UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

DOUTORADO EM CIÊNCIAS BIOLÓGICAS

NICOLAAS JOHANNES HAVER

DESENVOLVIMENTO, PURIFICAÇÃO E CARATERIZAÇÃO DE IgG ANTI LECTINA DE FOLHA DE *Bauhinia monandra*

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Autor: Nicolaas Johannes Haver

Orientadora: Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho

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Tese apresentada ao Curso de Doutorado em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do grau de Doutor em Ciências Biológicas, na área de Biotecnologia.

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Para minha esposa Patrícia **e para minha família.**
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HAVER, N. J. Desenvolvimento, purificação e caracterização de IgG anti-lectina de folha de *Bauhinia monandra*. Tese (Doutorado em Ciências Biológicas) – Centro de Ciências Biológicas, Universidade Federal de Pernambuco, 2002.

A lectina de folhas de *Bauhinia monandra* (BmoLL) foi previamente purificada por fracionamento com sulfato de amônio (F 0-60%) seguido por cromatografia de afinidade com gel de Guar. Para o estudo da BmoLL foi desenvolvido um anticorpo policlonal em coelho contra esta lectina; anti-BmoLL IgG foi purificada em coluna de Sepharose-Proteína A. Em imunodifusão, anti-BmoLL IgG apresentou reações cruzadas com lectinas de *Bauhinia purpurea* e *Ulex europaeus* I. Lectinas de *Bandeiraea simplicilofia* II e *Triticum vulgaris* reagiram inespecificamente com substâncias presentes no antissoro mas ausentes na preparação obtida de IgG. Precipitações de anti-BmoLL IgG com extratos de outros tecidos de *B. monandra*, revelaram outras formas moleculares da lectina. Anti-BmoLL IgG foi conjugada com peroxidase e aplicada em ELISA, confirmando os resultados de imunodifusão. Para a sua aplicação no biossensor piezoelétrico, o rendimento do método de imobilização foi testado imobilizando anti-BmoLL IgG em placas de ouro (4 x 4 x 0.5 mm^3) em quantidades de 0, 110 e 220 μg em 100 μl utilizando dextrana tratado com bromocianeto. As concentrações de IgG não imobilizada foram medidas pela quantificação de proteína após a imobilização e lavagens com PBS. As quantidades de 110 e 220 μg de anti-BmoLL IgG apresentaram respectivamente, rendimentos de imobilização de 46% e 33%. Anti-BmoLL IgG (110 μg/ml) foi imobilizada na superfície de ouro de Microbalança de Cristal de Quartzo (do inglês *Quartz Crystal Microbalance*, QCM). Na execução de ensaio de piezoelétrico, a QCM foi incubada com solução de BmoLL (91 μg/ml) seguido de uma redução de freqüência com 5 kHz, mostrando um grande reconhecimento da lectina pela IgG imobilizada. No potenciometro e no medidor de turbidez, anti-BmoLL IgG não provocou nenhuma alteração na ligação de galactose por BmoLL, indicando que a IgG purificada não tem afinidade pelos sítios de ligação de carboidrato de BmoLL.

ABSTRACT

HAVER, N. J. Desenvolvimento, purificação e caracterização de IgG anti-lectina de folha de *Bauhinia monandra*. Tese (Doutorado em Ciências Biológicas) – Centro de Ciências Biológicas, Universidade Federal de Pernambuco, 2002.

Bauhinia monandra leaf lectin (BmoLL) was purified previously by ammonium sulphate fractionation (F 0-60%) followed by guar gel affinity chromatography. To study BmoLL a polyclonal antibody against the lectin was raised in rabbits; anti-BmoLL IgG was purified on a Sepharose-Proteína A column. Cross-reactions of anti-BmoLL IgG with *Bauhinia purpurea* and *Ulex europaeus* I lectins were seen in immunodiffusion tests. Lectins of *Bandeiraea simplicilofia* II e *Triticum vulgaris* reacted unspecific with substances present in antiserum but absent in the IgG preparation. Precipitations of anti-BmoLL IgG with extracts of other *B. monandra* tissues revealed other lectin molecular forms. Anti-BmoLL IgG was conjugated with peroxidase e applied in ELISA, confirming the results of immunodiffusion. For application in piezoelectric biosensor, the immobilization yield was tested of anti-BmoLL IgG on gold plates (4 x 4 x 0.5 mm^3) in quantities of 0, 110 e 220 μg em 100 μl using dextran treated with bromocyanide. Concentrations of unbound IgG were measured by protein quantification after immobilization and PBS washsteps. A quantity of anti-BmoLL IgG (110 μg) showed an immobilization yield of 46% and 220 μg yielded 33%. Anti-BmoLL IgG (110 μg/ml) was immobilized on the gold surface of the Quartz Crystal Microbalance, QCM. During the piezoelectric assay, the QCM was incubated with BmoLL solution (91 μg/ml) following a frequency reduction of 5 kHz, showing a strong recognition of the lectin by the immobilized IgG. Potentiostatic analysis and tubidity measurements revealed that anti-BmoLL IgG didn't cause any alteration on galactose binding by BmoLL, indicating that purified IgG has no affinity for carbohydrate binding regions of BmoLL.

