPPH, 1.1-diphenyl-2-picrylhydrazyl; SDS–PAGE, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SejaBL, *Sebastiania jacobinensis* bark Lectin; SejaBF, *Sebastiania jacobinensis* bark Flavonoids. TLC, Thin Layer Chromatographic; %HA, percentage relative Hemagglutinating Activity.

Abstract: Flavonoids are ubiquitous plant secondary products with radical scavenger ability. *Sebastiania jacobinensis* bark lectin (SejaBL), protein capable of binding to carbohydrates, has their hydrophobic surface modified by free radicals produced via water radiolysis. In the present study the protective effect of *S. jacobinensis* bark flavonoids (SejaBF) on SejaBL activity and their antioxidant stability after gamma irradiation were evaluated. SejaBF showed fast scavenger ability measured by 2.2-diphenyl-1-picrylhydrazyl radical (DPPH) after high doses of radiation and reduced by 55% the damage caused by irradiation on SejaBL activity. The results showed radiostability of antioxidant capacity of the isolated flavonoids (SejaBF) protecting the lectin from free radicals formed after the irradiation.

Keywords: Lectin; Flavonoids; DPPH radical scavenger capacity; Gamma irradiation

1. Introduction

Radiation treatments of biological materials have been applied in various fields such as sterilization of materials and food for reducing microbial contamination. Gamma irradiation can damage biomolecule by initial rupture covalent bonds as a direct result of the depositing photon energy, and indirectly, by producing free radicals and other non-radical reactive oxygen species (Kempner, 2001). Investigations showed that free radicals cause lipid peroxidation (Terao et al., 1994), structural damage proteins with biological activity abolished (Cho and Song, 2000), DNA damage (Wallace, 1998), and oxidative stress (Aruoma, 1998) making the recovery of biologically active materials unlikely. However, chemical changes caused by irradiation can be prevented or reversed by use effective and radiostable antioxidant agents.

Flavonoids are among the most ubiquitous of plant secondary metabolites groups acting on reproduction and protection of plants (Winkel-Shirley, 2002). Their low toxicity (Zhang et al., 2005), high antioxidant activity (Garcia-Alonso et al., 2005), ultraviolet protection (Bieza and Lois, 2001), anti-inflammatory (Lin et al., 2008), antiviral (Yamaguchi et al., 2001) and antitumour activities (Hou, 2003) have been documented. Flavonoids are

subdivided into flavones, flavonol, flavanone, and flavanol types depending on the pattern of substitution: hydroxylation or methoxylation.

Irradiated flavonoid solutions produce chemical reactions between the solute and reactive species formed from the radiolyzed solvent operating in the fast repair of chemical modifications on biomolecules (Hanasaki et al., 1994; Kozlowski et al., 2007). One of the most important probes, to evaluate the fast repair antioxidant capacity is the 2.2-diphenyl-1-picrylhydrazyl radical (DPPH), a stable nitrogen synthetic radical (Brand-Williams et al., 1995).

Lectins constitute a class of proteins that possess a non-catalytic domain capable of specific recognition and binding with carbohydrates (Sharon and Lis, 2004). The number of roles has been proposed, including stimulation of cell proliferation (Peumans and Van Damme, 1998), carbohydrate transport (Hsu and Liu, 2004), plant-microbe interactions (Limpens and Bisseling, 2003), and in plant defense (Peumans and Van Damme, 1995). So far, lectins have been isolated and characterized on the basis of hemagglutinating activity.

S. jacobinensis bark lectin (SejaBL) is a protein which loses their activate front free radicals produced by water radiolysis due to modification in hydrophobic surfaces. *Sebastiania jacobinensis* Müll. Arg. (Euphorbiaceae family) is a tree which is resistant to the arid climate and common in tropical regions of Brazil. Therefore, the aim of our study was to evaluate the protective effect on SejaBL, free radical scavenging ability and radiostability of flavonoids isolated from *S. jacobinensis* bark after ample dose of γ-radiation.

2. Experimental

2.1. Materials

S. jacobinensis bark was collected from trees in the semi-arid region, state of Pernambuco, Brazil. Reference samples of (+)-catechin, 1.1-Diphenyl-2-picrylhydrazyl radical (DPPH), DEAE-Cellulose and ascorbate were purchased from Sigma Chemical Co., (St. Louis, MO). All the solvents and other chemicals used were of analytical grade from Merck (Darmstadt, Germany). All solutions were made with water purified by the Milli-Q system.

2.2. Flavonoids isolation

S. *jacobinensis* bark was mixed in a beaker with 250 mL of ethanol/acetic acid/water (70:4:26, v/v/v) for 2 h at 4 °C (this was carried out three times to extract all phenolic compounds; a fourth extraction did not extract any phenolic compounds detectable in the amount). The three extracts were combined (750 mL) vacuum-filtered through filter paper (Whatman N°. 1), concentrated to dryness under reduced pressure (40 °C) and re-dissolved in 10 mL ethanol/water (70:30, v/v). The extract containing flavonoids was chromatographed on an anion-exchange DEAE-Cellulose column (40 x 2.5 cm) equilibrated with ethanol-water (70:30, v/v). Elution was performed with ethanol-acetic acid-water (70:4:26, v/v/v) at a flow rate of 2 mL min^{-1}. Each fraction collected was checked by TLC for their compositions using ethyl acetate/formic acid/acetic acid/water (100:11:11:26, v/v/v/v) for development and vanillin dipping in chloridic acid was used for detection under UV 254 nm. TLC conditions were optimized using (+)-catechin standard. The obtained flavonoids were denominated SejaBF.

2.3. LC-ESI-MS

The analysis of SejaBF was revealed by LC analyses (Waters) with a diode-array detector (LC-UV), in Deltapak C18 column (2.0 mm x 150 mm; 3.5 µm size; 60 Å; Walters) at a flow rate of 0.4 mL min^{-1} keeping the column at a constant temperature (30 °C) and using 0.1% TFA/H_2O (solvent A) and 90% ACN/0.1% TFA/H_2O (solvent B) as solvents. A linear gradient elution was used with 5-95% B in A at 30 min. The molecular mass was determined by electrospray ionization ion-trap mass spectrometry on a Waters LC/ESI+/MS scanning of 200-2500 m/z.

2.4. Purified lectin from Sebastiania jacobinensis and Analyses

S. jacobinensis bark was homogenized overnight at 4 °C in 10 mM Tris–HCl buffer (pH 8.5) with 0.2% (v/v) Triton X-100 detergent. The homogenate was centrifuged 5,000g for

20 min (crude extract) followed by lyophilization. In brief, two volumes of cold acetone were added, mixed well and kept on ice for 10 min. The solution was centrifuged at 10,000g for 20 min at 4 °C. The acetone precipitate was separate. The supernatant was concentrated under vacuum until total evaporation of the acetone. The supernatant obtained was precipitated with 60% $(NH_4)_2SO_4$. The precipitate was dialyzed against Tris–HCl buffer. This fraction was applied to a CM-Cellulose column (2 x 20 cm) previously equilibrated with Tris–HCl buffer into 150 mM of NaCl. Following removal of the unadsorbed proteins, the column was eluted with 1 M acetic acid. The largest fractions of absorbance eluted with acetic acid were pooled, dialyzed and concentrated by ultrafiltration (Amicon ultra-15, Mr 10,000 cut-off) and chromatographed on a Sephadex G-100 column (2 x 70 cm) equilibrated with 150 mM NaCl. The column was eluted with the same solution at a flow rate of 0.5 mL min^{-1}. The primary unadsorbed fraction (SejaBL) was dialyzed by ultrafiltration against 10 mM phosphate buffer and stored. Hemagglutinating activity (HA) was evaluated as described by Correia and Coelho (1995), the lowest sample dilution showing hemagglutination. The percentage of remaining HA ($\%HA_{REM}$) was calculated according to equation:

$$\%HA_{REM} = [HA]_G / [HA]_{G0} \times 100$$

where G is the lectin HA of each irradiated dose (0.02-35 kGy) and G_0 is the non-irradiated lectin HA (control).

