UNIVERSIDADE FEDERAL DE PERNAMBUCO
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MESTRADO EM BIOQUÍMICA

CARACTERIZAÇÃO FÍSICO-QUÍMICA E BIOLÓGICA DA LECTINA DE SEMENTES DE *Eugenia uniflora L.*

MARIA DANIELLY LIMA DE OLIVEIRA

Orientadora: Profa. Dra. Maria Tereza dos Santos Correia

Co-orientadora: Profa. Dra. Nereide Stela Santos Magalhães

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do grau de Mestre em Bioquímica pela Universidade Federal de Pernambuco.

Aprovado por:

_____
Profa. Dra. Maria Tereza dos Santos Correia (Presidente)

_____
Prof. Dra. Luana Cassandra B. B. Coelho, UFPE

_____
Prof. Dra. Vera Lúcia de Menezes Lima, UFPE

_____
Prof. Dra. Sandra Rodrigues de Souza, FAFIRE

Data: 29 / 07 / 2005.

Ata da defesa de dissertação da Mestranda **Maria Danielly Lima de Oliveira,** realizada em 29 de julho de 2005, como requisito final para obtenção do título de Mestre em Bioquímica

Às 9:15 minutos do dia 29 de julho de 2005, foi aberto, no Auditório Prof. Marcionilo Barros Lins, Depto. de Bioquímica/CCB, o ato de defesa de dissertação da mestranda **Maria Danielly Lima de Oliveira**, aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Vera Lúcia de Menezes Lima, Coordenadora do Curso de Mestrado em Bioquímica, fez a apresentação da aluna, de sua orientadora, Profa. Dra. Maria Tereza dos Santos Correia, de sua Co-orientadora, Profa. Dra. Nereide Stela Santos Magalhães e da Banca Examinadora composta pelos professores doutores: Maria Tereza dos Santos Correia, Pressidente, Luana Cassandra Breitenbach Barroso Coelho, Vera Lúcia de Menezes Lima, ambas do Depto. de Bioquímica/CCB/UFPE, e Sandra Rodrigues de Souza da Faculdade Frassinete do Recife. Após as apresentações, a Profa. Vera Menezes passou a palavra para a Sra. Presidente que convidou a aluna para a apresentação de sua dissertação intitulada: **"Caracterização Físico-Química e Biológica da Lectina de Sementes de Eugenia uniflora L.",** e informou que de acordo com o Regimento Interno do Curso, o candidato dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 35 (trinta e cinco) minutos. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, Profa. Dra. Sandra Rodrigues de Souza, que agradeceu o convite, fez alguns comentários, e elogios à mestranda e à orientadora, iniciando sua argüição. Ao final, a referida professora parabenizou a mais uma vez a mestranda e a sua orientadora, dando-se por satisfeita. Em seguida, a Sra. Presidente passou a palavra para a Profa. Dra. Vera Lúcia de Menezes Lima, que agradecendo o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Finda a argüição, a referida professora parabenizou a candidata e sua orientadora e deu-se por satisfeita. Em seguida, a palavra foi passada para a Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho, que após agradecer e fazer alguns comentários, iniciou sua argüição. Ao final da sua argüição, a referida professora deu-se por satisfeita, parabenizando a candidata e sua orientadora. Daí a Sra. Presidente passou a palavra para a Profa. Dra. Nereide Stela Santos Magalhães que, na qualidade de co-orientadora, teceu alguns comentários e parabenizou a candidata pelo seu trabalho. Com a palavra, a Sra. Presidente teceu alguns comentários, agradeceu à Banca e parabenizou a candidata. Finalmente, a sessão foi suspensa para proceder o julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 29 de julho de 2005.

Maria Tereza Correia

Sandra N V

Vera Lúcia M. Lima.

Resumo

Lectinas são proteínas que se ligam especificamente aos carboidratos sobre a superfície celular. *Eugenia uniflora L*. é uma planta Myrtaceae nativa do sul da América, Sudeste da Ásia e África. *Triticum vulgaris* é uma aglutinina (36 kDa) constituída por duas subunidades. Proteínas antimicrobianas têm sido isoladas de uma variedade de espécies de plantas. O objetivo deste estudo foi purificar uma lectina de sementes de *Eugenia uniflora* e avaliar o comportamento interfacial da lectina EuniLS através de medidas da pressão de superfície Π e potencial de superfície ΔV em diferentes valores de pH do volume, detectar atividade e caracterizar a lectina de sementes de *E. uniflora*, EuniLS, bem como avaliar o efeito da lectina sobre as bactérias Gram-negativas e Gram-positivas. EuniLS foi purificada de um extrato de sementes (E) em tampão fosfato de sódio (pH 7,0) em cromatografia de DEAE-Sephadex. EuniLS (67 kDa) permaneceu estável na faixa de pH 2 a 9 e mostrou especificidade para açúcares complexos, assim como oligossacarídeos. Além do mais, EuniLS tem uma atividade hemaglutinante específica de 85,3. As isotermas de pressão de superfície (Π) × área molecular (A) evidenciaram que o comportamento interfacial foi fortemente dependente do pH do volume. EuniLS apresentou uma alta atividade superficial (Πc=40 mN/m e ΔV=440 mV) que a lectina WGA (Πc=34 mN/m e ΔV =340 mV) no pH 2. O momento dipolar de EuniLS ($\mu_\perp$) aumentou em 1,3 vezes com o incremento de pH de 2 a 9, enquanto que WGA o ($\mu_\perp$) aumentou 3,8 vezes para a mesma variação de pH. Ambas as lectinas apresentaram uma contribuição negativa da dupla camada elétrica Ψ_0=-68 mV a -64 mV para EuniLS e Ψ_0=-117 mV -144 mV para WGA. Uma relação linear para Ψ_0 e pH (faixa de 2 –6) foi observada para EuniLS. Um ponto de quebra foi detectado em pH 6,0 e um platô foi observado até o pH 7,0, que corresponde ao ponto isoelétrico da EuniLS. Um comportamento similar foi observado para valores de ζ –7,5 a –30 mV. As variações ocorridas em ΔV ocorreram devido a contribuição da orientação da molécula de lectina na superfície ($\mu_\perp$), e a dupla camada elétrica (Ψ_0). A eluição da proteína adsorvida foi realizada com TFS (pH 2,0) e sua atividade foi avaliada através de eritrócitos (coelho e humano), carboidratos e estabilidade em diferentes valores de pH (3,5-9,0). Uma eletroforese de EuniLS foi realizada através de gel de poliacrilamida (SDS-PAGE) para definir o peso molecular. A atividade antimicrobiana do extrato e EuniLS foram investigados utilizando o teste de disco. O meio de cultura (100mL, 43º C) e 0,5 mL de inoculo foram adicionados e a solução foi distribuída em placas de Petri estéril em porções de 10 mL. Discos de 6 mm de diâmetro foram impregnados com 15 µL de solução de lectina estéril e E em TFS (pH 7,0). A atividade de EuniLS foi parcialmente inibida pelas glicoproteínas (caseína e soro de coelho) e aglutinada por eritrócitos de coelho e humano (tipos A, B, AB e O). Eletroforeses das preparações de E. uniflora mostraram uma lectina de peso molecular de 67 kDa. A atividade de EuniLS foi aumentada no pH 6,5. EuniLS inibiu a maioria dos microrganismos testados: *Klebsiella sp*. (halo de 19,6 mm ± 2,5), *Pseudomonas aeruginosa* (halo de 18,6 mm ± 0,6), *Staphylococcus aureus* (20,0 mm ± 0,5) e *Corinebacterium sp*. (8,0 mm ± 0,1), com mínima concentração inibitória (MIC) de 1,5 e mínima concentração bactericida (MBC) de 16,5 µg/mL. Extrato de sementes não mostrou atividade antibacteriana contra os microrganismos testados. Estes resultados indicam uma purificação de uma nova lectina e sua atividade antibacteriana; EuniLS pode ser utilizada como um adjuvante em terapia antibacteriana.

ABSTRACT

Lectins are proteins that have specific binding to carbohydrates on cellular surface. *Eugenia uniflora L.* is a Myrtaceae plant native to South America, Southeast Asia and Africa. Triticum vulgaris is the wheat germ agglutinin (36 kDa) constituted by two identical proteic subunits. Antimicrobial proteins have been isolated from a wide range of plant species. The goal of the present study was to purify a lectin from seeds of *E. uniflora* and to evaluate the interfacial behavior of the EuniSL lectin by surface pressure Π and surface potential ΔV measurements at different pH bulk phase and to detect activity and characterize the lectin from *E. uniflora* seeds, *EuniSL*, as well as to evaluate the lectin effect on Gram-negative and Gram-positive bacteria. EuniSL was purified from a seed extract (E) in sodium phosphate buffer (PB, pH 7.0) in DEAE-Sephadex chromatography. EuniSL (67 kDa) remained stable at pH ranging from 2 to 9 and showed specificity for complex sugars such as oligosaccharides. Furthermore, EuniSL has an 85.3 specific haemaglutinating activity. Surface pressure (Π) × molecular area (A) isotherms evidenced that the lectin interfacial behavior was strongly dependent on the pH of the bulk phase. EuniSL presented a higher surface activity (Πc=40 mN/m and ΔV=440 mV) than WGA lectin (Πc=34 mN/m and ΔV =340 mV) at pH=2. The EuniSL dipole moment ($\mu_\perp$) increased 1.34 times as pH increased from 2 to 9, while the WGA $\mu_\perp$ increased 3.8 times for the same pH variation. Both lectins presented a negative contribution of the electric double layer Ψ_0=-68 mV to -64 mV for EuniSL and Ψ_0=-117 mV -144 mV for WGA. A linear relationship for Ψ_0 and pH (range 2-6) was observed for EuniSL. A breaking point was detected at pH 6 and a plateau was reached until pH 7, which corresponds to the EuniSL isoelectric point. A similar behavior was observed for ζ values from -7.5 to -30 mV. ΔV variations occurred due to the orientation of the lectin molecule at the surface ($\mu_\perp$), and the electric double layer (Ψ_0) contributions. Elution of adsorbed protein was performed with PB (pH 2.0) and activity was evaluated by erythrocytes (rabbit and human), carbohydrates and stability at a different pH range (3.5 – 9.0). An Eletrophoretic pattern of EuniSL was evaluated by polyacrylamide gel (SDS-PAGE) to define the molecular weight. Antimicrobial activity of extract and *EuniSL* were investigated using the disc method. Warmed medium (100 mL, 43°C) and 0.5 mL of inoculum were added and the solution was distributed in sterile Petri plates in portions of 10 mL. Discs of 6 mm diameter were impregnated with 15 μL de sterile lectin solution and E in PB (pH 7.0). *EuniSL* activity was partially inhibited by glycoproteins (casein and serum rabbit) and agglutinated rabbit and human erythrocytes (types A, B, AB and O). Eletrophoresis of *E. uniflora* preparations showed a lectin of molecular weight 67 kDa. The activity of EuniSL was improved at pH 6.5. *EuniSL* inhibited growth of tested microorganisms: *Klebsiella sp. (*19.6 mm ± 2.5 halo), *Pseudomonas aeruginosa* (18.6 mm ± 0.6 halo), *Staphylococcus aureus* (20.0 mm ± 0.5) and *Corinebacterium sp*. (8.0 mm ± 0.1), with minimal inhibitory concentration (MIC) of 1.5 μg/mL and minimal bactericide concentration (MBC) of 16.5 μg/mL. Extract of seeds did not show antibacterial activity against tested microorganisms. These results indicated a purification of a new lectin and its antibacterial activity; *EuniSL* can be used as an adjuvant to antibacterial therapy.

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INTRODUÇÃO

1. LECTINAS

1.1 Denominação e Definição

O isolameno de ricina marcou o início das pesquisas envolvendo lectinas, extraída de *Ricinus communis* (mamona) por Stilmark em 1988, quando observaram-se os efeitos de toxicidade e capacidade de aglutinar eritrócitos. Desde então, ampliaram-se as pesquisas e aplicações destas proteínas (Gabor *et al*., 2001). Proteínas presentes em plantas capazes de aglutinar eritrócitos foram inicialmente nomeadas como fitohemaglutininas, hemaglutininas, fitoaglutininas ou aglutininas de plantas (Sharon e Lis, 1988).

O termo "lectina" (originado do latim "lectus", que significa selecionado, escolhido) foi proposto por Boyd e Shapleigh (1954) para fazer designação desse grupo de proteínas que apresentam uma característica comum: seletividade na interação com carboidratos. Lectinas são proteínas ou glicoproteínas que possuem a habilidade de se ligar especificamente a mono ou oligossacarídeos de forma reversível (Sato *et al.,* 2000; Hong *et al.,* 2001; Souza *et al.,* 2001).

A origem não imune das lectinas serve para distingui-las de anticorpos anticarboidratos que aglutinam células. Enquanto os anticorpos são estruturalmente similares, as lectinas diferem entre si quanto à composição aminoacídica, requerimentos de metais, peso molecular e estrutura tridimensional. Além disso, as lectinas não são apenas encontradas em animais, mas também em outros organismos que não possuem sistema imune, como plantas e bactérias (Moreira *et al*; 1991), possuindo dois ou mais sítios de ligação a carboidrato.

Peumans e Van Damme (1995) definiram lectinas de plantas como todas as proteínas que possuem no mínimo um domínio não-catalítico que se liga reversivelmente a um mono ou oligossacarídeo específico (Figura 1), estendendo o conceito para proteínas que se comportam de forma completamente diferente com relação às suas propriedades de aglutinação e/ou precipitação de glicoconjugados.

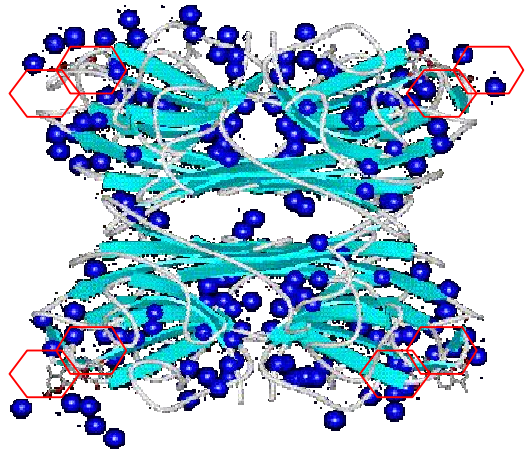

Figura 1 - Especificidade de ligação de lectinas de plantas a carboidratos.