PALAVRAS-CHAVE

- *Bauhinia monandra*
- Lectina de folha
- Purificação de IgG
- Caracterização
- Imobilização
- Propriedades eletroquímicas

SUMÁRIO

1 INTRODUÇÃO

1.1 Características e uso medicinal de *Bauhinia* spp.

O gênero Bauhinia foi criado em 1753, por Carolus Linnaeus, em homenagem ao botânico franco-suíço Gaspar Bauhin. Compreende cerca de 300 espécies (LEWIS, 1987), sendo que 64 podem ser encontradas no Brasil. Em Pernambuco, podem ocorrer espécies nativas como *Bauhinia acuruana* Moric, *B. breviola* Benth, *B. cheilantha* Stend, *B. forticada* Link, *B. heterandra* Benth, *B. monandra* Kurz, entre outras.

B. monandra é uma planta nativa da Ásia, embora possa ser achada em muitos lugares como a Índia, Nigéria e outras regiões da África e América do Sul (BADAMI; DAULATABAD, 1969; BALOGUN; FETUGA, 1985). No Brasil é conhecida vulgarmente como "Pata-de-vaca", por causa das suas folhas bifoliadas, podendo também ser conhecida como unha-de-vaca, casco-de-vaca, unha-de-boi, unha-de-anta e mororó (BADAMI; DAULATABAD, 1969; BALOGUN; FETUGA, 1985; CARIBÉ; CAMPOS, 1991). Figura 1 mostra as folhas, flores e sementes de *B. monandra*.

As propriedades medicinais do gênero Bauhinia foram amplamente estudadas. Dois flavonóides da casca de *B. manca*, (2S)-7,4'-di-hidroxiflavan e (2S)-3',4'-di-hidroxi-7-metoxiflavan, demonstraram atividades significantes de antifungo em *Coprinus cinereus* e *Saprolegnia asterophora* (ACHENBACH *et al.*, 1988). O extrato metanólico de gemas de *B. racemosa* (2,0 g/kg) reduziu significantemente a produção de ácido e pepsina em ratos com úlceras induzidas por aspirina (AKHTAR; AHMAD, 1995). Extratos de parreiras de *B. cumanensis* ou de *B. excisa* são utilizadas para tratar mordidas de cobra nos caçadores indígenas e nos seus cães em Trinidad, para evitar gangrena (LANS *et al.*, 2001). Extrato de casca de *B. guianensis* (50 mg/kg) demonstrou atividade anti-malárica *in vivo* em ratos com *Plasmodium vincket*, mas *in vitro* foi inativo contra *P. falciparum* (MUÑOZ *et al.*, 2000). Extrato de casca de *B. purpurea* estimulou a função tiroidal em ratos aumentando os níveis de T_3 e T_4 livres no soro (PANDA; KAR, 1999). Extrato metanólico de raiz de *B. vahlii* (25 μg/ml) demonstrou uma atividade antiviral total *in vitro* contra Sindbis vírus (SINV), mas não teve atividade contra Pólio virus 1 e Herpes simplex vírus 1 (TAYLOR *et al.*, 1996). Russo *et al.* (1990) demonstraram uma redução de nível de insulina na plasma

em pessoas normais e diabéticos de tipo II, bebendo chá de folhas de *B. forficata.* Entretanto, os níveis de glicose na plasma não foram alterados, concluindo que não houve efeito hipoglicemiante. Atividade hipoglicemiante foi demonstrada em folhas de *B. divaricata* (ROMAN-RAMOS *et al.*, 1992), em *B. candicans* (LEMUS *et al.*, 1999), e mais recentemente em *B. forficata* (PEPATO *et al.*, 2002).