The molecular mass profile of irradiated lectin was determined using SDS-PAGE (Laemmli, 1970). Equal amounts of protein samples (200 µg) were loaded, resolved on a 10% separation gel and stained with silver kit (Bio-Rad). Protein Markers: Rabbit muscle myosin (205 kDa), E. coli β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), rabbit muscle fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), egg albumin (45 kDa), rabbit muscle gliceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybeam trypsin inhibitor (20 kDa), bovine milk α-lactalbumin (14.2 kDa) and bovine lung aprotinin (6.5 kDa).

2.5. Gamma irradiation

The lectin 0.5 mg mL^{-1} in 10 mM phosphate buffer (pH 7.0) was added in borosilicate glass vials (16-125 mm) with and without SejaBF (1 mg mL^{-1}). The mixtures were frozen and irradiated into atmospheric O_2, using a ^{60}Co gamma ray irradiator Gammacell 220 Excel (Ontario, Canada). The doses were 0.020; 0.050; 0.075; 0.1; 0.2; 0.4; 0.6; 0.8; 1.0; 3.0; 6.0; 12.5; 25; 35 kGy. The dose rate was 8.8 kGy/h. Each sample was analyzed 1 h, 24 h and 7 days after exposition to HA.

2.6. Determination of antioxidant activity

Spectrophotometric analysis was used to determine the inhibition concentration (IC_{50}) and the inhibition percentage (IP). IC_{50} is the amount of antioxidant necessary to decrease the initial concentration of DPPH radical (75 µM) by 50%. IP is the percentage of total DPPH radical (75 µM) which reacted with antioxidant at the steady state (20 min). The experiments were performed using the SmartSpec 3000 spectrophotometer (Bio-Rad) according to the methods of Soler-Rivas et al. (2000) and Moure et al. (2001). An aliquot (20 µL) of SejaBF irradiated in doses of 0.02; 0.8; 3; 35 kGy and not irradiated (control) at 0.5 mg mL^{-1} were mixed separately with 75 µM methanolic DPPH radical solution to a final volume of 1 mL. Pure methanol was used as negative control. (+)-Catechin and ascorbate in methanol (1 mg mL^{-1}) were used as positive control. The disappearance of the DPPH radical was monitored by the decrease in absorbance at 515 nm, which was recorded after 0; 1; 2; 3; 4 and 5 min, and subsequently every 5 min up to 30 min, during which time the radical was stable. The DPPH radical concentration in the reaction mixture was calculated by the calibration curve according to the following nonlinear regression equation (R= 0.997): $A_{515\ nm} = 0.01029\ [DPPH] - 0.0177$, where [DPPH] is expressed in mg mL^{-1}. The percentage of remaining DPPH ($\%DPPH_{REM}$) was calculated according to Brand-Williams et al. (1995), as follows:

$$\%DPPH_{REM} = [DPPH]_T / [DPPH]_{To} \times 100$$

where T is the time when absorbance was determined (1–30 min) and T_0 is the time zero.

IC_{50} was calculated by plotting the $\%DPPH_{REM}$ at the steady state (20 min) against various concentrations of each sample (1, 0.5, 0.25, 0.125, 0.0625 mg mL^{-1}). The results were expressed by mg antioxidant/g DPPH ± standard deviation. For determination of IP, an aliquot (50 µL) of SejaBF was added to 2 mL of 75 µM methanolic solution of the DPPH radical and the absorbance was determined at 515 nm at the steady state (20 min). IP was calculated according to the expression:

$$IP = [(A_{To} - A_{Ts})/A_{To} \times 100$$

where A_{T0} is the absorbance at time zero and A_{TS} is the absorbance at the steady state (defined as the state when the absorbance of the sample remained stationary). The results were expressed by % of inhibition.

2.7. Data Analysis

Data are presented as mean ± S.E.M. The curve and its 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GraphPrism® (GraphPad Software Inc., San Diego, CA, USA). Student's t-test for paired data was developed and significant difference between groups was analyzed at $p < 0.05$.

3. Results and Discussion

Two flavonoids were isolated from *S. jacobinensis* bark extracts by combination of anion-exchange DEAE-Cellulose, HPLC and TLC chromatographys (Fig. 1), denominated *S. jacobinensis* bark flavonoids (SejaBF). Figure 2 shows a positive molecular ion [(m/z) = $M+H^+$] to 613 m.u. corresponding to epigallocatechin dimmer and a non-identified peak [(m/z) = $M+H^+$ 702 m.u.] that can be heterogeneous dimer of monogalloylated procyanidin. The identification of heterogeneous dimers by antioxidant capacity and HPLC/MS protocol was demonstrated for analysis of the procyanidins (Ferreira and Slade, 2002).

SejaBF exhibited high antioxidant capacity by DPPH method when compared to ascorbate, antioxidant widely used as standard, however, its antioxidant activity decreased after irradiation, in relation to the IC_{50} and IP, available in 20 min (Table I). IP reduction (19.2% to high dose) was significant although the IC_{50} reduction was not.

DPPH radical scavenger capacity ($\%DPPH_{REM}$) was significantly reduced only in the initial 5 min after high dose of radiation (Fig. 3). The DPPH radical is scavenged by antioxidants through donation of hydrogen to form stable reduced DPPH molecule. The radical scavenger is reduced after high doses of gamma radiation due to displacement from the B-ring to the 2.3-double bond, indicating a relatively small reactivity (because of the absence of high density spin on a given site) for these intermediate species (Zhao et al., 2002; Marfak et al., 2003). Our result is in good agreement with the presence of stabilized quinones, which has been observed after H-abstraction from the B-ring of flavonoids in radiolytic solutions (Pannala et al., 2001).

Gamma irradiation of SejaBL aqueous solution showed significant loss ($p < 0.05$) of HA with high dose of gamma rays compared to the control (non-irradiated), but the damage caused by irradiation on the activity of lectin was minimized by 55% in the presence of SejaBF in high dose (Fig. 4). The lectin activate protecting can be explained by the high radical scavenger capacity of flavonoids always after irradiation. This antiradical mechanism in phenol-protein aggregates might be due to the ability of phenolic compounds to transfer oxidative damage from one phenolic site to another, protecting proteins and amino acids from oxidation (Franzini et al., 1993; Xu et al., 2003; Štajner et al., 2008). In a study with 42 lectins, Barre et al. (2002) evidenced common hydrophobic domains, suggesting strong structural conservation, with secondary metabolites binding nature of these domains. Thus, flavonoids might have a protective effect on hydrophobic domains easily attacked by active species of water radiolysis (Steinhart, 1991).