 -Lectina  -Carboidrato

1.2 Detecção e Especificidade

A presença de lectinas pode ser facilmente detectada através de testes efetuados com extratos, verificando-se, se os mesmos aglutinam eritrócitos e demonstrando-se, se a aglutinação é inibida por um carboidrato simples ou complexo. O ensaio de hemaglutinação (Figura 2 a) é o mais comumente utilizado por promover a visualização desta propriedade de aglutinação de eritrócitos por lectinas (Goldstein *et al*., 1980). Os eritrócitos utilizados no ensaio podem ser de origem humana ou de outros animais, enzimaticamente ou quimicamente tratados (Coelho e Silva, 2000) ou não tratados (Mo *et al*., 2000).

As lectinas também induzem a precipitação de polissacarídeos ou glicoproteínas em solução, sendo as reações de aglutinação por lectinas inibidas por seus carboidratos específicos (Moreira *et al*., 1991). Para assegurar que o agente hemaglutinante é uma lectina, são necessários ensaios subseqüentes de inibição da AH (Figura 2 b) utilizando uma solução do carboidrato ligante (Cavada *et al.,* 2000; Kawagishi *et al.,* 2001).

Muitas lectinas são metaloproteínas; precisam de cátions divalentes tais como Ca^{+2} e Mn^{+2} para exibir sua atividade total. A presença de cátions na estrutura da proteína promove termoestabilidade e uma relativa resistência à ação enzimática (Moreira *et al*., 1991).

Exemplos de lectinas dependentes de metais são a lectina isolada de sementes a de *Pitilota filicina* (Sampaio *et al*., 1998).

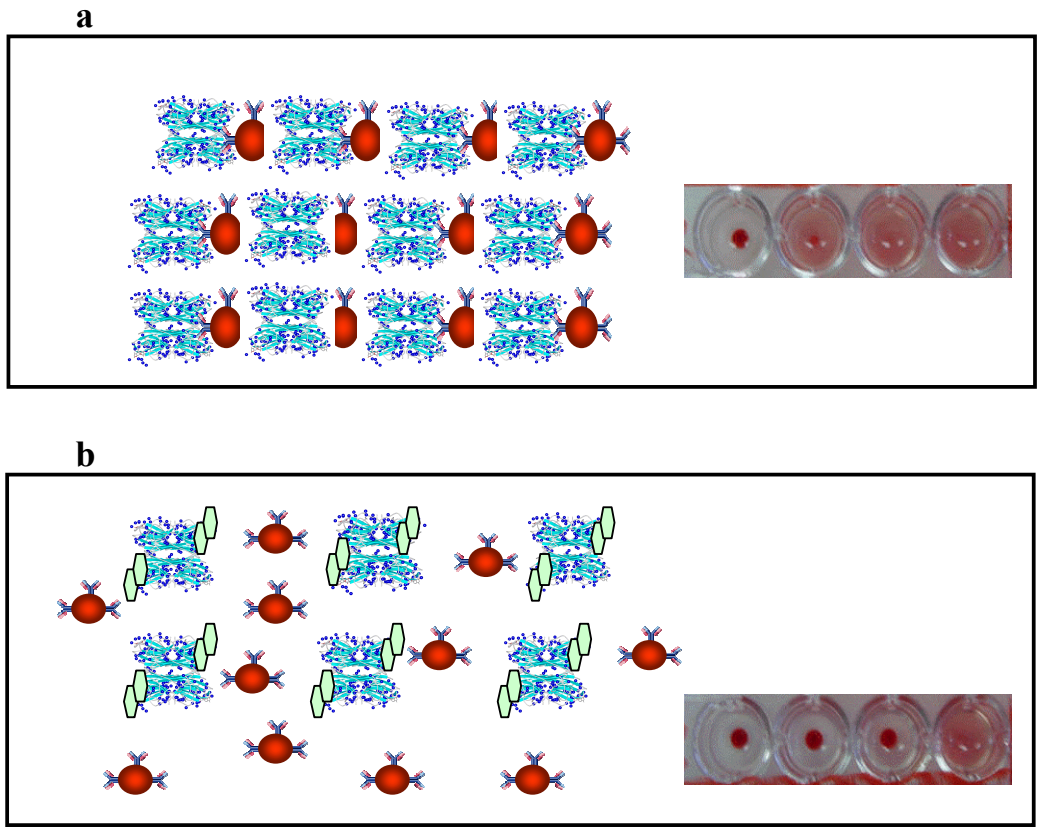

Figura 2 – Atividade hemaglutinante (a) e inibição da atividade hemaglutinante (b).
- Lectina, - Eritrócitos, – Carboidrato.

1.3 Características Estruturais e Classificação

Em geral, lectinas de plantas são oligômeros formados de duas ou mais subunidades, idênticas ou não; geralmente possuem estrutura tetramérica (Moreira *et al.,* 1991). As diferenças estruturais entre as diversas lectinas se devem à variação do número de subunidades por molécula e pela natureza dos polipeptídios. Pontes dissulfeto, pontes de hidrogênio e também as interações hidrofóbicas podem estar presentes nas subunidades de associação (Kennedy *et al*., 1995). As especificidades e afinidades dos sítios associados são alcançadas principalmente por pontes de hidrogênio, com a ajuda de forças de van der Walls e interações hidrofóbicas com resíduos de aminoácidos aromáticos que estão

próximos às porções hidrofóbicas de monossacarídeos (Sharon, 1993) contribuindo para a estabilidade e especificidade dos complexos formados.

Estas lectinas exibem uma elevada homologia, possuindo um total de 20% de resíduos de aminoácidos invariáveis, dentre os quais estão incluídos aqueles envolvidos na ligação a monossacarídeos e, a maioria dos que coordenam os íons metálicos muitas vezes necessários para a atividade da lectina. Em relação à estrutura global das lectinas de plantas, estas podem ser divididas em três principais tipos distintos: as merolectinas, as hololectinas e as quimerolectinas. As merolectinas são proteínas formadas exclusivamente por um domínio de ligação a carboidrato; são proteínas pequenas, formadas por um único polipeptídeo e, por conta de sua natureza monovalente, são incapazes de precipitar glicoconjugados ou aglutinar células. As hololectinas também são exclusivamente formadas de domínios de ligação a carboidratos, mas contêm dois ou mais destes domínios que são idênticos ou muito semelhantes; este grupo compreende todas as lectinas que possuem múltiplos sítios de ligação, sendo capazes de aglutinar células ou precipitar glicoconjugados. As quimerolectinas são a fusão de proteínas contendo um domínio não relacionado, tal domínio possui uma atividade catalítica bem definida ou não apresenta nenhuma atividade biológica que atua de forma independente (Peumans e Van Damme, 1995).

1.4 Caracterização estrutural

A caracterização de lectinas envolve vários métodos, tais como técnicas eletroforéticas (Davis, 1964; Laemmli, 1970), que servem para indicar basicidade ou acidez de uma lectina, assim como para determinar sua estrutura quanto ao número de subunidades, peso molecular (Correia e Coelho, 1995) ou ainda para caracterizá-la como glicoproteína através da coloração com reativo de Shiff (Coelho e Silva, 2000).

Testes para a determinação da estabilidade térmica são importantes para delinear a capacidade de lectinas de suportar determinadas temperaturas, mantendo sua atividade biológica, e para indicar as condições térmicas ideais para se trabalhar com a molécula, de forma que esteja apresentando sua melhor atividade na interação com carboidratos. Algumas lectinas apresentam uma atividade acentuada, depois de submetidas a temperaturas relativamente altas (Correia e Coelho, 1995). Também, testes quanto a

modificações de pH e de soluções tampão são importantes para a determinação da estabilidade das moléculas, quanto à sua propriedade de ligação a carboidratos e glicoconjugados.

Ensaios de atividade hemaglutinante com eritrócitos de diferentes animais e de inibição por carboidratos e/ou glicoconjugados são excelentes meios de caracterização lectínica, promovendo a descoberta quanto à ligação específica a eritrócitos, quanto à especificidade a mono, di ou oligossacarídeos e quanto a capacidade de interação da lectina em estudo a outras moléculas como glicoproteínas, glicopeptídeos ou polissacarídeos (Gupta e Srivastava, 1998, Machuka *et al*., 1999, Sharon e Lis, 2001).

Outro ensaio importante na caracterização é a determinação da dependência ou não de tais moléculas por íons metálicos porque, como já mencionado algumas lectinas precisam da presença destes íons para promover sua atividade biológica (Konozi *et al*, 2002), outras não.

A imunodifusão dupla tem sido bastante utilizada para a caracterização de soros antilectinas, servindo igualmente para estudos preliminares de homologia entre lectinas de mesma espécie ou de espécies diferentes (Ashford *et al*., 1982). O sequenciamento aminoacídico é outra ferramenta valiosa no estudo das lectinas, expondo informações valiosas sobre a estrutura e função das moléculas, como foi verificado para a lectina de *Erytrina indica*, sendo verificado que modificações no aminoácido tirosina desta lectina causam uma inativação parcial da sua função (Konozy *et al*., 2002). O sequenciamento da lectina de *Cratylia mollis* forneceu informações a cerca de sua estrutura terciária (De Souza *et al*., 2003).

1.5 Eugenia uniflora L.

A pitanga (*Eugenia uniflora L*.) é uma planta originária da região que se estende desde o Brasil Central até o Norte da Argentina (Fouqué, 1981), no entanto sua distribuição se fez ao longo de todo o território nacional. A planta conhecida popularmente como pitangueira (Figura 3a), pitanga ou pitanga-vermelha tem seu nome derivado do tupi, que quer dizer vermelho, em alusão à cor do seu fruto (Figura 3b); a Figura 3c ilustra as sementes de *E. uniflora*. Pertence à ordem *Myrtales*, Família das *Myrtaceae* e à espécie *E. uniflora L* (Fouqué, 1981; Villachica *et al*., 1996).

Devido a sua adaptabilidade às mais distintas condições de clima e solo, a pitangueira foi disseminada e é atualmente cultivada nas mais variadas regiões do globo. Segundo descrições de Fouqué (1981) e Villachica *et al*. (1996), a pitangueira é um arbusto denso de 2 a 4 m de altura, ramificada, com copa arredondada de 3 a 6 m de diâmetro, com folhagem persistente ou semidecídua. Apresenta um sistema radicular profundo, com uma raiz pivotante e numerosas raízes secundárias e terciárias. O fruto é uma baga globosa, deprimida nos pólos, com 7 a 10 sulcos mais ou menos marcados no sentido longitudinal, de 1,5 a 5,0 cm de diâmetro, coroado com as sépalas persistentes. A planta suporta poda forte e repetida, cresce lentamente, tem copa densa e compacta, sendo por essas razões empregada como cerca viva e planta ornamental. O seu potencial de utilização é ressaltado quando se considera que seu fruto de sabor exótico é rico em vitaminas, principalmente em vitamina A. Além disso, a promoção de campanhas de educação nutricional pode aumentar o consumo de pitanga como alimento rico e saudável.

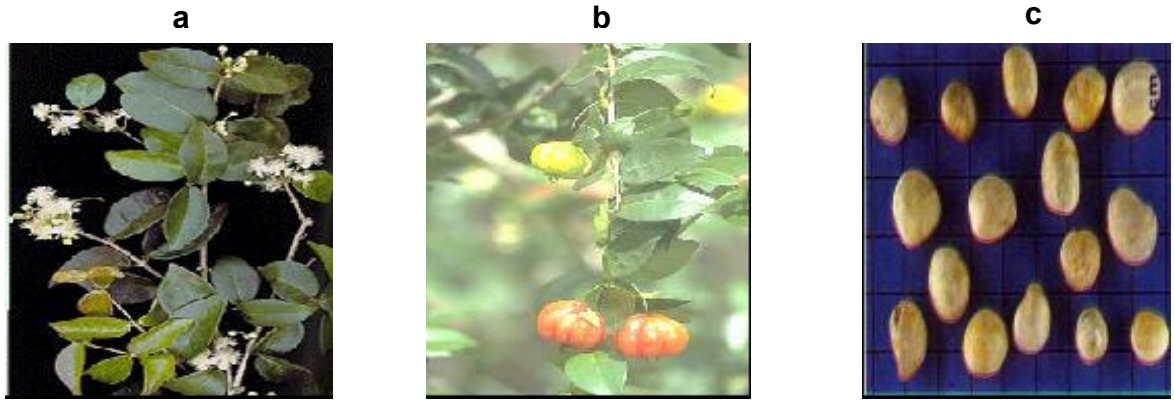

Figura 3 - Visão geral da planta *Eugenia uniflora*: Floração (a), frutos (b) e sementes (c).

2. ATIVIDADE ANTIMICROBIANA

A disponibilidade de um grande número de lectinas com diferentes especificidades para carboidratos tem levado à sua extensiva utilização como reagentes para estudar carboidratos simples e complexos em solução e sobre superfícies celulares (Lis e Sharon, 1986). A lectina de *Canavalia ensiformis* demonstrou aglutinação para certas espécies de micobactérias, sendo específica para a α-arabinogalactona presente na superfície de

Mycobacterium bovis (Goldstein *et al*., 1970). A capacidade das lectinas em aglutinar as bactérias tem por finalidade estudar a constituição sacarídica da superfície de bactérias, para a tipagem de bactérias e para a determinação de receptores para bacteriófagos (Archibald *et al*., 1972).

Conseqüentemente as lectinas são moléculas que atuam no sistema de defesa imunológico desde que elas podem seqüestrar várias bactérias, outros microinvasores celulares, bem como substâncias que eles secretam (Yeaton *et al*., 1981). A opsonização que ocorre quando a lectina se liga com a bactéria, é considerada a primeira etapa que promove a aderência, ingestão e subseqüentemente a ingestão do microrganismo. Considerando sua especificidade para o conteúdo de carboidratos na estrutura, muitas análises têm caracterizado os componentes estruturais das bactérias pelo uso de lectinas que possuem especificidade para açúcares neutros. Apenas poucas investigações têm mostrado a especificidade de lectinas para ácido siálico (Doyle *et al*., 1994).

Muitas lectinas de planta têm sido estudadas por suas interações com várias bactérias e a específica simbiose entre plantas e bactérias. No entanto pouco tem sido sugerido sobre a ação em reduzir a infectividade das bactérias patogênicas. Uma lectina de sementes de maçã, *Datura stramonium*, bloqueou a motilidade normal da bactéria na interface ar-água (Broekaert *et al*., 1985). Enquanto que a lectina de *Cyphomandra betacea* inibiu o crescimento de bactérias patogênicas (Nieva *et al*., 1997).

Um peptídeo catiônico isolado de sementes de *Robinia pseudoacacia* foi testado contra sete bactérias (*Corynebacterium michiganense*, *Staphylococcus aureus*, *Bacillus subtilis*, *Erwinia carovora subsp*. *Carotovor*a, *Pseudomonas syringae pv syringae*, *Xanthomonas campestris pv campestris*, and *Escherichia coli*). O peptídeo inibiu a maioria das cepas testadas, sendo que o *Staphylococcus aureus* demonstrou ser o mais sensível para o peptídeo (Talas-Ogras *et al*., 2005). Também a lectina de *Morus alba* (MLL1) foi estudada por sua ação antibacteriana contra *Pseudomonas syringae pv mori*, onde a MLL1 induziu a aglutinação de *P. syringae pv mori*, sendo inibida por N-glicolilneuramínico, N-acetilgalactosamina e mucina de submaxilar bovino (Ratanapo *et al*., 2001).

3. FÍSICO-QUÍMICA DE INTERFACE

Caracterização através da técnica de Langmuir

As moléculas orgânicas se orientam entre a fase gasosa e líquida para minimizar sua energia livre. Estas moléculas são substâncias insolúveis na subfase líquida e são chamadas de filme de Langmuir. O filme superficial formado é chamado de camada monomolecular ou simplesmente monocamada. O limite entre um líquido e um gás, como o ar e a superfície aquosa, marca uma transição entre a composição e as propriedades dos dois volumes de fases. Uma monocamada na superfície existirá com diferentes propriedades (Adamson, 1982; Gaines, 1966). Se as moléculas são eletricamente neutras, as forças entre elas serão de fraca extensão e a camada de superfície não será mais que um ou dois diâmetro molecular. Em contraste, as forças Coulombianas associadas com as cargas das espécies podem se estender da região de transição acima das distâncias consideráveis.

As monocamadas de Langmuir formadas por compressão dinâmica podem ser transferidas para substratos sólidos, de modo que nos estudos de monocamadas o principal objetivo é a capacidade de mensurar a diferença na tensão superficial entre a superfície do líquido limpo ou puro e uma recoberta com o filme, permitindo uma interpretação simples dos efeitos da tensão superficial em termos de forças intermoleculares, bem como em estudos de propriedades dielétricas de lectinas (Andrade *et al*., 2005), sendo a força mensurada denominada de pressão de superfície.

As moléculas na monocamada são orientadas de tal forma que esta técnica torna-se extremamente atrativa na preparação de sistemas altamente organizados e com espessura controlada (ordem de grandeza de angstrons). Portanto, como várias moléculas de importância biológica (fosfolipídios e proteínas) possuem propriedades anfifílicas, esta técnica se mostra muito efetiva em alguns processos de estudos biológicos (Philiphs e Chapman, 1968).

A técnica de formação de monocamadas de Langmuir é o primeiro passo para a produção de estruturas moleculares de alta qualidade – filmes de Langmuir-Blodgett (LB). Os filmes de Langmuir-Blodgett, em homenagem aos cientistas americanos Irving Langmuir e Katherine-Blodgett, são obtidos através da transferência de monocamadas para um substrato sólido. Para essa transferência, o substrato é imerso e retirado da subfase

aquosa, passando pela monocamada que se transfere para o substrato (Pett *et al*., 1996). A repetição desse procedimento permite a deposição de várias camadas em um mesmo substrato. Esta técnica apresenta-se como uma das mais importantes na área da tecnologia de ponta por permitir a fabricação de filmes ultra-finos (ordem de nm), o controle da espessura em angstroms, e a obtenção de uma grande ordem estrutural (Gaines, 1966; Ulman, 1991). Estudos com a lectina de *Bauhinia monandra* mostraram sua interação com monocamadas lipídicas (Rosilio *et al*., 2004).

OBJETIVOS

- **Objetivo Geral**

Purificação e caracterização físico-química de lectina de sementes de *Eugenia uniflora L*. (EuniLS), bem como avaliação da atividade antimicrobiana.

- **Objetivos Específicos**

- Avaliação da atividade hemaglutinante em extratos de sementes de *E. uniflora* para a extração de lectinas;

- Purificação da lectina de sementes de *E. uniflora* (EuniSL) através de cromatografia de troca-iônica;

- Avaliação da especificidade de EuniSL para eritrócitos, carboidratos e glicoproteínas;

- Avaliação da atividade hemaglutinante de EuniSL frente a diferentes temperaturas, íons e a variações de pH;

- Avaliação de interação lectina/glicoproteína pelo teste de difusão dupla;

- Avaliação da atividade antimicrobiana de EuniSL através do teste de difusão em disco;

- Determinação da Concentração mínima bactericida (CMB) e da concentração mínima inibitória (MIC);

- Medidas da pressão de superfície e do potencial de superfície através da cuba de Langmuir.

- Medidas do potencial Zeta e de impedância eletroquímica.

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ARTIGO A SER SUBMETIDO AO PERIÓDICO FITOTERAPIA

PURIFICATION OF LECTIN FROM SEEDS OF A *Eugenia uniflora* AND ITS POTENTIAL ANTIMICROBIAL ACTIVITY

PURIFICATION OF LECTIN FROM SEEDS OF A *Eugenia uniflora* AND ITS POTENTIAL ANTIMICROBIAL ACTIVITY

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Introduction

Lectins are proteins that bind reversibly to monossacharides and glycoproteins with high specificty (Sharon and Lis, 1995). They are abundant in seeds, roots, fruits, flowers and leaves. Vegetable lectins are the more studied and the most of these proteins have been isolated and extensively investigated in relation to its chemical and physico-chemical characteristics, structural and biological properties. The interactions of plant lectins with human pathogenic bacteria have been extensively studied (Schauer *et al*., 1982, Slifkin *et al*., 1994). These proteins have been purified and characterized mainly from mature seeds of leguminous, as only or multiple molecular forms (Sharon and Lins, 1990; Paiva and Coelho, 1992; Suslelam *et al.*, 1997).

The ability of plant lectins to react with exposed carbohydrates in the microorganism surfaces has promoted the identification of pathogenic bacteria based on the reaction of selective agglutination between lectins and bacteria (Pistole, 1981; Slifkin and Doyle, 1990; Calderon *et al*., 1998; Munoz-Crego *et al*., 1999). The study of interaction of lectins with cellular wall carbohydrates from Gram-positive and Gram-negative bacteria and specialized forms, can demonstrate the binding capacity of these proteic molecules to a wide variety of complex carbohydrates such as teicoic acid, teicuronic acids, peptidoglycans and lipopolissacharides present in the cellular walls (Ratanapo *et al*., 2001).

Eugenia uniflora L. is a Myrtaceae plant distributed in South of African and Brazil; this plant has been used to treat hypercholesterolemia, gout and hypertension (Schiro *et al*., 1999). Extracts of *E. uniflora* have been reported to reduce corporal pressure in hypertense patients (Consolini *et al*, 1999), as well as performed to inhibit DNA polymerase of Epstein-Barr virus (Lee *et al*., 2000).

This work reports the purification and characterization of an antibacterial lectin (*EuniSL)* obtained from *E. uniflora* seeds.

Materials and methods

Purification of EuniSL

Lectin purification was developed by ionic-exchanged chromatography in DEAE-Sephadex G-50. Chromatographies were made in columns equilibrated with sodium phosphate buffer pH 7.0 (PBS), at a flow rate of 20 mL/h, using a peristaltic pump and assays were performed at room temperature. Sample of 10 % (w/v) crude extract (CE), in PBS, dialysed with three changes of 10 mM PBS (Coelho *et al*., 2000), containing 11.3 mg of protein/mL was applied to the support. Then, the column was washed with PBS. Elution of adsorbed proteins was performed with PBS adjusted to pH 2.0, until absorbance 280 nm was zero. Hemagglutinating activity (HA) was determined and protein concentration measured (according to Lowry *et al*., 1951 by absorbance at 720 nm) and the samples was stored at -20 ºC.

Hemagglutination activity and inhibition assays

Fresh erythrocytes were obtained as described by Bukantz et al. (1946) from human (A, B, O and AB types), rabbit, chicken and quail and treated with glutaraldehyde (Bing *et al*., 1967). HA assays were performed according to Correia and Coelho (1995). HA was defined as the lowest sample dilution which showed haemagglutination (different from control); specific HA (SHA) corresponded to HA divided by protein concentration.

EuniLS carbohydrate specificity was determined by HA inhibition assay using glycoproteins (bovine serum fetal, fetuin, tyroglobulin, asialofetuin, casein and rabbit serum) and sugars (+)-arabinose, D(+)-galactose, D(+)-raffinose, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, N-acetyl-D-galactosamione, D(+)-lactose, D(+)-mannose, D(+)-glucose, N-acetyl-D-glucosamine, D-glucuronic acid, L(+)-rhamynose, trehalose, D(+)-cellobiose, D(-)-fucose, L-fucose, D(-)-ribose, D(-)-fructose, D(+)-xylose, sucrose and D(+)-maltose. The inhibition assays were performed in microtitre plates: 100 μL of a solution containing either carbohydrate (0.19 – 400 mM) or glycoprotein (0.005 – 1,000 μg/mL), in 0.15 M sodium chloride, was mixed with 100 μL/mL), and an aliquot (50 μL) of the mixture was distributed in the wells. After 15 min at

room temperature, 50 μL of a 2.5 % (v/v) suspension of rabbit erythrocytes was added in a final volume of 100 μL. The result was recorded visually after 45 min at room temperature.

Effect of pH, temperature and metal ions on hemagglutinating activity

The effects of pH and temperature on EuniSL HA were evaluated by incubating EuniSL samples at different pH values for 1 h at room temparature in selected buffers (10 mM Tris-hydrochloric acid buffer at pH values 7.5, 8.0, 8.5, and 9.0 and 10 mM citrate phosphate buffer at pH values 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) or at 30, 40, 50, 60, 70, 80, 90 or 100 ºC for 30 min. The effect of Mg^{2+} and Ca^{2+} was performed with incubation (15 min) at same volume of a solution containing either metal ions $CaCl_2$ and $MgCl_2$ (5, 10 and 20 mM) in 0.15 M NaCl and EuniSL preparation (136 μg/mL). An aliquot (50 μL) of the mixture was distributed in microtitre plate wells and the HA was proceeded as described to inhibition assays.

Polyacrylamide gel electrophoresis (PAGE) of denatured protein

Denatured and reduced samples were evaluated as described by Laemmli (1970). The standard marker proteins were bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibition (20.1 kDa) and α-lactalbumin (14.4 kDa), purchased from Pharmacia Fine Chemicals (Pharmacia Biotechnology, Uppsala, Sweden). The gels were stained with 10 % Coomassie Brilliant Blue (Laemmli, 1970).

Radial simple diffusion

Radial simple diffusions were carried out according Ashford *et al* (1982) using 1% agarose gels prepared with 0.15 M NaCl, containing 0.02 % NaN_3 and 0.1 M glucose. The lectin (20 μg) was applied in well in a circular distribution, around a central well containing *Cratylia mollis* lectin (Cramoll 1,4). Samples were allowed to diffuse for 48 h at room temperature and the precipitated are detected by 0.005 % Coomassie Brilliant Blue staining.

Antimicrobial activity assay

Antibacterial activity assay (Bauer *et al*., 1966, Ahmed *et al*., 2001) was performed in sterile plates (90 mm x 15 mm) containing 10 ml of nutrient agar (NA), 0.7 %, w/v. Warm NA (3 mL) containing bacteria (*Pseudomonas aeruginosa, Baccilus subbitilis, Streptococcus aureus, Staphylococcus aureus, Corinebacterium* sp*., Escherichia coli* and *Klebsiella* sp*.)* were poured into the plates and sterile blank paper disks (6 mm diameter) impregnated with 20 μL of sterile EuniSL solution (2 mg/mL) in 0.15 M NaCl was placed on the NA containing bacteria; negative control disk and positive control disk containing 0.15 M NaCl and Amoxicilin (1mg/mL), respectively. Plates were incubated at 25 ºC for 20-24 h. A transparent ring around the paper disk signified antibacterial activity.

Determination of the minimal inhibitory concentration (MIC) and of the minimal bactericide concentration (MBC)

Seriate dilutions of EuniLS in PBS were prepared and added to cultures with 10^7 cell/mL of the microorganisms in the exponential phase of growth to certain the MIC according to Courvalin *et al*. (1988). The samples were incubated by 24 h at 37 ºC. This test consisted of a series of assay tubes (13 x 100 mm) containing 1.8 mL of Nutrient agar (NA) and 0.2 mL of microorganism suspensions with 1.5 x 10^8 CFU/mL (turbidity equivalent to 0.5 of McFarland scale). To the first tube 0.2 mL of lectin solution was added, (2 mg/mL) resulting in a final concentration 0.2 mg/mL. After homogenation, successive dilutions were proceeded in way to obtain the same final volume of 2.0 mL in all the tubes. Control tube just contained NB medium and microorganism. MIC corresponds to the smallest lectin concentration capable to inhibit the visible growth of the microorganism.