The change in terms of molecular weight pattern in irradiated lectin was significant, with the formation of degraded small molecular weight to high dose of gamma radiation (Fig. 5). The degradation of lectin (MW 52,000 Da) was lower in the samples with the antioxidant SejaBF. Usually, breakage of covalent bonds into irradiated proteins shows new bands below the major band (Le Maire et al., 1990; Xu and Chance, 2005).

4. Conclusions

SejaBF prevented the lectin oxidation by free radicals, due to possible binding to the hydrophobic domain and fast repair of groups sensitive to ROS. In summary, this study demonstrated the special protective effect on protein and the high antioxidant capacity of the obtained flavonoids after irradiation.

Acknowledgement

This research was financially supported by the Brazilian Council for Scientific and Technological Development (CNPq) for research grants and Ministry of Science and Technologies of Brazil, Higher Education Improvement of Coordination (CAPES). We are very thankful to Dr. Antônio Miranda from the Federal University of São Paulo (UNIFESP) for the mass spectrometry measurements.

References

Aruoma, O.I., 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. J. Am. Oil Chem. Soc. 75, 199-212.

Barre, A., Hervé, C., Lescure, B., Rougé, P., 2002. Lectin receptor kinases in plants. Plant Sci. 21, 379-399.

Bieza, K., Lois, R., 2001. An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. Plant Physiol. 126, 1105-1115.

Brand-Williams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol. 28, 25-30.

Cho, Y., Song, K.B., 2000. Effect of γ-irradiation on the molecular properties of BSA and β-lactoglobulin. J. Biochem. Mol. Bio. 33, 133-137.

Correia, M.T.S., Coelho, L.C.B.B., 1995. Purification of a glucose/manose specific Lectin, isoforma 1, from seeds of *Cratylia mollis* Mart. (Camaratu bean). App. Biochem. Biotech. 55, 261-273.

Ferreira, D., Slade, D., 2002. Oligomeric proanthocyanidins: naturally occurring O-heterocycles. Nat. Prod. Rep. 19, 517–541.

Franzini, E., Sellak, H., Hakim, J., Pasquier, C., 1993. Oxidative damage to lysozyme by the hydroxyl radical: comparative effects of scavengers. Biochim. Biophys. Acta. 1203, 11-17.

Garcia-Alonso, M., Rimbach, G., Sasai, M., Nakahara, M., Matsugo, S., Uchida, Y., Rivas-Gonzalo, J.C., de Pascual-Teresa, S., 2005. Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins. Mol. Nutr. Food Res. 49, 1112-1119.

Hanasaki, Y., Ogawa, S., Fukui, S., 1994. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radic. Biol. Med. 16, 845-850.

Hou, D.X., 2003. Potential mechanisms of cancer chemoprevention by anthocyanins. Curr. Mol. Med. 3, 149-159.

Hsu, D.K., Liu, F.T., 2004. Regulation of cellular homeostasis by galectins. Glycoconj. J. 19, 507-515.

Kempner, E.S., 2001. Effects of high-energy electrons and gamma rays directly on protein molecules. J. Pharm. Sci. 90, 1637-1646.

Kozlowski, D., Marsal, P., Steel, M., Mokrini, R., Duroux, J.L., Lazzaroni, R., Trouillasa, P., 2007. Theoretical Investigation of the Formation of a New Series of Antioxidant depsides from the Radiolysis of Flavonoid Compounds. Radiation Res. 168, 243–252.

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

Le Maire, M., Thauvette, L., De Foresta, B., Viel, A., Beauregard, G., Potier, M., 1990. Effects of ionizing radiations on proteins: evidence of non-random fragmentations and a caution in the use of the method for determination of molecular mass. Biochem. J. 267, 431-439.

Limpens, E., Bisseling, T., 2003. Signaling in symbiosis. Cur. Op. Plant Biol. 6, 343-350.

Lin, J.Y., Li, C.Y., Hwang, I.F., 2008. Characterisation of the pigment components in red cabbage (*Brassica oleracea* L. var.) juice and their anti-inflammatory effects on LPS-stimulated murine splenocytes. Food Chem. 109, 771-781.

Marfak, A., Trouillas, P., Allais, D.P., Champavier, Y., Calliste, C.A., Duroux, J.L., 2003. Radiolysis of kaempferol in water/methanol mixtures. Evaluation of antioxidant activity of kaempferol and products formed. J. Agric. Food Chem. 51, 1270-1277.

Moure, A., Franco, D., Sineiro, J., Dominguez, H., Núñes, M.J., Lema, J.M., 2001. Antioxidant activity of extracts from *Gevuina avellana* and *Rosa rubiginosa defatted* seeds. Food Res. Int. 34, 103-109.

Pannala, A.S., Chan, T.S., O'Brien, P.J., Rice-Evans, C.A., 2001. Flavonoid B-ring chemistry and antioxidant activity: Fast reaction kinetics. Biochem. Biophys. Res. Commun. 282, 1161-1168.

Peumans, W.J., Van Damme, E.J.M., 1995. Lectins as plant defense proteins. Plant physiol. 109, 347-352.

Peumans, W.J., Van Damme, E.J.M., 1998. Plant lectins versateli proteins with important perspectives in biotechnology. Biotech. Gen. Eng. Rev. 15, 199-228.

Sharon, N., Lis, H., 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycob. 14, 53-62.

Soler-Rivas, C., Espın, J.C., Wichers, H.J., 2000. An easy and fast test to compare total free radical scavenger capacity of foodstuffs. Phytochem. Anal. 11, 1-9.

Štajner, D., Milošević, M., Popović, B.M., 2008. Irradiation Effects on Phenolic Content, Lipid and Protein Oxidation and Scavenger Ability of Soybean Seeds. Int. J. Mol. Sci. 8, 618-627.

Steinhart, H., 1991. Stability of tryptophan in peptides against oxidation and irradiation. Adv. Exp. Med. Biol. 294, 29-40.

Terao, J., Piskulaand, M., Yao, Q., 1994. Protective effect of Epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. Arch. Biochem. Biophys. 308, 278-284.

Wallace, S.S., 1998. Detection and repair of DNA base damages produced by ionizing radiation. Environ. Mol. Mutagen. 12, 431-477.

Winkel-Shirley, B., 2002 Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. 5, 218-223.

Xu, G., Chance, M.R., 2005. Radiolytic Modification of Sulfur-Containing Amino Acid Residues in Model Peptides: Fundamental Studies for Protein Footprinting. Anal. Chem. 77, 2437-2449.

Xu, G., Takamoto, K., Chance, M.R., 2003. Radiolytic Modification of Basic Amino Acid Residues in Peptides: Probes for Examining Protein-Protein Interactions. Anal. Chem. 75, 6995-7007.

Yamaguchi, K., Honda, M., Ikigai, H., Hara, Y., Shimamura, T., 2002. Inhibitory effects of (−)-epigallocatechin gallate on the life cycle of human immunodeficiency virus type 1 (HIV-1). Antiviral Res. 53, 19-34.