Minimum bactericidal concentration (MBC) was performed starting from the coming tubes MIC assay. Dilutions of 1:10.000 of the content of each tube were proceeded and aliquots (10 μL) were removed and sowed in plates of Petri containing NA medium. The readings were made through the count of CFU grown in plates. The low bactericidal concentration corresponds to the smallest concentration of the sample capable to reduce the number of CFU for 0.1 % of the initial inoculum (Courvalin *et al.*, 1988).

Bacterial agglutination test

Agglutination activity was made for quantitative determination of minimum agglutinating concentration (MAC), and minimum concentration of lectin which promotes bacterial aggregation, bacteria were fixed in 0.5% formaldehyde (Vazquez *et al*., 1996). Agglutination assays were performed in microplates by two-fold serial dilutions of lectin in 0.15 M NaCl. To each wall 50 µL of diluted bacterial suspension were added to a final volume of 200 µL. MAC was determined by visual agglutination after overnight incubation of plates at 37 ºC. EuniSL carbohydrate inhibition of induced microorganism agglutination was performed in microplates. The lectin (50 µL) was mixed with equal volume of diluted carbohydrate (50 mM). After incubation at room temperature for 30 min, 50 µL of microorganism was added and the mixture was further left standing for additional 30 min.

Results and Discussion

Crude extract of *E. uniflora* seed has a high hemagglutination activity (8192) and when was applied to ion-exchanged column showed one adsorbed protein peak (Figure 1).

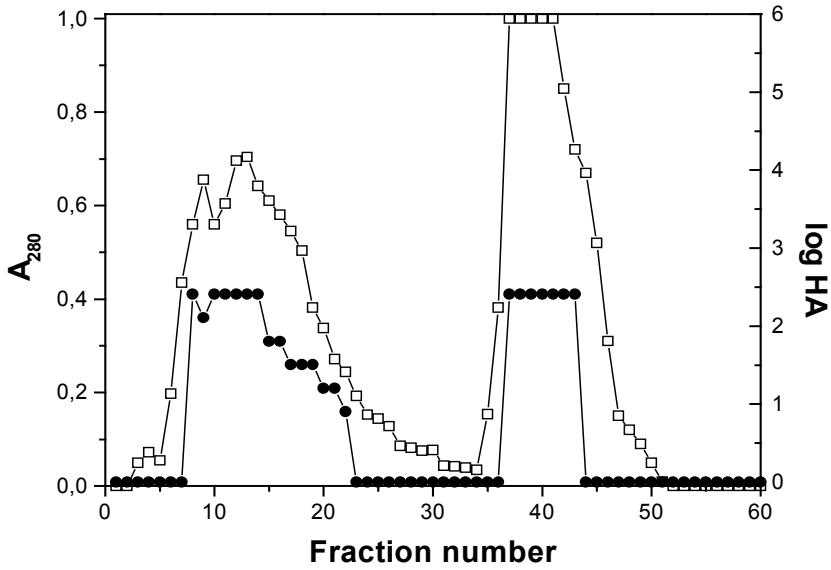

Figure 1: Chromatography of DEAE-Sephadex G50, eluted with TFS, pH 2.0 (○-○ absorbance at 280 nm; ●-● log of HA).CE was applied to a 10 mL column, and fractions (2 mL) were colleted at 20 mL/h.

Results showed the lectin purification with an increase of specific activity (85.3) and a purification time of 11.68 (Table 1). DEAE-Sephadex was choice as a matrix of to ion-exchanged chromatography because its action as exchanged promoting an ionic interaction between the effective charges of proteins with the matrix, showed adsorption of interesting molecule. One lectin from *Robinia pseudoacacia* (Duverger *et al*., 1997) also was purified by ion-exchanged chromatography (CM-trysacryl) in one step.

Table 1 – Yields of EuniSL obtained during the purification of seed lectin from *E. uniflora.*

Sample	**Volume (mL)**	**Total protein (vol x mg/mL)**	**Specific HA (titre/protein)**	**Purification (times)**
E. uniflora Extract	200	14,000	7.3	1
Adsorbed (EuniSL)	73	129	85.3	11.68

Polyacrilamide gel electrophoresis of EuniSL treated with SDS and 2-mercaptoetanol revealed one band of 67 kDa (Figure 2).

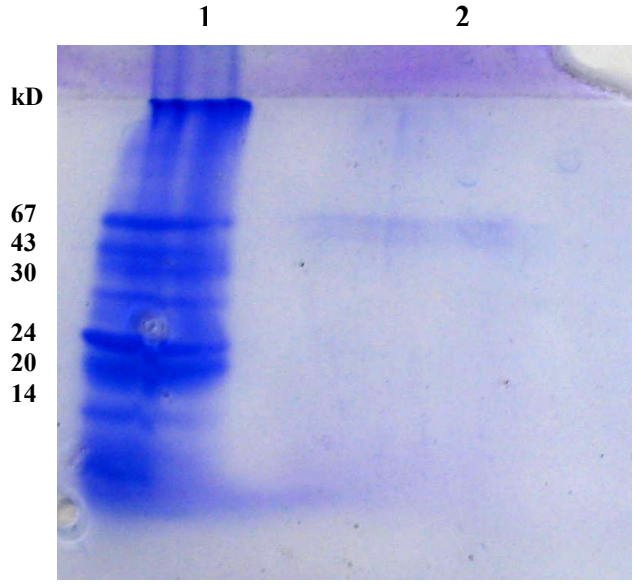

Figure 2: SDS-PAGE of EuniSL.

1- Standard marker proteins bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibition (20.1 kDa) and α-lactalbumin (14.4 kDa), 2- EuniSL.

CE and EuniSL demonstrated agglutination to human and rabbit erythrocytes, however CE showed a higher SHA to type O human erythrocytes while EuniSL agglutinating more intensely rabbit erythrocytes (Table 2). The difference in HA between lectin preparations is due to the nature of superficial glycoproteins of red cells, pure lectins can have their binding sites modified after the purification process. EuniSL can be potentially inhibited by glycoproteins present in bovine fetal serum (BFS) and rabbit serum (RS) and by tyroglobulin, casein and fetuin and is not specific to simple carbohydrates.

Isolectins from *Acacia constricta* and *Phaseolus vulgaris* were inhibited only by complex carbohydrates present in fetuin and tyroglobulin (Guzmán-Partida *et al*, 2004); glycoproteins which inhibited EuniSL activity These findings characterize EuniSL and the isolectins from *Acacia constricta* and *Phaseolus vulgaris* as to belong to complex lectins´s class. The determination of which glycans are well-recognized lectins is important to characterize the lectins`s carbohydrate binding sites. Consider that EuniSL did not bind mono and disaccharides and that many lectins can be settling to oligosacharides, the natural binding to these proteins are mainly complex glycans.

Table 2 - SHA with different erythrocytes and glycoproteins

Samples	**Erythrocytes**					**Glycoproteins**				
	A	**B**	**O**	**AB**	**R**	**BFS**	**F**	**T**	**RS**	**C**
			SHA			**SHA Inhibition**				
Seed Extract	14.6	14.6	58.5	-	7.3	0	11.0	1.5	3.0	0
Adsorbed (EuniSL)	21.3	10.6	10.6	21.3	85.3	5.3	10.6	2.6	0	0

SHA: Specific hemaglutinante activity; - no determinated.
The SHA inhibition was accomplished with rabbit erythrocytes.
R – Rabbit, BFS – bovine fetal serum, F- fetuin, T- tyroglobulin, RS- rabbit serum, C- casein.
EuniSL concentration = 3 mg of protein/mL and Seed extract concentration = 70 mg/mL.

This observation pattern is exclusive for many lectins inhibited by complex oligossacharides in branch glycoproteins of animals (Peumans and Van Damme, 1998). PNA (*Peanut agglutinin*) and ABA (*Agaricus bisporus agglutinin*) lectins demonstrated precipitation bands with samples that have glycolipeptides (Zeng *et al*, 2000); showed by

simple radial diffusion, a method used to qualitative detection of lectin interactions with glycidic compounds. By this method EuniSL, also, recognized glycoproteins present in *E. uniflora* seed and leaf extracts (Figure 3); different from Cramoll 1,4, a lectin that belonging from the glucose/mannose family obtained from *C. mollis* seeds (Correia and Coelho, 1995), that only recognized the glycoproteins present in *E. uniflora* seed extract. Furthermore, EuniSL was not recognized by Cramoll 1,4 (Figure 3), that demonstrated that *E. uniflora* lectin does not possess sacharide residues (glucose, mannose or its derivatives) in its structure, observed by the diffusion assay, once Cramoll 1,4 was not a glycoprotein and can be used as a pattern to determine the natural glycoproteic nature recognized by glucose/mannose lectins (Lima *et al*, 1997).

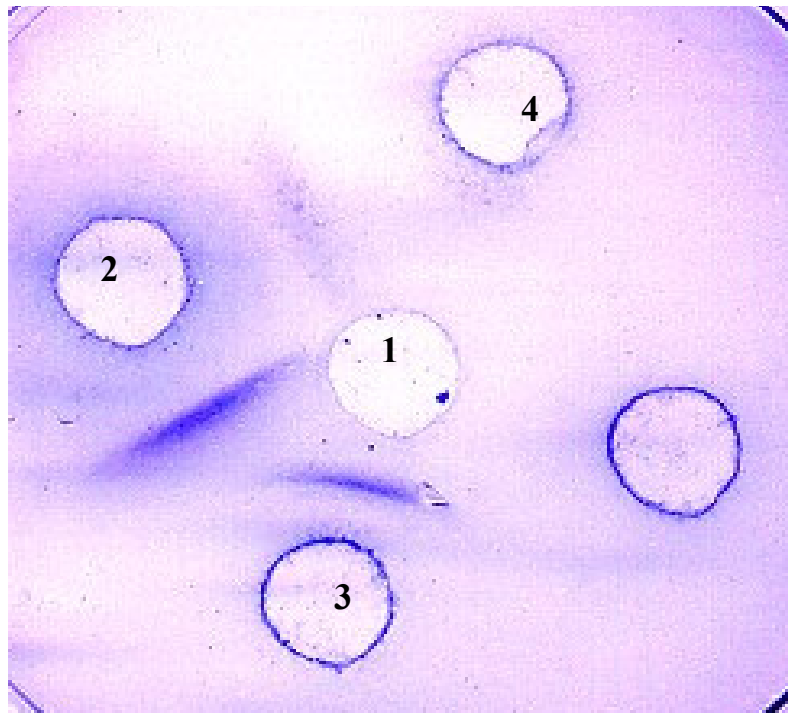

Figure 3: Simple diffusion of lectin from *E. uniflora:* (1) Cramoll 1,4 lectin; (2) EuniSL; (3); Extract of seeds of *E. uniflora* (4) Extract of the leaves of *E. uniflora.*

In relation to EuniSL stability it could be observed that the results of thermic denaturing demonstrated a significant stability at 100 ºC for 60 min, without lost its HA. This result was similar by demonstrated by *Ganoderma capense* with complete retention at

100°C for 1 h , its thermostability is more pronounced than what has been presviously reported for other lectins from leguminous and mushroom (Ngai *et al*, 2004), showing that some lectins are thermoresistent. Still, EuniSL is more stable at pH 6.5 (Figure 4), at pH 8.5 or higher, a reduction was observed of lectin activity. Divalent tested ions, did not affected or increase the activity of *E. uniflora* lectin.

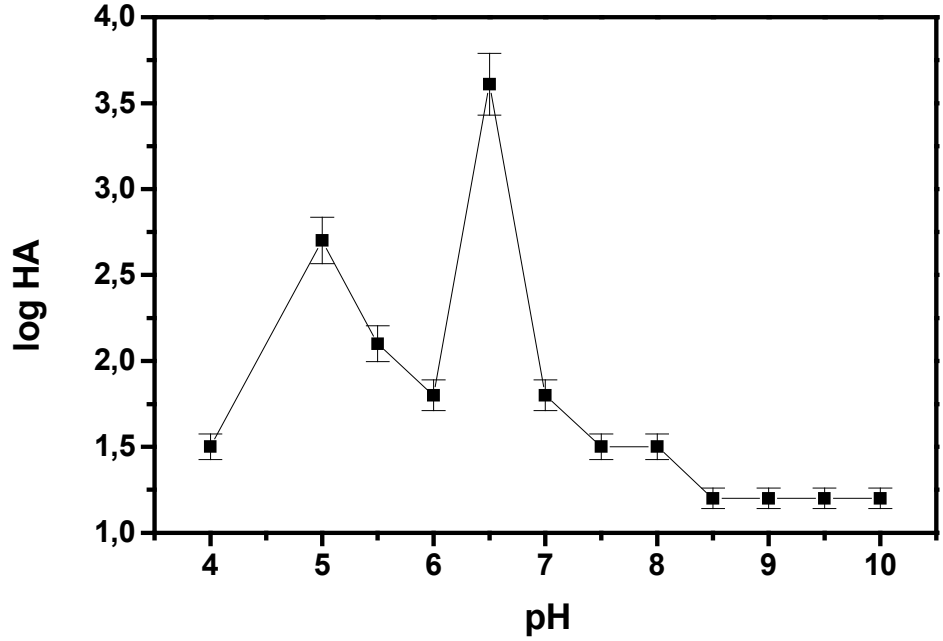

Figure 4: Influence of the pH on hemagglutinating activity of EuniSL.

EuniLS exhibited an antibacterial action on *Staphylococcus aureus*, *Streptococcus sp.*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Corinebacterium sp*. and *Klebsiella sp.* revealing the binding ability of this lectin to bacterial wall (Figure 5).

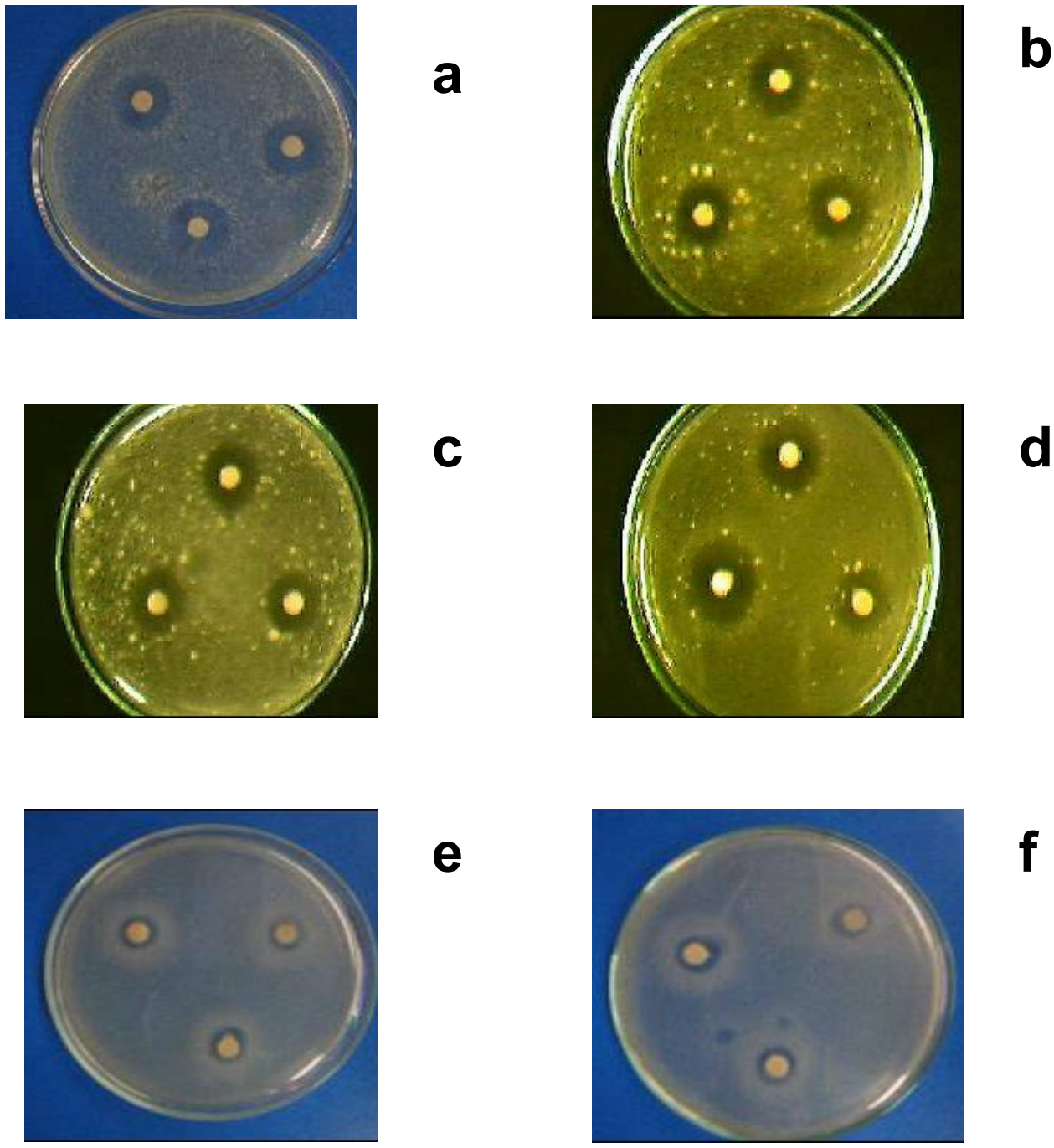

Figure 5: Diffusion assay of EuniSL front the bacteria (a) *Streptococcus sp.*, (b) *Sthaphylococcus aureus*, (c) *Pseudomonas aeruginosa*, (d) *Klebsiella sp.*, (e) *Bacillus subtilis* and (f) *Escherichia coli*.

The inhibition halos obtained through the diffusion test in disk are summarized in Table 3; CE did not exhibit antibacterial activity, demonstrating that the purified protein is responsible for the antibacterial action.

Table 3 - Antimicrobial activity of EuniSL.

Microrganism	**Diameter of clearing zone (mm)***
Staphylococcus aureus (+)	20.0 ± 0.57
Streptococcus sp. (+)	17.3 ± 0.57
Bacillus subtilis (+)	12.0 ± 0
Klebsiella sp. (-)	19.6 ± 3.3
Pseudomonas aeruginosa (-)	18.6 ± 0.6
Corinebacterium sp. (-)	14.0 ± 0.1
Escherichia coli (-)	12.0 ± 1.0

Gram – positive (+); Gram – negative (-) bacteria.
* Diameter of a paper disc

The MAC of EuniLS with the tested bacteria was expressed as a degree of precipitation of the solution lectin-bacteria, in which it was read the titre of the activity in agreement with the bacterial control. The activity, verified visually after incubation overnight, was indicated the minimum lectin concentration capable to agglutinate the bacteria (Table 4).

Table 4 - Agglutination activity of EuniSL on the bacterial cells.

Microorganism	**MAC (mg/mL of EuniSL)**
Gram negative	
Klebsiella	0.0625
Pseudomonas aeruginosa	0.25
Gram positive	
Staphylococcus aureus	0.25
Streptococcus sp.	0.25

Lectin initial concentration = 1 mg/mL

The differences in MAC were observed in agreement with the types of bacteria, however, for our study the largest MAC went to the bacteria *Klebsiella sp*. that exhibited a MAC of 0.0625 (titre of 64). It is known that the lectins does not just bind with sugars of glycoprotein reduced terminals, but some also react with internal components of the carbohydrate chains or with non carbohydrates (Goldstein *et al*., 1986). Inhibition of EuniSL and bacteria agglutination using O-methyl-α-D-glucopyranoside, trehalose, raffinose and bovine fetal serum showed that EuniSL did bind specifically to tested bacteria through bacteria surface carbohydrates (Table 5). A lectin of *Morus alba* was studied by exhibited an antibacterial activity against *P. syringae pv mori*; was inhibited agglutination by fetuin and tyroglobulin (Ratanapo *et al* ., 2001).

Table 5 – Agglutinating inhibition by sugars.

Microorganism	o-metil-α–D-glucopyranoside	Trehalose	Raffinose	Bovine fetal serum
Staphylococcus aureus (+)	4	4	2	0
Streptococcus sp. (+)	4	8	8	8
P. aeruginosa (+)	16	16	8	4
Klebsiella sp.(+)	4	8	16	0

Numbers represent the titre of agglutination activity inhibition; EuniLS agglutination was 64.

The concentrations necessary to determined the MIC and MBC values are summarized in Table 6. EuniSL exhibited a notable antibacterial activity against Gram-positive and Gram-negative bacteria, being the results are in agreement with the diffusion assay. The highest MIC values were obtained to *S. aureus*, *P. aeruginosa* and *Klebsiella* sp. Equivalentely the results indicate that EuniLS have bactericide activity against tested bacteria. *Robinia pseudoacacia* seed lectin demonstrated action against *S. aureus*, *B. subbtilis* and *E. coli* exhibiting MIC of 180, > 200 and> 200 respectively (Talas–Ogras *et al*., 2005) higher than the data obtained for EuniSL. A secretion lectin from the fish *Sebastes schlegeli* (Y. Nagashima *et al*, 2003) demonstrated antibacterial action for *B. subtilis* and *E. coli* with value of MIC (> 200). The mechanism of action of peptides is not very elucidated, but it has been proposed that the proteins with antibacterial action form a channel on the cell membrane and these for its time die due to exit of the cellular content, being this mechanism different from the antibiotics (Talas–Ogras *et al*., 2005).

Table 6 - MIC and MBC of EuniLS against bacteria.

	MIC value (μg/mL)	**MBC value (μg/mL)**
	Gram positive	
Staphylococcus aureus	1.5	16.5
Bacillus subtilis	16.5	180
Streptococcus sp.	16.5	180
	Gram negative	
P. aeruginosa	1.5	16.5
Klebsiella sp.	1.5	16.5
Escherichia coli	16.5	180
Corinebacterium sp.	16.5	180

Lectin initial concentration = 2 mg/mL

Conclusions

In the present study thermoresistant lectin (EuniSL) was obtained , purified in only one step by ion-exchanged chromatography, showing activity by radial simple diffusion. The results evidenced a potent antibacterial lectin in *E. uniflora* seeds (demonstrated by the *in vitro* inhibition of the growth of some important pathogenic bacteria. Future researches of the application of lectins, obtained from medicinal plants, in biological systems, can be of great importance for clinical microbiology and possible therapeutic applications.

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ARTIGO A SER SUBMETIDO AO PERIÓDICO LANGMUIR

INTERFACIAL PROPERTIES of *Eugenia uniflora* and *Triticum vulgaris* LECTIN MONOLAYERS

INTERFACIAL PROPERTIES of *Eugenia uniflora* and *Triticum vulgaris* LECTIN MONOLAYERS

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Introduction

Lectins are proteins capable of recognizing carbohydrates that are involved in several cellular processes due to their structural characteristics and interaction principles between carbohydrates and the specific site of lectin (Sharon, 1993; Elgavish and Shaanan, 1997; Syed *et al*., 1999). Although they are widely distributed in the plant kingdom, seeds of leguminous are a particularly rich source of them. *Eugenia uniflora L*. is a Myrtaceae plant native of South America, Southeast of Asia and Africa. In Southern Africa the leaves of this plant have been used to treat hipercholesterolemy, gout and arterial hypertension (Arai *et al*., 1999). It is claimed that extracts of *Eugenia uniflora* contribute to the reduction of blood pressure in hypertense patients (Consolini *et al*, 1999), as well as acting as inhibitors of DNA polymerase of the Epstein-Barr virus (Lee *et al*., 2000). *Triticum vulgaris lectin* (WGA) it is a wheat germen agglutinin with a molecular weight of 36kD that consists of two identical subunits. WGA contains a group of isolectins, with isoelectric point at a pH close to 9. The sugar receptor of WGA is N-acetylglucosamine, that binds preferentially to dimers and trimers of this sugar. WGA can bind to oligosaccharides containing a chitobiose or N-acetylglucosamine terminal, structures that are common in several Peptideglycans from the wall of bacterial cells, chitin and cartilage glucosaminoglycans can also bind to WGA.

Due to their amphiphilic nature, protein molecules are known to spontaneously organize themselves in a water-air interface (Rosilio *et al.*, 2004). This phenomenon is widely found in the science of proteins and it has been largely used in the food and pharmacological industries (el Kirat *et al*., 2004). In this regard, since the study of organized monolayers floating in a water-air interface is an important working model in the search of a better understanding of the structural characteristics and relative stability of protein molecules, (el Kirat *et al*., 2004), the interfacial properties of proteins have been subject of intensive study for several years. However, proteins present a complex structural morphology, and it is well-known that the extent with which proteins adsorb is influenced by; at the same time, once adsorbed they unfold and rearrange themselves in secondary and tertiary structures (MacRitchie, 2000) since the exposure of their lipophilic residues to the hydrophobic phase reduces the free energy of the system. The high concentration of proteins at the surface favors the mutual interactions and the formation of aggregates

(Wilde *et al*., 2004) and, as result, the mechanical properties of the floating monolayers depend on the structure of the adsorbed protein, and by nature and extent of the interactions among their molecules (Bos and van Vliet, 2001). The observed differences in behavior arise because films of biopolymers in general, and of proteins in particular, have special characteristics due to the peculiar nature of the groups present in these molecules. Proteins usually have a high molecular weight, a large relative size, and a very flexible structure so that its conformation can change accordingly to the experimental conditions. In addition, protein molecules contain a large number of apolar groups that prefer to avoid the contact with water and other high number of polar group that can become molecule highly soluble and this way reduce the number of molecules in the subphase air-water (desorption process). The combination of groups with opposite nature in the same molecule allows that thermodynamically stable films of biomacromolecules could be formed in interfaces between two media of opposite polarities (Roberts, 1990; MacRitchie, 1997; Sánchez-González, 2003). When dissolved protein molecules migrate to the air-water interface to form a monolayer, the free energy of the system can increase and therefore an energy barrier is formed during the process (Donohue and Aranovich, 1998); as a consequence, structural rearrangements through the adjustment of the conformation of the adsorbed protein molecules can increase the surface pressure to counterbalance the energetic barrier (de Jongh *et al*., 2004). Hence, under adsorption at the interface, the molecules of proteins tend to expand and unfold their active intrinsic structure (Cornec et al., 1999). Due to the prevailing interactions at the interface, the existing cohesive forces acting inside the protein molecule (such as those of hydrophobic and van of Waals nature) are reduced (Miller et al., 2000).

In this study, we present results of the characterization of a new lectin (EuniLS) obtained from seeds of *Eugenia uniflora* and demonstrate how relate the interfacial behavior of EuniLS and WGA lectins measuring the surface pressure (Π),surface potential (ΔV) and calculus of double electric layer of floating monolayers of this protein prepared in a Langmuir trough at different pH values to measurements of bulk behavior of lectins using potential ζ and impedance spectroscopy.

Materials and Methods

Chemicals

In all experiments in the Langmuir trough, pure water obtained by osmosis from an NANOpure- water system (Barnstead, USA) (with a pH value of 7.0 and a surface tension of typically 72.2 $mN.m^{-1}$ at 20ºC) was used. For the experiments performed at different pH values in the range 2-9, a citrate (citrate of sodium)-phosphate (sodium phosphate) buffer was used. All needed glassware was cleaned by using a freshly prepared sulfochromic solution and then abundantly rinsed with ultrapure water. WGA lectin was purchased from Sigma (Saint Louis, USA).

Purification of lectin

Lectin purification was performed by ion - exchange chromatography in DEAE-Sephadex G-50 Chromatographic assays were made in columns at a rate of 10 mL/h, using a peristaltic PUMP, at room temperature. Samples of crude extract (11.3 mg of protein/mL) were applied directly on the support. After passage of the samples, columns were washed with phosphate sodium phosphate (PSB) at neutral pH. The elution of the adsorbed material was performed with PSB adjusted to pH 2.0, until vanishing of the absorbance band at 280 nm. Hemagglutinanting activity was determined to evaluate the lectin-binding activity to carbohydrates and the protein concentration was determined by Lowry method (1951) and the material was stored at -20º C.