Zhang, Y., Vareed, S.K., Nair, M.G., 2005. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. Life Sci. 76, 1465-1472.

Zhao, C., Shi, Y., Wang, W., Lin, W., Fan, B., Jia, Z., Yao, S., Zheng R., 2002. Fast repair activities of quercetin and rutin toward dGMP hydroxyl radical adducts. Radiat. Phys. Chem. 63, 137-142.

Table 1

Table 1 Radical scavenging performance.

Samples	IP (%)	IC_{50} (mg/ g DPPH)
SejaBF 20 Gy	89.1313[a#]	0.115 ± 0.002[a]
SejaBF 800 Gy	88.3073[a#]	0.112 ± 0.002[a]
SejaBF 3 kGy	79.6359[b#]	0.180 ± 0.002[a]
SejaBF 35 kGy	70.886[c#]	0.300 ± 0.004[a]
SejaBF	90.1122[a]	0.126 ± 0.004[a]
(+)-catechin	90.8577[a]	0.206 ± 0.000[a]
Ascorbate	39.2215[d]	1.317 ± 0.002[b]

Values with the same superscript letter are not significantly different at $p < 0.05$ (Student's T-test). IC_{50} and IP (0.5 mg mL^{-1}) of SejaBF were calculated at the steady state (20 min). [#] Values of IP (%) are significantly different at $p < 0.05$ (Student's T-test) in relation to non-irradiated SejaBF.

Figure

Figure captions

Fig. 1. Isolation of flavonoids from *S. jacobinensis* (SejaBF). **(a)** Analysis by thin-layer chromatography: (1) Extract ethanolic, (2) SejaBF isolated from DEAE-Cellulose chromatography and (3) (+)-catechin standard. **(b)** SejaBF reverse phase chromatography in C-18 column by HPLC system (Waters).

Fig. 2. *S. jacobinensis* flavonoids (SejaBF) ESI-MS spectra. Purified flavonoids in LC/MS with retention time of 10.04 min (a) and 9.59 min (b).

Fig. 3. Kinetic behaviour of irradiated antioxidants. The error in the determination of $\%DPPH_{REM}$ to relative values of different doses is approximately ± 1%, smaller than the size of the symbols. * Values significantly different at $p < 0.05$ (Student's T-test) in relation to (○) non-irradiated *S. jacobinensis* flavonoids (SejaBF). SejaBF after irradiation by: (x) 35 kGy; (▲) 3 kGy; (■) 800 Gy; ( ) 20 Gy; (●) Ascorbate and (+)-catechin.

Fig. 4. Effect of γ-radiation on *S. jacobinensis* lectin (SejaBL) activity. %HA (▲) 01 h; (O) 24 h and ( ) 7 days after irradiation. SejaBL with absence **(a)** and presence **(b)** *S. jacobinensis* flavonoids (SejaBF). * Significant difference ($p < 0.05$) in relation to the non-irradiated (i.e., the control). # Significant difference ($p < 0.05$) in relation to the groups without **(a)** and with **(b)** antioxidant (SejaBF).

Fig. 5. SDS–PAGE profile of irradiated *S. jacobinensis* lectin (SejaBL). (1) Protein markers. Irradiated SejaBL without SejaBF (even numbers): (2) 35 kGy; (4) 3 kGy; (6) 800 Gy and (8) 20 Gy. Irradiated SejaBL in SejaBF presence (odd numbers): (3) 35 kGy; (5) 3 kGy; (7) 800 Gy and (9) 20 Gy. (10) Non-irradiated SejaBL. Arrows indicate the change on irradiated SejaBL molecular weight with formation of degraded small peptide, after high dose of gamma radiation.

Figure

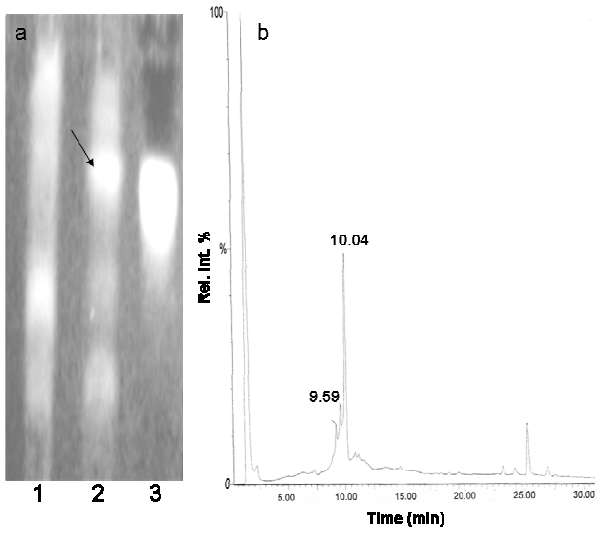


Figure 1

Figure

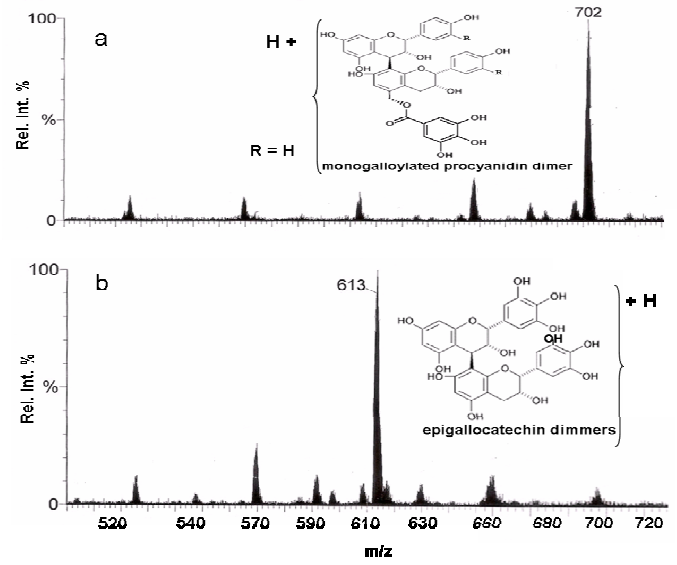


Figure 2

Figure

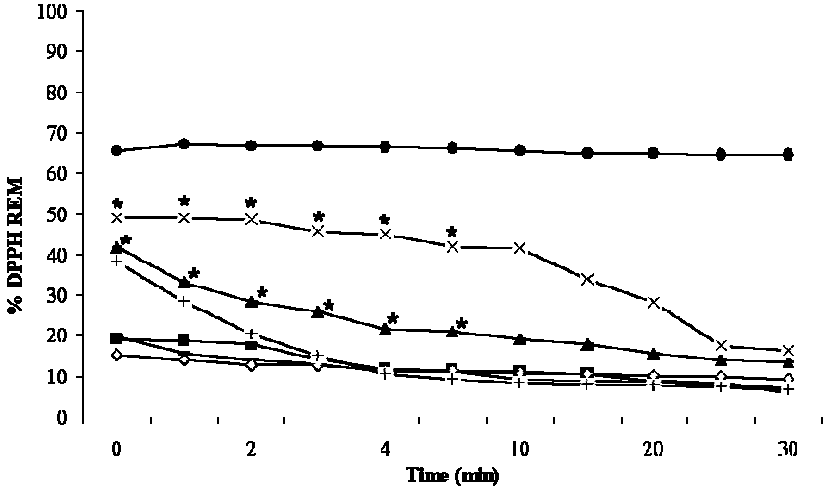


Figure 3

Figure

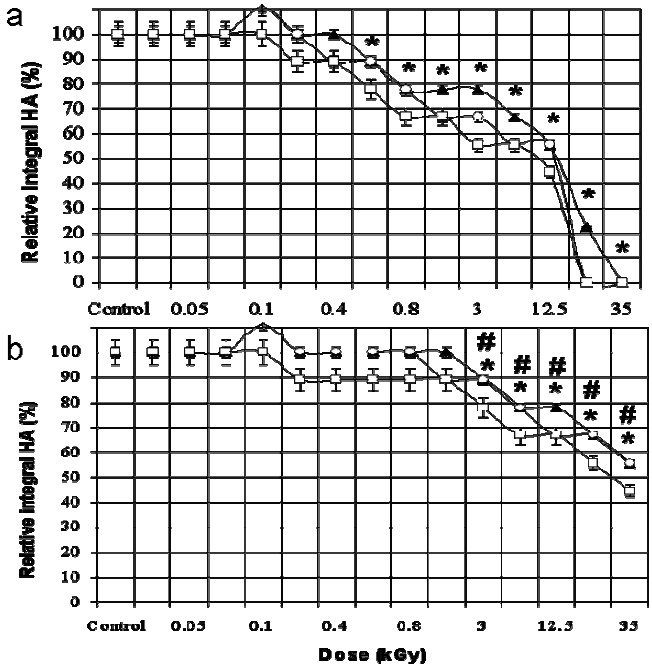


Figure 4

Figure

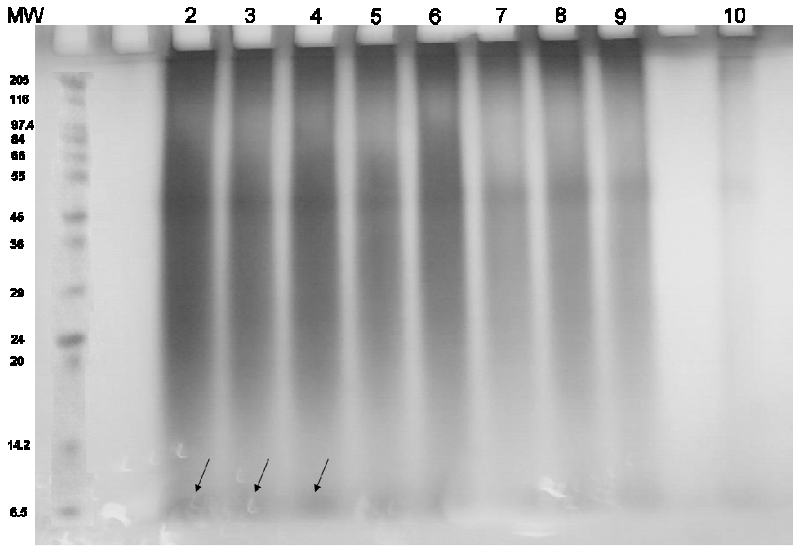


Figure 5

6. CONCLUSÃO

- Num protocolo eficiente, cromatografias em CM-Celulose e Sephadex G-100 isolam uma lectina da entrecasca de *Sebastiania jacobinensis* (SejaBL);
- SejaBL é termoresistente, não dependente de íons, estável em pH ácido, glicosilada, com resistência a proteinases e inibe fortemente tripsina;
- SejaBL possui massa molecular (52 kDa) composta por duas subunidades de 24 kDa não homologas, superfície hidrofóbica e perfil homogêneo;
- A lectina em baixa concentração promove ação antifúngica, atuando como um inibidor do crescimento de espécies de Fusarium; SejaBL também promove ação antifúngica seletiva, pois organismos não alvos não foram afetados podendo ser uma ferramenta útil em estudos de biotecnologia vegetal sem agredir a diversidade ambiental;
- SejaBL perdeu totalmente AHE após exposição a altas doses de radiação gama;
- SejaBL exibiu a partir da dose de 6 kGy desestruturação molecular e perda de AHE;
- A fluorescência intrínseca mostrou uma eliminação da emissão de fluorescência quando excitada a 280 e 295 nm, indicando alterações químicas em aminoácidos aromáticos;
- A fluorescência do bis-ANS mostrou uma emissão blue shifts a 490 nm em altas doses de radiação evidenciando a ação de radicais livres sobre superfície hidrofóbica protéica;
- Dois flavonóides foram isolados exibindo alta capacidade para capturar radicais;
- Os flavonóides exibiram boa estabilidade frente irradiação gama;
- SejaBL exibiu um menor queda da AH na presença dos flavonóides após irradiação;
- A lectina mostrou diferentes perfis de oxidação protéica na presença do antioxidante pela possível conservação de domínios hidrofóbicos.

ANEXOS

Anexo 1: Normas do periódico especializado, ao qual o trabalho da dissertação será submetido (BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS).

Anexo 2: Normas do periódico especializado, ao qual o trabalho da dissertação será submetido (ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS).

Anexo 3: Normas do periódico especializado, ao qual o trabalho da dissertação será submetido (RADIATION PHYSICS AND CHEMISTRY).

7.1. Anexo 1: BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

Guide for Authors
Biochemical and Biophysical Research Communications (BBRC) is devoted to the rapid dissemination of timely and significant observations in the diverse fields of modern experimental biology. Articles submitted should be written to emphasize clearly the novel aspects of the information reported.

Submission of Manuscripts
It is a condition of publication that all manuscripts must be written in clear and grammatical English and be submitted to the BBRC Web site at http://ees.elsevier.com/bbrc. Authors are requested to transmit the text and art of the manuscript in electronic form to this address. No exceptions will be allowed; manuscripts submitted via any other method will be returned. Each manuscript must also be accompanied by a cover letter outlining the basic findings of the paper and its significance. Complete instructions, including instructions for proper artwork format, are available at the submission Web site. Authors who have questions regarding the electronic submission process should contact the Editorial Office prior to submission (e-mail: bbrc@elsevier.com; telephone: (619) 699-6359; or fax: (619) 699-6859).

There are no submission fees or page charges.

Peer Review Policy
The practice of peer review is to ensure that good science is published. It is an objective process at the heart of good scholarly publishing and is carried out on all reputable scientific journals. Our Editorial Board therefore plays a vital role in maintaining the high standards of BBRC while ensuring that it retains the speed of publication necessary for a rapid communication journal.

Type of Peer Review
BBRC is a rapid communications journal. As such, the decision to publish an article rests entirely with the handling Editor. Manuscripts are assigned to members of the Editorial Board based on expertise. This Editor may accept the manuscript as it is, send it to a colleague for review, or reject it. Requests for revisions are rare. Should the Editor request revisions, the manuscript will be treated as a new submission.