Surface pressure and surface potential measurements

A 100 µl Hamilton micropipet was used to disperse a lectin solution a top the surface of a PSB volume contained in a LB-5000 Langmuir trough (KSV, Finland) equipped with a Wilhelmy plate for measuring the surface pressure. The compression of the floating monolayer was a constant rate of 10 mm m^{-1}. The spread lectin monolayers solution at the air-buffer interface had approximately 2.85×10^{13} to 1.65×10^{13} molecules/µL for EuniLS and WGA, respectively. All results are mean values of at least three measurements. The surface potential of spread lectins monolayers was measured using the vibrating plate method (KSV, Finland). The formation of monolayer brings a

change in surface potential which is proportional to the change of the vertical component of the dipole density of the spread molecule with respect to the pure water surface. It was considered that equilibrium was established when the value of ΔV did not change after 15 min. All reported surface potential values are mean values of at least three measurements.

Measures of potential ζ

Measures of potential ζ were accomplished by the eletrophoresis methods using an instrument Zetasizer Nano-ZS90 (Malvern, United Kingdom). The lectin (1 mg/mL) it was dissolved in citrato-phosphate buffer and the values ζ they were recorded as function of the variations of the pH. The results were the averages of at least three measures accomplished in the samples to each pH.

Impedance spectroscopy measurements

The impedance analyses were accomplished using an impedance analiser of gain/phase SI 1260 (Solartron Instruments, Farnborough, UK). The absolute values of impedance were recorded in a frequency range of 0.1-10^7 Hz, with the data obtained equally spaced in logarithmic scale and with ten points per decade. The impedance measurements were performed with two parallel plates of steel (20 mm × 5 mm) placed inside of a becker of 25 ml contends the solutions of proteins.

Results and Discussion

The isotherms characteristics of surface pressure (Π)-surface area (A) of the dispersed monolayers of EuniSL and WGA they are presented in Fig. 1 (a, b). Starting from the evaluation of these isotherms it is evident that the profiles Π-A of both lectins depends strongly on the pH. With the passage of the acid pH the alkaline (pH 2 to 9) a displacement of the mean molecular area is observed for higher values, therefore significant differences in the interfacial behavior of these two lectins can be observed. The Π-A isotherm of EuniSL at pH 2, showed that the mean molecular area (mma) increased of 8750$Å^2$/molecule a Π =0 had a gradual increase in this point until a maximum pressure of 18mN/m at 7600$Å^2$/molecule.

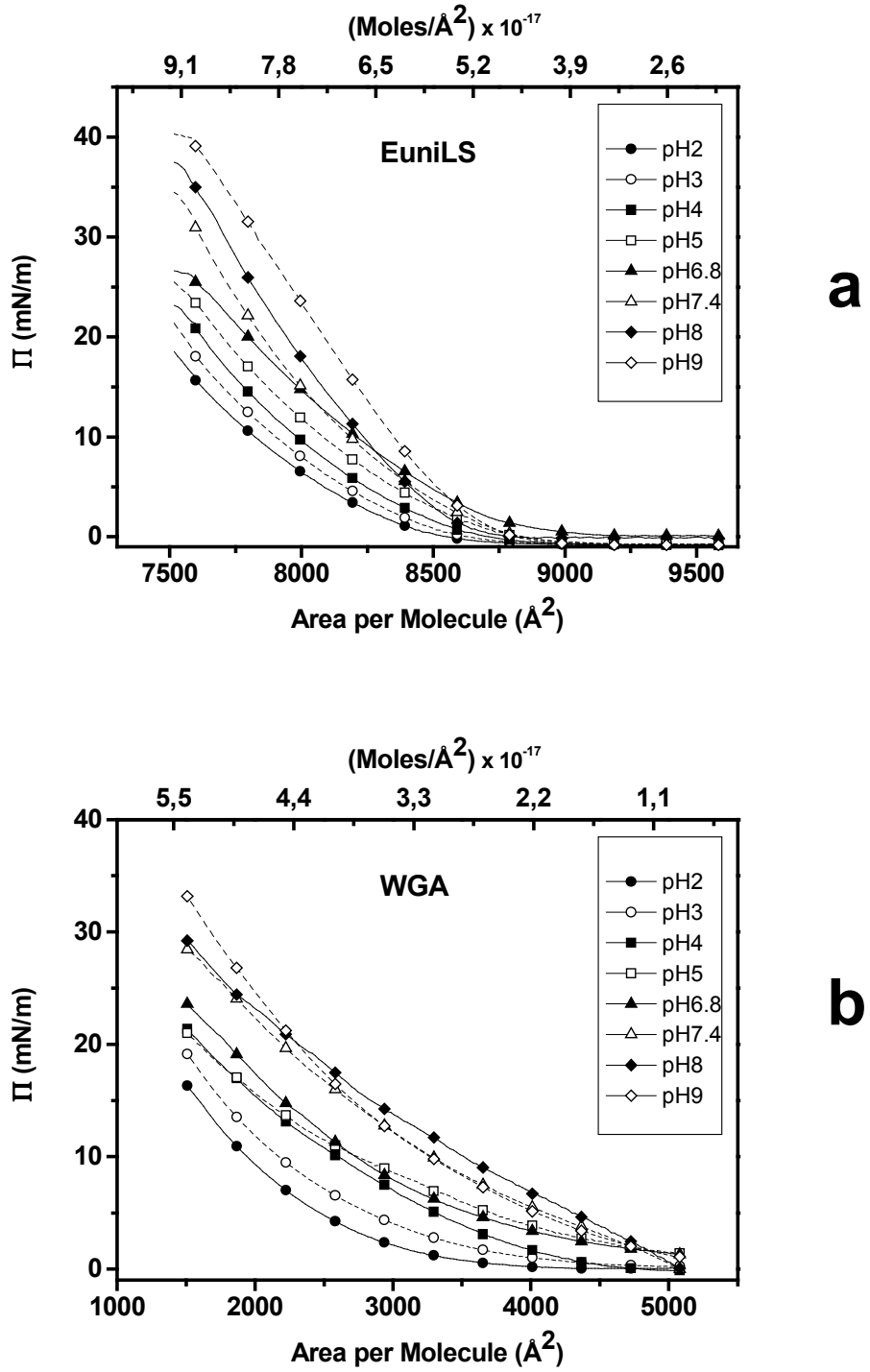


Figure 1: Variation of the surface pressure (Π) as a function of the mean molecular area (A) for monolayers of: (a) EuniSL and (b) WGA lectins spread at the air-water interface at different pHs.

For WGA the increase began in area of 3600Å^2/molecule with similar increase EuniSL, presenting a smaller surface pressure, Π_{max}=16mN/m. A notable difference between EuniLS and WGA was also observed in the pH range from 7,4 to 9. EuniSL presented a condensed contour beginning in 8750Å^2/molecule for this pH range (7,4-9) and finishing in 7600Å^2/molecule. WGA presenting contours expanded for the pH 7,4 and 8 and a change in the Π-A isotherm characteristics is already observed in the profile (at pH 9) demonstrating the beginning of a transition for the condensed phase. Since the isoelectric point of WGA that is close of the pH 9, this easily would explain the changes in the contours of the isotherms of WGA when they approach of the alkalinity (pH 7,4 to 9) and presenting surface areas contained among 5000Å^2/molecule and 1600Å^2/molecule for this pH range.

The conformation of proteins depends on the environment, and the interface is very special because it imposes bidimensional energy conditions that are not present in solution (Borioli *et al*., 2001; Borioli *et al*., 2004). Such restrictive conditions result in an orderly orientation of all the molecules, including lipides and proteins, in the interface. This concept should be considered when works with monolayers since such an orderly organization means that the protein generally adopts a different conformation than that found in the bulk, but it is not necessarily considered denaturated. Besides, in most of the cases this conformation at the interface is not irreversible. On the other hand, it was found that the thermal aggregation in solution can be totally reversible while the changes in the interfacial properties of the protein β-casein presented in the superficial film were irreversible (Dauphas *et al*., 2004). However, molecules on the surface are confined than in solution as well as they come in larger concentration in just certain local. As a consequence of the change of the conformation, some proteins amphitropics can lose the biological activity depending on the composition interfacial and/or organization, as shown for several associated enzymes the membrane (Muderhwa and Brockman, 1992; Laux *et al*., 2000).

Molecules of proteins or other polymeric ones can reduce its free energy for the displacement of segments of the interface (Mackitchie, 2000). This activated segment of proteins in the liquid interface generally consists of some 6-10 residues of amino acids and, as a result of such displacement, the monolayer will not be stable. The activated state of the

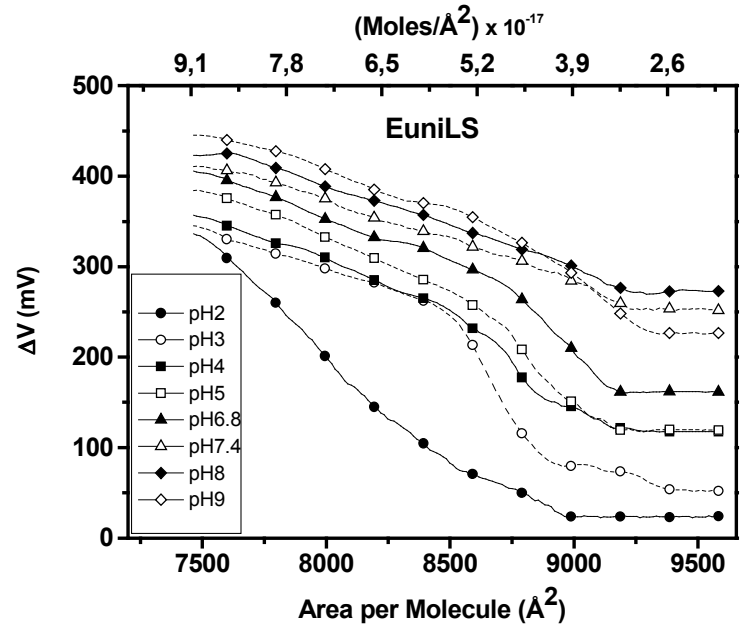
protein molecule can be gotten by surface compression or flotations in the free energy on the conformation balance governed by the distribution of Boltzmann.

With the increase of the lateral pressure it increases the statistical probability of desorption of the segments of protein. Even so for Π contained among 5 and 20 mN/m this probability is low. Studies of protein monolayers of bovine serum albumin (BSA) under aqueous subphase in different pHs, they determined that superficial pressures among 10 e 15 mN/m the proteins films was highly orderly and good quality for transfers of the films for supports (Sanchez-González *et al*., 2003). Therefore, starting from these indications the estimate of the dependence of the ΔV with different pHs was determined in superficial areas corresponding to Π=15mN/m, where the monolayers of the two studied lectins were considered stable and in this pressure the process of desorption of the interface air-subphase will not be happening (Mac Ritchie, 2000).

A careful analysis of the Π-A isotherms of EuniSL and WGA indicate that the variation in the pH had a different effect on its respective profiles. Two important observations can be made in this consideration: (i) for EuniLS and WGA the maximum surface pressure increases with the increase of the pH, for EuniSL of the pH 2 to 9 the Π_{max} it varied of 18 the 40mN/m while for WGA in this pH range the Π_{max} it varied of 16mN/m to 34mN/m; (ii) For WGA, the curves of Π-A (pHs 4 and 5) they are put upon in regimes of superior pressures the Π=16mN/m e nos pHs 7,4 e 9 they are put upon in regimes of inferior pressures the 16mN/m. While there was superposition of the contours of Π-A in the case of EuniSL.

In agreement with Andrade *et al*. (2005) this superposition particularity can be attributed to a reduction of the charges present in the lectin molecules. Possibly for these specific pHs the lectin molecules in the interface repel some less strongly the other ones and as consequence, it should happen an increase in the packing (Andrade *et al*., 2005). However, it is conceivable that such inversion in the isotherms of Π-A for certain lectins they result of conformation changes of its structure (McKenzie et al., 1972).

The potential isotherms surface (ΔV-A) area for EuniSL and WGA is presented in the Fig. 2 (a, b). Accompanying the decrease in the molecular areas a notable tendency can be observed for elevation of the surface potential values.



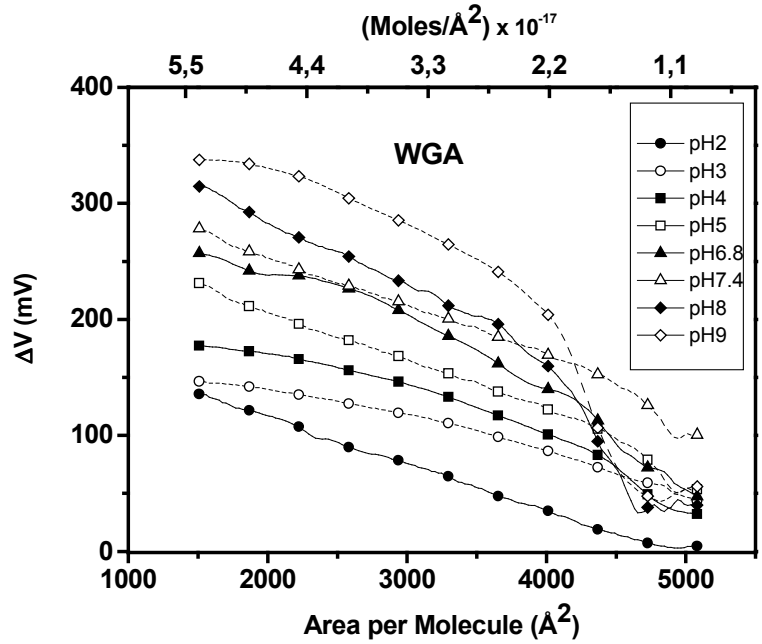


Figure 2: Variation of the surface potential (ΔV) as a function of the mean molecular area (A) for monolayers of: (a) EuniSL and (b) WGA lectins spread at the air-water interface at different pHs.

However, in high molecular areas and specifically for WGA, the beginnings of the curves of the pHs 8 and 9 tend putting upon or they cross due to the disorder phenomenon. The complexity of factors for the surface potential of the monolayers hinders the explanation of the reasons for this behavior disordered in small molecular areas. However some authors (Oliveira Jr *et al*., 2004) affirm that two main causes exist for the surface potential to be different from zero in higher molecular areas, (i) the formation of great clusters after the spreading of the monolayers, (ii) the existence of a strong contribution for ΔV of the double layer formed in the interface monolayer/subphase.

However it should be reminded that one of the contributions of the values of ΔV in this area comes from the existence in the interface of intersticiais connections of the water filling the spaces among the dispersed molecules. The molecules of water besides the small mass, possess high multipolar moments that contribute to the formation of hydrogen bonds (Degrève and Blum, 1996). For great extensions of air-water interfaces, the break of the bonds is inevitable and the local distribution of the hydrogen bonds becomes asymmetric, in this order to minimize the potential energy of the system, the hydrogen bonds are redistributed in the direction of the bulk (Rocha *et al*., 2004). This great effect inside of this plane of water structured among the spaces it should mask other global contributions of ΔV, doing with that they are less capable of controlling the influence of the pH on ΔV in this area of the isotherm of ΔV-A.

Fig. 3 illustrate the dependence of ΔV in function of pH for EuniSL and WGA in Π=15mN/m. It is evident that with the increase of the pH brought a great linear increase in the values of ΔV. Besides, whole of course the values of ΔV of EuniLS went superiors to the from WGA. As expected, the high pHs where the molecules of these lectins are with larger packing density the isotherm it tends to a condensed type (Fig. 2a, b) and high values of ΔV were observed. Behavior this inverse for two galactose-specific lectins (BmoLL and Con A), already studied by our group (Andrade *et al*., 2005), once the unfolding characteristic is characteristic for each protein molecule. In order to have a better insight on how the monolayers influence the surface potential values, we have calculated the effective dipole moments using the classical equation

$$\mu_{\perp} = \frac{\Delta V}{12\pi\delta} \qquad \textbf{Eq. 1}$$

where $\delta = 1/A$ is the surface density expressed in molecules/cm^2, A the molecular area in Å^2, ΔV the maximum surface potential in mV, and $\mu_{\perp}$ the vertical component of the total dipole moment expressed in mD (Gaines, 1966). The above equation is the Helmholtz equation expressed in international units considering ε_0 equal to the unity. The dipole moments were calculated from Eq. 1.

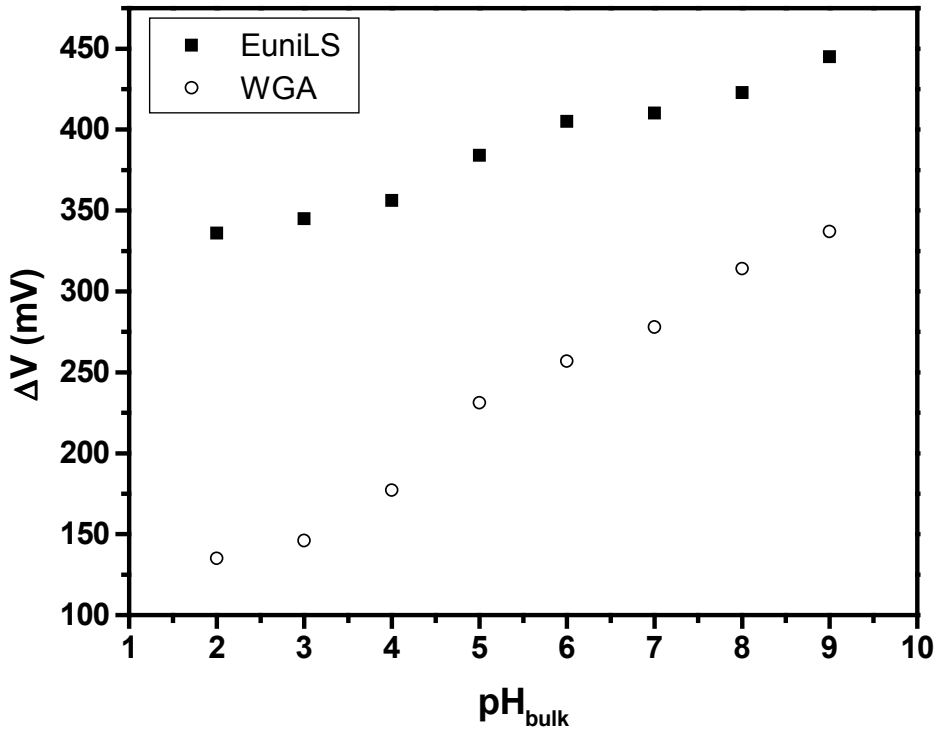


Figure 3: Variation of the maximum surface potentials (ΔV) as a function of pH_{bulk} for monolayers of EuniLS and WGA lectins spread at the air-water interface.

The plot of the $\mu_{\perp}$ versus pH_{bulk} (Fig. 4) demonstrates the behavior in the dependence of the pHi (inset Fig. 4). It observes that for EuniSL the increase of the pH from 2 to 9 produced an increase of three folds in the value of $\mu_{\perp}$. While, that for WGA in the same pH range, this increase in the $\mu_{\perp}$ it was of 3,6 times.

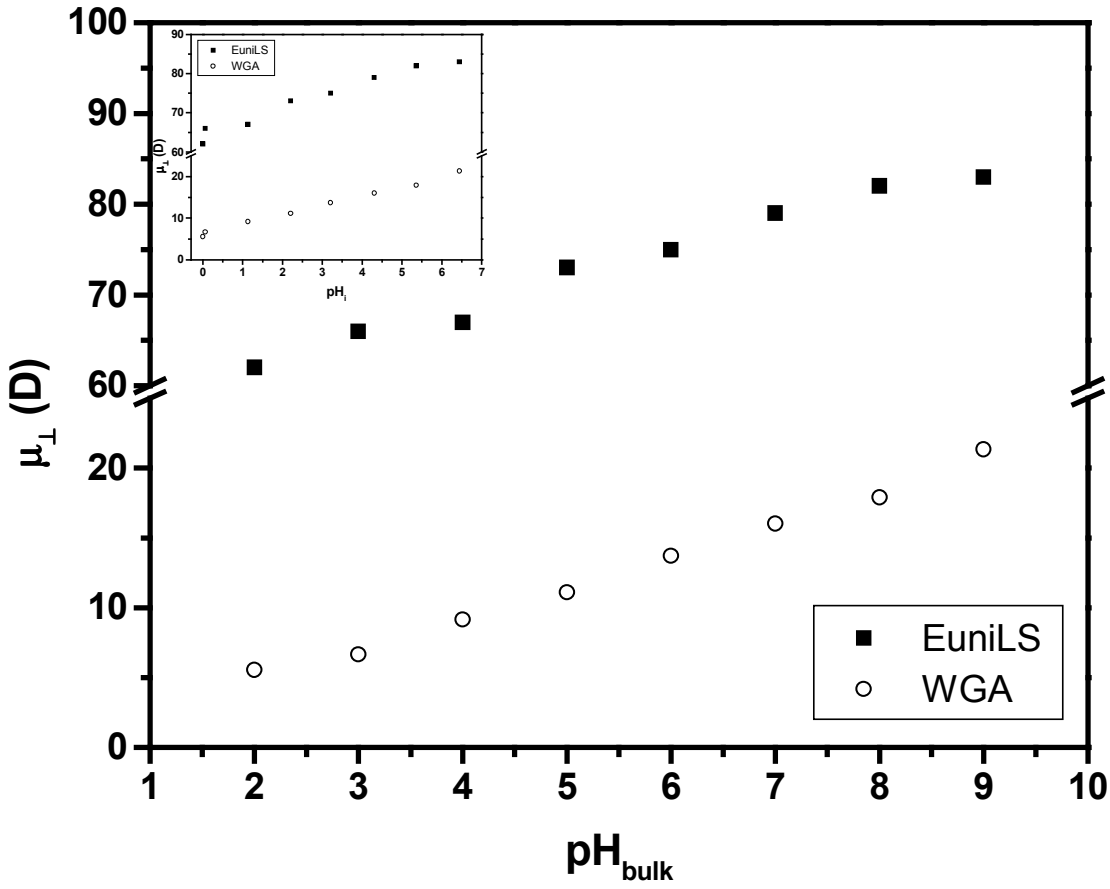


Figure 4: Variation of the effective dipole moments ($\mu_{\perp}$) as a function of pH_{bulk} for monolayers of EuniSL and WGA lectins spread at the air-water interface. (Inset: Maximum surface potential versus pH_i for EuniLS and WGA).

The proteins in solution possess structural flexibility and a great number of different configurations. Therefore, the dipole moments found by some authors (Porschke, 1997; Takashima and Yamaoka, 1999) they are the average of the dipole moment of different configurations documented in databases. Consequently, these values cannot be compared with the found values of $\mu_{\perp}$ for EuniLS and WGA. However, to determine the molecular structure of a protein, techniques as electronic microscope or scattering of X-Ray (Porschke, 1997) they are involved and used typically them database formed for the calculation of the dipole moment of the proteins in the form of crystals. With the innovation of the technique of RMN became possible the verification of the contribution of the proteins structures in solution, even so new techniques have been proposed to promote the growth of crystals 2-D in interface air-water funcionalizada (Drazek *et al*., 2005). Evidently, the number of available conformations for these two lectins confined in small molecular areas is considerably reduced.

Therefore, the surface potential depends on the dipole moments of the molecules of the monolayer and double electric layer. If a monolayer is partially charged, a double electric layer is formed in the interface monolayer/water, therefore for analyzes of interfacial behavior of the lectins we used the Gouy-Chapman theory (Gouy, 1910; Davies, 1951; Andrade *et al*., 2005) whose contribution of the surface potential can be estimated using,

$$\Psi_0 = \frac{2kT}{e} sinh^{-1}\left[\frac{e\alpha}{A(5.88\times10^{-7} c\varepsilon T)^{1/2}}\right] \qquad \textbf{Eq. 2}$$

where *K* is the constant of Boltzmann, *T* it is the temperature, and it is the proton charge, α is the degree of polar groups dissociation, c is the ionic force of the subphase, *A* the area for molecule and ε is the dielectric constant in the area of the double layer. The model of Davies considers the charges as spherical electric points and it assumes that they are distributed on the superficial monolayer with charge density σ in ions/cm^2 where σ=e/A. The pH of the interface (pH_i) it depends inside on the distribution of the double layer and it can be calculated (Davies, 1951; Andrade *et al*., 2005),

$$pH_i = pH_{bulk} + \frac{e\Psi_0}{2.3kT} \qquad \textbf{Eq. 3}$$

Fig. 5 present the potential of the double layer (Ψ, mV) as a function of pH_{bulk}. Another contribution of the dielectric properties of the monolayers of the studied lectins can be obtained through the calculations of the contributions of the double electric layer (Ψ) for global ΔV the several pHs. Clearly an increase is observed in the Ψ with the increase of the pH. Both proteins presented a negative contribution of the double layer, calculated for all the pHs.

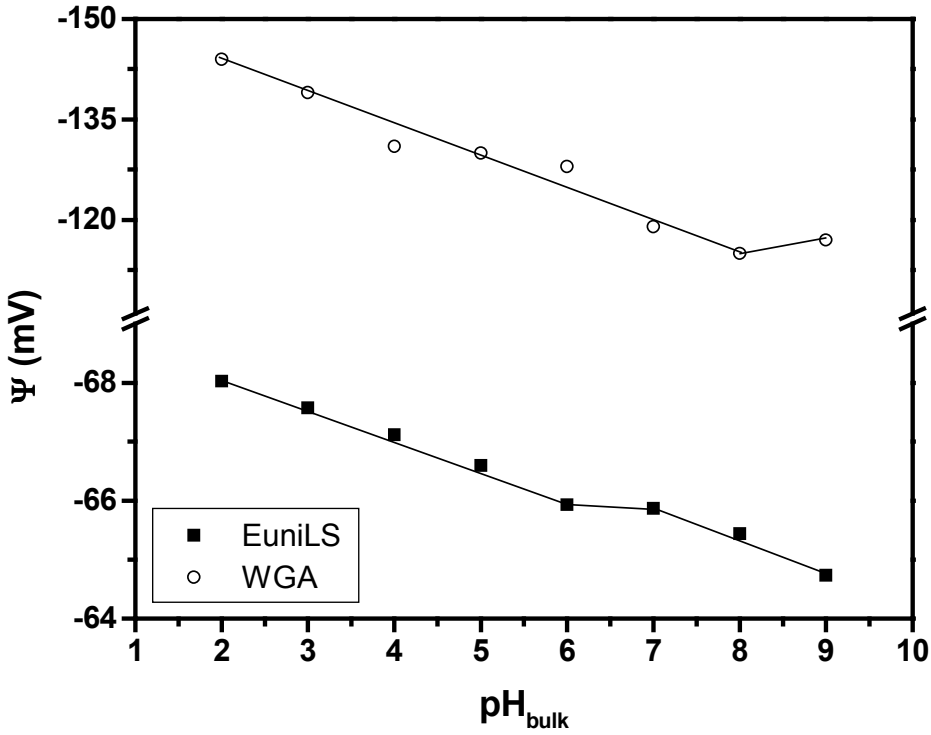


Figure 5: Variation of the double electric layer (ψ) as a function of pH_{bulk} for monolayers of EuniSL and WGA lectins spread at the air-water interface.

It is observed from Fig. 5 a decrease of the value of the Ψ (-148mV a -175mV, for EuniSL and -117mV to -144mV for WGA), even so demonstrating that both lectins was sensitive for pH variation in these conditions and that for EuniSL the value exceeded the value calculated for WGA. Besides, it was obvious that the pH in the interface (pH_i) it was significant decreased by the presence of the double electric layer. It is important to notice

that inside of all the pH range studied (pH 2 to 9), the negative charge of the lectin was strongly reduced in pHs below of 7 (Fig. 6). Presenting the behavior of the potential ζ similar to the behavior of the Ψ calculated starting from the ΔVs. It can be observed that happens a linear increase in the Ψ of pH 2 to 6 for EuniLS where in the pH 7 an inflection point can be observed that corresponds the characteristic change of a profile expanded for a more condensed in to Π-A isotherm demonstrating that this can be due to a decrease of effective charges of the lectin.