How long does the review process take?
Authors of manuscripts can expect an accept or reject decision normally within 2 weeks of receipt. Publication will then take place immediately unless the author has, upon submission, requested an embargo.
Manuscripts are accepted for review with the understanding that no substantial portion of the study has been published or is under consideration for publication elsewhere and that its submission for publication has been approved by all of the authors and by the institution where the work was carried out. Redundant or duplicate publication in another peer-reviewed primary research journal of the same paper (or substantially similar paper)

reporting the same results arising from the same research work does not serve the research or library community well, and any such publication will be subject to review by the editor and to the retraction and removal policies of the journal. Manuscripts that do not meet the general criteria or standards for publication in BBRC will be immediately returned to the authors, without detailed review.
Any manuscript rejected by one editor may not be resubmitted to a different editor; any revised version should be sent to the original editor. Note that all revisions will be processed as new submissions.
Upon acceptance of an article, authors will be asked to transfer copyright (for more information on copyright, see http://www.elsevier.com/copyright). This transfer will ensure the widest possible dissemination of information. A letter will be sent to the corresponding author confirming receipt of the manuscript. A form facilitating transfer of copyright will be provided after acceptance.
If material from other copyrighted works is included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: contact Elsevier Global Rights Department, P.O. Box 800, Oxford OX5 1DX, UK; phone: (+44) 1865 843830, fax: (+44) 1865 853333, e-mail: permissions@elsevier.com.

Minireviews
This section includes minireviews, commentaries, and views on relevant subject matter, not simply short papers. Authors submitting a manuscript for this section should, in a cover letter, indicate that they are "submitting a manuscript for the Minireviews section." Minireviews generally are invited; however, authors may inquire about potential interest in a minireview. Manuscripts for the Minireviews section should be indicated as for the attention of William J. Lennarz. Articles should not exceed 9 to 10 double-spaced, typewritten pages (including tables, figures, and references). One page of figures in articles in Minireviews can be published in color, free of charge. The authors of minireviews may, if they wish, include a limited number of annotated references. Two hypothetical examples follow:
[1] J.Y. Smith, S.S. Doe, A novel retinoid-response gene set in vascular smooth muscle cells, J. Biol. 280 (2000) 5-8. [A very concise review of recent findings.]
[2] J.Y. Black, R.J. Blue, Magnetic field exposure induces DNA degradation, Biol. Acta 120 (2001) 20-29. [The first study presenting detailed information on the enzyme activity.]

Preparation of Manuscript
Manuscripts must be written in clear, concise, grammatical English. Authors unfamiliar with English usage are encouraged to seek the help of English-speaking colleagues in preparing their manuscripts or to use the services described below.
Language Editing: English language and copyediting services for authors who want to publish in scientific, technical and medical journals and need assistance before they submit their article or, before it is accepted for publication should visit http://www.elsevier.com/wps/find/authorshome.authors/languagepolishing. For more information about language editing services, please contact authorsupport@elsevier.com who will be happy to deal with any questions. Please note Elsevier neither endorses nor takes responsibility for any products, goods or services offered by outside vendors through

our services or in any advertising. For more information please refer to our terms and condition (http://www.elsevier.com/wps/find/termsconditions.cws_home/termsconditions).

Manuscript length: Manuscripts should be double-spaced throughout, with a minimum of 1-inch margins. The length of the article when published will not exceed 6 printed journal pages, including all figures and tables. To achieve this the submitted article length must be no greater than 4,200 words (or 19,000 characters with spaces, whichever is the greater) and 4 figures (n.b. any figure larger than half a page will be counted as two figures). For information on article length for Breakthroughs and Views, refer to the Minireview paragraph. Pages should be numbered consecutively and organized as follows:
The Title Page (p. 1) should contain an article title of less than 75 characters, the authors' names and complete affiliations, any footnotes to the title, and the name and address for manuscript correspondence (including e-mail address and fax numbers). Note that the title should not contain any jargon or nonstandard abbreviations. The corresponding author must confirm that the correspondence address in the manuscript agrees with the address in the accompanying letter.
Please ensure that all authors are properly listed and identified. No additions or deletions to the author line will be permitted after the initial submission of the paper.
The Abstract (p. 2) must be a single paragraph that summarizes the main findings of the paper in fewer than 150 words. After the abstract a list of up to 10 keywords that will be useful for indexing or searching should be included.

The Introduction should be as concise as possible, without subheadings.

Materials and methods should be sufficiently detailed to enable the experiments to be reproduced.

Results and Discussion may be combined and may be organized into subheadings.

Acknowledgments should be brief and should precede the references.

References to the literature should be cited by numbers in square brackets in the text and listed in numerical order with full article titles included. Use the most recent edition of the Chemical Abstracts Service Source Index for abbreviations of journal titles.
Reference to a journal publication:
[1] A.G. Mueller, H.G. Joost, A. Schurmann, Mouse ARF-related protein 1: genomic organization and analysis of its promoter, Biochem. Biophys. Res. Commun. 292 (2002) 113-120.
Reference to a book: [2] W. Strunk Jr., E.B. White, The Elements of Style, third ed., Macmillan, New York, 1979.
Reference to a chapter in an edited book: [3] O.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: E.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 1999, pp. 281-304.

Figures

Number figures consecutively with Arabic numerals. Please visit our Web site at http://www.elsevier.com/artworkinstructions for detailed instructions on preparing electronic artwork.

Color illustrations should be sized close to the size expected in publication. If, together with your accepted article, you submit usable files of color figures, Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.
Note: Because of technical complications that may arise in converting color figures to gray scale, for the printed version should you not opt for color in print, please submit in addition usable black-and-white files corresponding to all the color illustration files.

Tables
Tables should be numbered consecutively with Arabic numerals in order of appearance in the text. Type each table double-spaced on a separate page with a short descriptive title typed directly above and with essential footnotes below.

Protein or Gene Databases
The Editors encourage the use of databases as repositories for detailed data on protein structure (e.g., X-ray crystallographic coordinates) and on DNA sequences and mapping assignments. Verification of submission and, when available, pertinent accession numbers should be provided with the manuscripts. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should type this information in the following manner:
For each and every accession number cited in an article, authors should type the accession number in bold, underlined text. Letters in the accession number should always be capitalized. (See example below.) This combination of letters and format will enable the typesetter to recognize the relevant texts as accession numbers and add the required links to GenBank sequences.
Example: "GenBank accession nos. AI631510 , AI631511 , AI632198 , and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)."
Authors are encouraged to check accession numbers used very carefully. An error in a letter or number can result in a dead link. In the final version of the printed article, the accession number text will not appear bold or underlined. In the final version of the electronic copy, the accession number text will be linked to the appropriate source in the NCBI databases, enabling readers to go directly to that source from the article.

Chemical or Biological Hazards
Chemical or biological hazards, if any, in carrying out the experiments described should be clearly noted in the Materials and Methods section. Any relevant safety precautions should be described. If an accepted code of practice has been followed, a reference to the relevant standards should be given.

Ethics

When conducting scientific research using human tissue and which is intended for publication in BBRC, authors should follow procedures that are in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983). When conducting experiments on animals, authors should adhere to the local or national requirements for the care and use of laboratory animals.

Preparation of Supplementary Material
Elsevier now accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer additional possibilities for publishing supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips, and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect (http://www.sciencedirect.com). To ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. Please note, however, that supplementary material will not appear in the printed journal. For more details, please see http://ees.elsevier.com/bbrc.