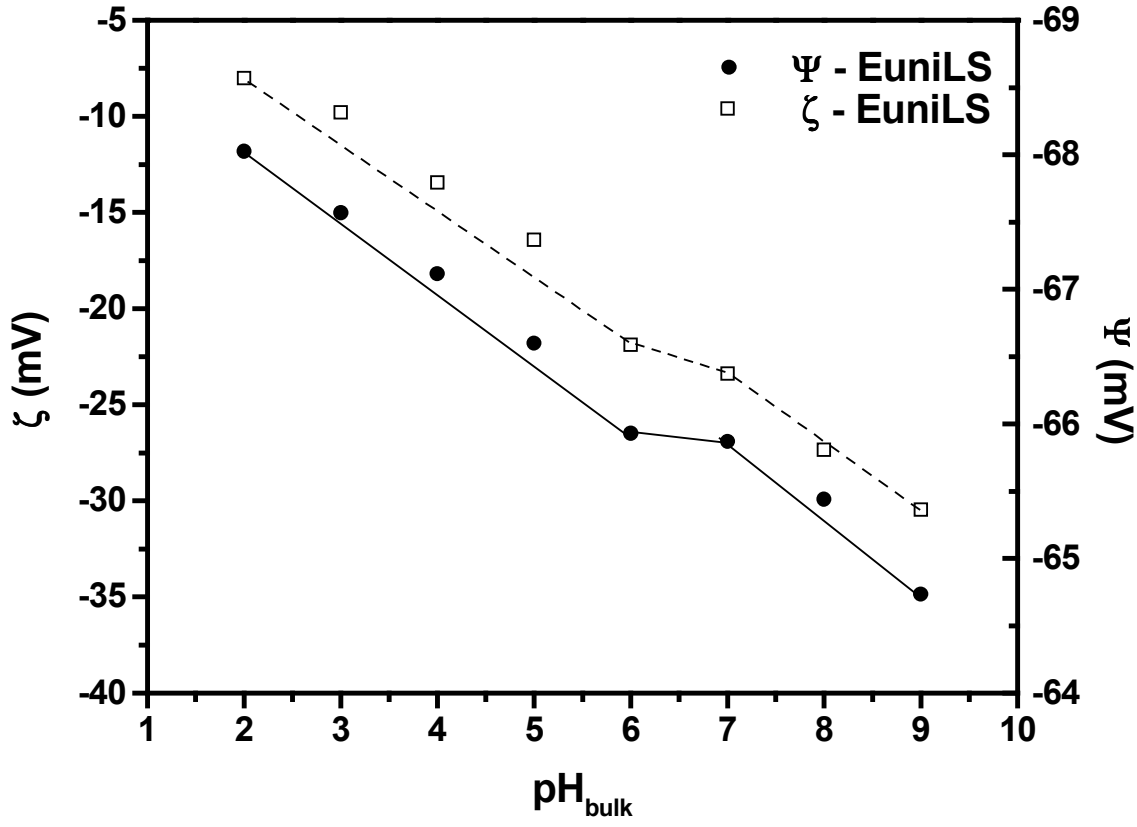


Figure 6: Variation of the zeta potential (ζ) and double electric layer (ψ) as a function of pH_{bulk} for monolayers of EuniSL lectin spread at the air-water interface.

For WGA we observed a similar behavior in the pH 8, for this case it can be affirmed that this change happens due to total decrease of the effective charges and its proximity of the isoelectric point. The collapse pressure of the Langmuir film of EuniLS is quite high, revealing a notable tensoative properties and stability for the collapse, being about of Π_c=40mN/m.

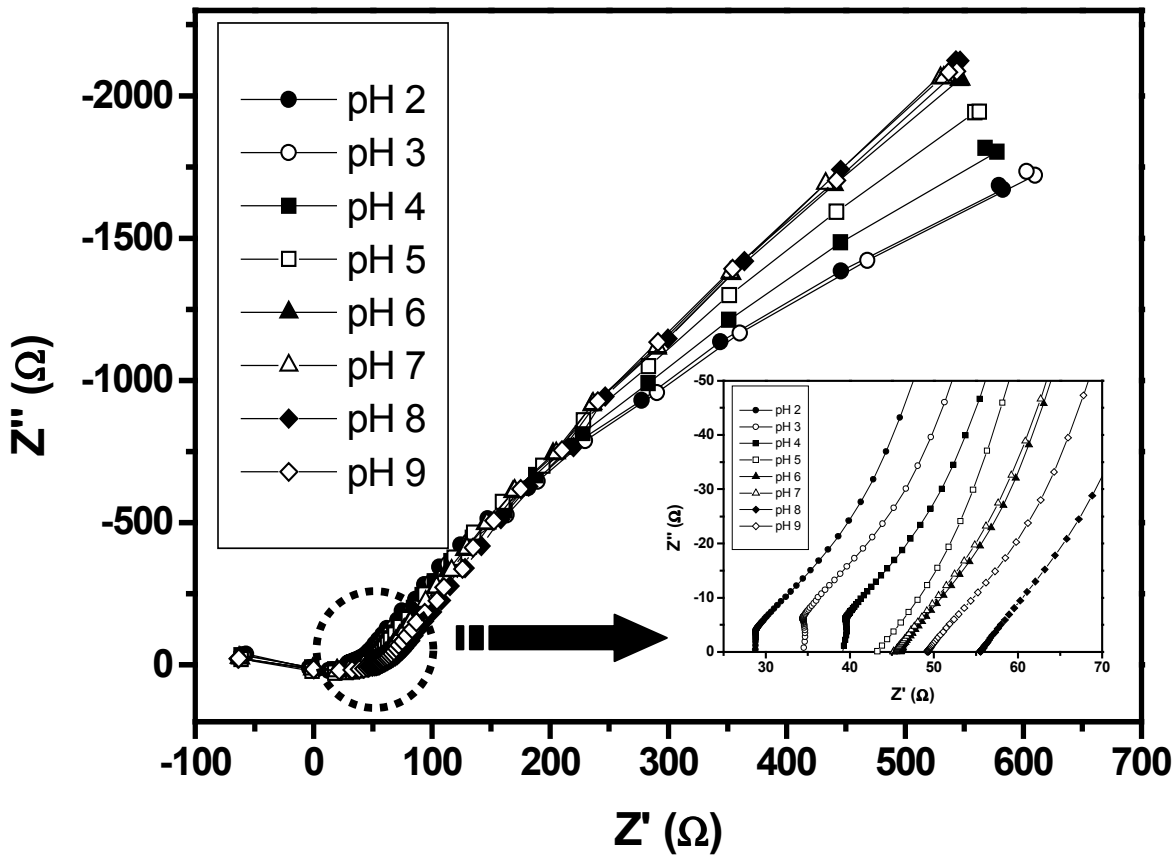


Figure 7: RX diagram for EuniSL.

Starting from Fig. 7 can be observed the diagram of resistance-reactance (RX) for the solution of proteins. To high applied external fields when the answer of the bulk should become dominant the for the different pHs they tend to converge. The detail of Fig. 7, resultant of an amplification of the circulated area, indicates the variation observed for the

real part, that will be plot in function of the pH (Fig. 8). Impedance measurements clearly can be noticed that there is a break of the derived in the pH varying between 6 and 7, indicating a direct correlation with the values of Ψ calculated and the potencial ζ .

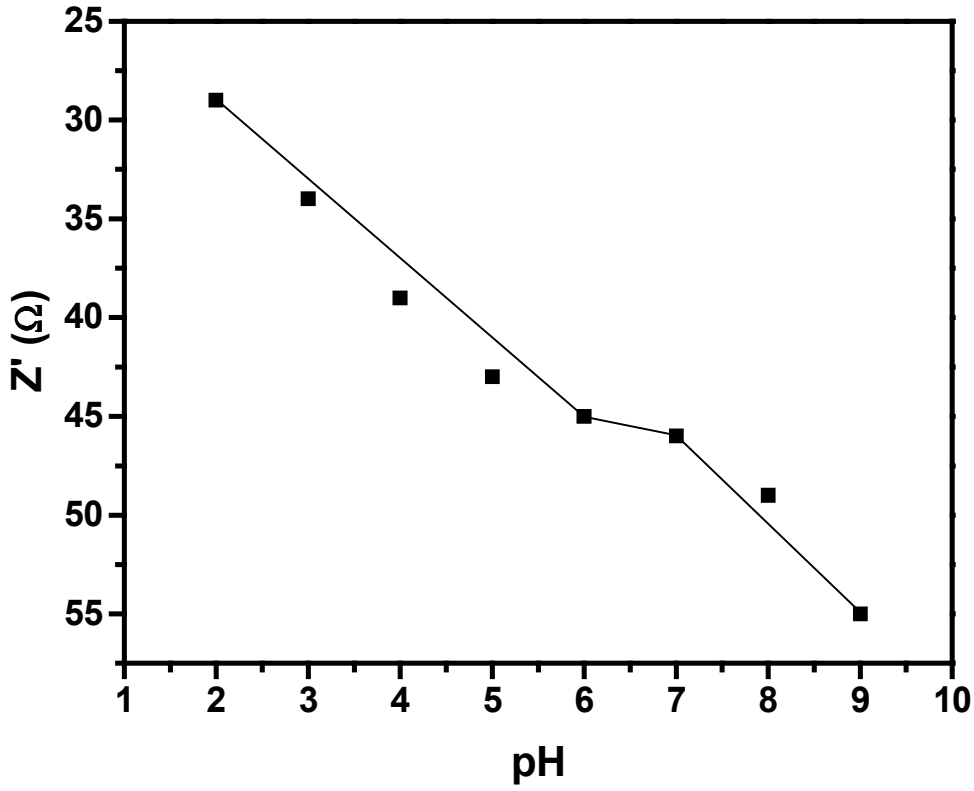


Figure 8: Real part versus pH for EuniSL.

Conclusions

In the purification study it was obtained a lectin (EuniSL) thermorresistent, of acid character, purified in an only stage by exchanged-ionic chromatography, showing visible activity in simple diffusion assay could be used potentially for biotechnological applications. The results indicated the formation of stable Langmuir films for both studied lectins. The studied properties indicated that the Π-A and ΔV-A isotherms are dependents of the pH variation of subphase. These results indicate that the tensoative properties of

EuniLS is enough strong for thermodynamically to drive and to retain the protein in the interface. The values of the Π-A e ΔV-A isotherms increased with the increase of the pH. The dielectric properties of EuniLS and WGA were evaluable from dipolar and double electric layer (Ψ) contributions calculated for global ΔVs. Starting from the equation of modified Davies the contribution of the double layer was calculated (Ψ) for Π=15mN/m, assuming that in this condition the lectin molecules did not suffer desorption of the interface during the compression. Since the value of Ψ, for both lectins it was demonstrated to be dependent of the pH that it contributes to variation of the effective protein charges, it can be concluded that the variation of its respective ones ΔVs in function of the pH it was not just owed the reorientation of the molecules of the lectins film that happen during the compression of the monolayers that act directly in its effective dipoles ($\mu_{\perp}$), and also due to contribution of the electric double layer. The results of the Ψ (from ΔV), ζ and dielectric measurements demonstrated that all the techniques are complemental and effective for determination of the isoelectric point of protein solutions and monolayers, denoting the dielectric spectroscopy as an useful tool for detection of isoelectric of proteins in solution.

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CONCLUSÕES

• EuniLS foi purificada de extrato de sementes por cromatografia de troca-iônica em uma única etapa;

• EuniLS apresentou 12 vezes de purificação;

• A AH de EuniLS não foi inibida por monossacarídeos, mas foi inibida pelas glicoproteínas, caseína, fetuína e pelas presentes no soro de coelho, soro fetal bovino e colostro;

• SDS-PAGE revelou a purificação de EuniLS apresentando peso molecular de 67 kDa;

• O teste de difusão em gel de agarose revelou que EuniLS não é reconhecida pela lectina de *Cratylia mollis* (CramoLL 1,4), mostrando que EuniLS não é uma glicoproteína;

• EuniLS é resistente a temperatura e sua AH não afetada pela presença de íons;

• No pH 6,5 EuniLS apresentou uma maIor AH, sendo mais estável neste pH;

• EuniLS apresentou a capacidade de aglutinar bactérias via interação com as células bacterianas;

• A atividade aglutinante de EuniLS foi inibida totalmente por soro fetal bovino demonstrando que a aglutinação ocorre pelo sítio de interação da lectina para o carboidrato;

• EuniLS exerceu uma ação antimicrobiana sobre a maioria das bactérias testadas, sendo que para o *S. aureus*, *Klebsiela sp*. e *P. aeruginosa*, observaram-se maiores halos de inibição;

• O melhor valor de MCI (1,5 mg/mL) e MCB (16,5 mg/mL) foi verificado para as bactérias *S. aureus*, *P. aeruginosa* e *Klebsiela sp.,*

• EuniLS apresentou propriedades tensoativas, formando filmes estáveis de Langmuir;

• As isotermas Π-A e ΔV-A de EuniLS são dependentes do pH da subfase, ocorrendo o aumento da pressão, do potencial de superfície e da área de superfície com o aumento do pH;

• A dupla camada elétrica (Ψ) modifica com o pH da subfase, apresentando um ponto inflexão entre o pH 6,0 e 7,0, correspondendo provavelmente ao ponto isoelétrico da EuniLS;

• O potencial zeta (ζ) confirmou a região de inflexão entre o pH 6,0 e 7,0, observada para o Ψ;

• A espectroscopia de impedância confirmou os resultados obtidos para a dupla camada elétrica (ψ) e para o potencial zeta;

• A espectroscopia de impedância é proposta como uma técnica alternativa para a determinação do ponto isoelétrico de proteínas.