Proofs
Corresponding authors will receive proofs by e-mail (PDF proofs). Because of the rapid publication schedule of BBRC, authors must check the proofs and return any corrections within 48 hours. Corrections should be restricted to typesetting errors, and all queries should be answered. Please proofread the manuscript carefully before initial submission.

Distribution of Material
Authors who publish a research article in BBRC must be prepared to freely distribute to academic researchers for their own use any cell lines, DNA clones, monoclonal antibodies, or genetically engineered mice described in the article.

Author Inquiries
Visit http://www.elsevier.com/authors for the facility to track accepted articles and set up e-mail alerts to inform you when an article's status has changed. The Elsevier site also provides detailed artwork guidelines, copyright information, frequently asked questions and more. Contact details for questions arising after acceptance of an article, especially those relating to proofs, are provided after registration of an article for publication.

March 2008 version

7.2. Anexo 2: ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS

Guide for Authors

Archives of BIOCHEMISTRY AND BIOPHYSICS is an international journal dedicated to the dissemination of fundamental knowledge in all areas of biochemistry and biophysics. Manuscripts that contain new and significant information of general interest to workers in these fields are welcome. Sufficient detail must be included to enable others to repeat the work. The journal also invites timely reviews.

- Decisions in under one month
- No page charges
- Completely electronic submission and review process
- One free page of color per article in print; all color is free in online version

Submission of manuscripts

It is a condition of publication that all manuscripts must be written in clear and grammatical English and be submitted to the Archives of Biochemistry and Biophysics Web site at http://ees.Elsevier.com/yabbi. Minimal exceptions will be allowed. If you are unable to provide an electronic version of your paper, please contact the Editorial Office prior to submission (e-mail: abb@elsevier.com; telephone; (619) 699-6218; fax: (619) 699-6211.

Each manuscript is to be accompanied by an electronic cover letter outlining the basic findings of the paper and their significance. PDFs of all related manuscripts under consideration for publication must also be included with the submitted manuscript.

Authors should suggest at least four competent reviewers in their field and may also suggest individuals whom they wish to have excluded from the review process. The list of suggested reviewers should be compiled as a separate document.

The corresponding author will receive a decision letter from the Executive Editor. Revised manuscripts are to be submitted electronically.

Many acceptable papers require minor revision or condensation. It is in the mutual interest of both the authors and the journal that amended manuscripts be returned promptly. A revised paper will retain its original date of receipt only if it is received by the Executive Editor within 2 months of the date of return to the author.

Acceptable file types. Most word-processing packages are acceptable; however, we prefer that authors use a recent version of Microsoft Word or Corel WordPerfect. Manuscripts saved with formatting intact are preferred. Rich-text format (.rtf extension) is acceptable, but plain text (.txt extension) files are discouraged. Submit each figure as a separate TIFF or EPS file.

Once a paper is accepted, ABB cannot use PDF or PostScript files because they do not allow editing of the text. Files created in layout programs such as Adobe FrameMaker or PageMaker, QuarkXPress, and Corel Ventura are unacceptable. Artwork should not be embedded within the manuscript. It must be supplied in electronic files separate from the manuscript file.

Manuscripts are accepted for review with the understanding that no substantial portion of the study has been published or is under consideration for publication elsewhere and that its submission for publication has been approved by all of the authors and by the institution where the work was carried out. Manuscripts that do not meet the general criteria or

standards for publication in Archives of Biochemistry and Biophysics will be immediately returned to the authors, without detailed review.
US National Institutes of Health (NIH) voluntary posting ("Public Access) policy. Elsevier facilitates author response to the NIH voluntary posting request (referred to as the NIH "Public Access Policy"; see http://www.nih.gov/about/publicaccess/index.htm) by posting the peer-reviewed author's manuscript directly to PubMed Central on request from the author, 12 months after formal publication. Upon notification from Elsevier of acceptance, we will ask you to confirm via e-mail (by e-mailing us at NIHauthorrequest@elsevier.com) that your work has received NIH funding and that you intend to respond to the NIH policy request, along with your NIH award number to facilitate processing. Upon such confirmation, Elsevier will submit to PubMed Central on your behalf a version of your manuscript that will include peer-review comments, for posting 12 months after formal publication. This will ensure that you will have responded fully to the NIH request policy. There will be no need for you to post your manuscript directly with PubMed Central, and any such posting is prohibited.
Copyright and permissions. Upon acceptance of an article, authors will be asked to transfer copyright (for more information on copyright, see http://authors.elsevier.com). This transfer will ensure the widest possible dissemination of information. A letter will be sent to the corresponding author confirming receipt of the manuscript. A form facilitating transfer of copyright will be provided after acceptance.
If material from other copyrighted works is included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: contact Elsevier Global Rights Department, P.O. Box 800, Oxford OX5 1DX, UK; phone: (+44) 1865 843830, fax: (+44) 1865 853333, e-mail: permissions@elsevier.com.

Preparing your manuscript
Manuscripts should be double-spaced throughout. Symbols and foreign characters can be set with word-processing software by altering typefaces to a corresponding font that displays the appropriate character. Use the Symbol font for Greek characters whenever possible. When special characters are unavailable, please note them in a separate cover letter. Use the software's spell-checking and page-numbering capabilities before final transmission. The manuscript will be edited according to the style of the journal, and authors must read the proofs carefully. Pages should be numbered consecutively and organized as follows:
The title page (p. 1) should contain the article title, authors' names and complete affiliations, footnotes to the title, a short title of less than 65 characters, and the address for manuscript correspondence (including e-mail address and telephone and fax numbers).
The abstract (p. 2) must be a single paragraph that summarizes the main findings of the paper in less than 150 words. After the abstract a list of up to 10 keywords that will be useful for indexing or searching should be included.

The introduction should be as concise as possible, without subheadings.

Materials and methods should be sufficiently detailed to enable the experiments to be reproduced.

Results and Discussion may be combined and may be organized into subheadings.

Acknowledgments should be brief and should precede the references.

References to the literature should be cited by numbers in square brackets in the text and listed in numerical order at the end. Use the most recent edition of the Chemical Abstracts Service Source Index for abbreviations of journal titles. Only articles that have been published or are in press should be included in the references. Unpublished results or personal communications should be cited as such in the text. Please note the following examples.
[1] W.D. Strayhorn, B.E. Wadzinski, Arch. Biochem. Biophys. 400 (2002) 76-84.
[2] R. Hesketh, The Oncogene FactsBook, Academic Press, San Diego, 1995.

[3] O.R. Mettam, L.B. Adams, in: E.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 1999, pp. 218-304.
Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves on a separate sheet at the end of the article.
Endnote users should use the reference format template for Biochemical and Biophysical Research Communications.

Figures
Each individual figure or graphic must be supplied as a separate, stand-alone file. Figure files must be named with their respective numbers and graphic types such as SmithFig1.tif, SmithFig2a.tif, etc. Long file names are acceptable.Use EPS or TIFF file formats; TIFF is preferred. Artwork submitted in TIFF should adhere to the following resolution settings: half tones (color/gray scale): 300 dpi; line art (black and white) and mixed images (halftones with text or line art): 600 to 1200 dpi. If it is necessary to import graphics from a vector-based drawing program (e.g., Adobe Illustrator) into a raster-based program (e.g., Adobe PhotoShop) in order to produce a TIFF file, a resolution of at least 600 dpi is required for quality reproduction. For further information on the preparation of electronic artwork, please see http://authors.elsevier.com/artwork.
When creating your figures, use font sizes and line weights that will reproduce clearly and accurately when figures are sized to the appropriate column width. The minimum line weight is one-half point (thinner lines will not reproduce well). Eliminate all excess white space from the borders of each figure. Do not include figure legends or other extraneous text in a graphic file; figure legends should be provided as text, placed after the reference section in the main manuscript file. Number figures consecutively with Arabic numerals.

Color figures
One piece of color art per article will be published free of charge. If together with your accepted article, however, you submit usable color figures, then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether these illustrations are reproduced in color in the printed version.

Please note: Because of technical complications that can arise in converting color figures to "gray scale" (for the printed version should you not opt for color in print), please submit in addition usable black-and-white files corresponding to all the color illustrations.
Cover art. Suggestions for cover art are welcome and 4-process color is encouraged. This artwork may be a figure from the paper or a complementary figure. Submit art in electronic form (TIFF and EPS files only) to the Editorial Office via e-mail (abb@elsevier.com). A short (one-line) legend should accompany each photograph.

Tables
should be numbered with Arabic numerals and cited consecutively in the text. Each table should be titled and typed double-spaced. Units must be clearly indicated for each of the entries in the table.

X-ray crystallography and NMR spectroscopy. Factors and coordinates for structures determined by X-ray crystallography and NMR spectroscopy should be deposited in the Protein Data Bank (PDB; http://www.rcsb.org/pdb/; info@rcsb.org) at the Research Collaboratory for Structural Bioinformatics or at the European Bioinformatics Institute (EBI; http://autodep.ebi.ac.uk/). Manuscripts submitted to ABB should include the file name for the macromolecule and a reference to the database holding the coordinates.
Nucleotide sequence data. For nucleotide sequence data authors are required to submit original nucleotide or amino acid sequence data to a databank such as GenBank or EMBL. A footnote that includes the accession number should be included on the title page. It is preferable that the sequence data be deposited either prior to submission or by the time of acceptance of the manuscript. However, a footnote can be added at the proof stage if the material is deposited after acceptance. For further information please contact the Editorial Office or GenBank or EMBL directly.
GenBank/DNA sequence linking. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources should type this information in the following manner:
For each accession number cited in an article, authors should type the accession number in bold, underlined text. Letters in the accession number should always be capitalized (see example below). This combination of letters and format will enable the typesetter to recognize the relevant texts as accession numbers and add the required link to GenBank sequences.
Example: GenBank accession nos. AI631510 , AI631511 , AI632198 , and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117).
Authors are encouraged to check accession numbers used very carefully. An error in a letter or number can result in a dead link.
In the final version of the printed article, the accession number text will not appear bold or underlined. In the final version of the electronic copy, the accession number text will be linked to the appropriate source in the NCBI databases, enabling readers to go directly to that source from the article.

Animal studies
Policy regarding publication of experiments on unanesthetized animals conforms with the standards for use of laboratory animals established by the Institute of Laboratory Animal

Resources, U.S. National Academy of Sciences. Experiments in which curariform agents are used must be justified and details of the steps taken to reduce or avoid distress to the animal must be provided, particularly with regard to electrical stimulation.

Preparation of supplementary material
Elsevier now accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer additional possibilities for publishing supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips, and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect (http://www.sciencedirect.com). To ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. Please note, however, that supplementary material will not appear in the printed journal. Files can be stored on 3.5-inch diskette, ZIP disk, or CD (either MS-DOS or Macintosh). For more detailed instructions, please contact the Editorial Office (e-mail: abb@elsevier.com; telephone; (619) 699-6757; fax: (619) 699-6211).

Proofs
Proofs will be sent to the corresponding author as a PDF file by e-mail. To avoid delay in publication, only necessary changes should be made, and proofs should be returned promptly. Authors will be charged for alterations that exceed 10% of the total cost of composition.

E-Offprints
The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail or, alternatively, 25 free paper offprints. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use.

Author inquiries
For inquiries relating to the submission of articles (including electronic submission where available) please visit http://authors.elsevier.com.

Sponsored Articles:
Archives of Biochemistry and Biophysics offers authors or their institutions the option to sponsor non-subscriber access to their articles on Elsevier's electronic publishing platforms.

March 2008 version

7.3. Anexo 3: Radiation Physics and Chemistry

The Journal for Radiation Physics, Radiation Chemistry and Radiation Processing

Guide for Authors

PREPARATION

Language

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Use decimal points (not decimal commas); use a space for thousands (10 000 and above).

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. Do not embed "graphically designed" equations or tables, but prepare these using the wordprocessor's facility. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Do not import the figures into the text file but, instead, indicate their approximate locations directly in the electronic text and on the manuscript. See also the section on Electronic illustrations.
To avoid unnecessary errors you are strongly advised to use the "spell-check" and "grammar-check" functions of your wordprocessor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to "the text". Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Subdivision

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2,), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text.' Any subsection may be given a brief heading. Each heading should appear on its own separate line. Follow this general outline when writing manuscripts:
Title, Authors, Affiliations, Abstract, Keywords.
Introduction, Experimental, Results, Discussion, Conclusion.
Acknowledgements, Appendix, References, Figure legends, Tables. The introduction should briefly describe the background and reason for doing the work described. The experimental should provide enough information for readers to repeat the experiment. The discussion should evaluate the results and their relationship to comparable works. The conclusion should briefly summarize the obtained results.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on.

Essential title page information

Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each author.
Corresponding author. Clearly indicate who is willing to handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.
Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a "Present address"' (or "Permanent address") may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or

uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.
Table footnotes
Indicate each footnote in a table with a superscript lowercase letter.

Electronic artwork

General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Save text in illustrations as "graphics" or enclose the font.

• Only use the following fonts in your illustrations: Arial, Courier, Helvetica, Times, Symbol.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Produce images near to the desired size of the printed version.
• Submit each figure as a separate file.

A detailed guide on electronic artwork is available on our website:
http://www.elsevier.com/artworkinstructions
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
Regardless of the application used, when your electronic artwork is finalised, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as "graphics".
TIFF: color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF: Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications please supply "as is".
Please do not:
• Supply embedded graphics in your wordprocessor (spreadsheet, presentation) document;
• Supply files that are optimised for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. Please indicate your preference for color in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to "gray scale" (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either "Unpublished results" or "Personal communication" Citation of a reference as "in press" implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: All citations in the text should refer to:
1. Single author: the author's name (without initials, unless there is ambiguity) and the year of publication;
2. Two authors: both authors' names and the year of publication;
3. Three or more authors: first author's name followed by "et al." and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: "as demonstrated (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown ...."
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2000. The art of writing a scientific article. J. Sci. Commun. 163, 51-59.
Reference to a book:
Strunk Jr., W., White, E.B., 1979. The Elements of Style, third ed. Macmillan, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 1999. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281-304.

Journal abbreviations source

Journal names should be abbreviated according to
Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
List of serial title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

Supplementary material

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please ensure that data are provided in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. Video files: please supply 'stills' with your files: you can choose any frame from the video or make a separate image. These will be used instead of standard icons and will personalize the link to your supplementary information. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

January 2009 version