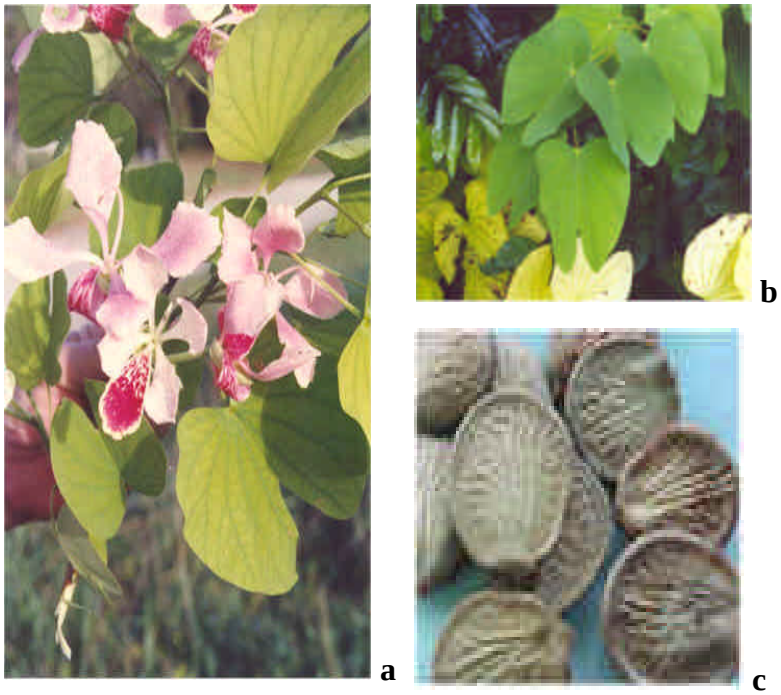

Figura 1. Flores (a), folhas (b) e sementes (c) de *Bauhinia monandra*.

Fontes (a) e (b): http://www.hear.org/pier_v3.3/bamon.htm

(c): http://www.driftwoodgardens.com/rainforestbauhiniamonandrabutterflyflower.htm

1.2 Generalidades sobre lectinas

Lectinas são proteínas ou glicoproteínas com um ou mais sítio(s) de ligação a açúcar por subunidade. Estas moléculas se ligam seletiva e reversivelmente a carboidratos e precipitam polissacarídeos, glicoproteínas e glicolipídeos; elas agem como reconhecedoras de células (SINGH *et al.*, 1999). A primeira descrição da atividade atualmente atribuída às lectinas foi feita por Stillmark em 1888 (KOCOUREK, 1986). Ele descobriu que extratos

de mamona, *Ricinus communis*, continham uma proteína que aglutinava eritrócitos; essa proteína foi chamada de ricina. Pouco tempo depois, uma outra hemaglutinina foi descoberta em sementes de *Abrus precatorius*, a lectina abrina.

Boyd e Shapleigh (1954) chamaram este grupo de proteínas de lectinas, do particípio passado do verbo latino *legere*, ou seja *lectus* que significa escolhido. Proteínas que têm atividade de hemaglutinação foram nomeadas hemaglutininas ou fito-hemaglutininas (ALLEN; BRILLIANTINE, 1969). Goldstein *et al*. (1980) definiram lectinas como proteínas de origem não-imunológica, com no mínimo dois sítios de ligação a carboidratos. Entretanto, Dixon (1981) incluiu neste grupo proteínas com apenas um sítio de ligação, chamando-as "lectin-like". Barondes (1988), bem como Sharon e Lis (1990) reportaram a existência de sítios hidrofóbicos adicionais. Hidrofobicidade é a força principal de interação entre lectinas e carboidratos através de sítios de ligação (QUIOCHO, 1986), e com proteínas ou outras substâncias através de sítios hidrofóbicos (ROBERTS; GOLDSTEIN, 1983; KELLA *et al.*, 1984; ROBERTS *et al.*, 1986; BARONDES, 1988). Na opinião de Peumans e Van Damme (1995) a presença de pelo menos um domínio de ligação não-catalítico, que liga reversível mas especificamente um mono- ou oligossacarídeo, já é suficiente para uma proteína obter o nome lectina. Por exemplo, as proteínas de orquídea que ligam manose são muito parecidas com as lectinas de orquídea específicas para manose, exceto que elas são monômeros (VAN DAMME *et al.*, 1994).

Uma vez que as lectinas leguminosas demonstram similaridades estruturais e de seqüência, suas especificidades de ligação a carboidratos são muito diferentes. Através da especificidade, lectinas são classificadas em cinco grupos, baseado no monossacarídeo que demonstra a maior afinidade, apesar que estas ligações são mais fracas que as estruturas complexas de carboidratos reconhecidas pelas lectinas. Os grupos são: Glc/Man, Gal/GalNac, Fuc, quitobiose (GlcNac), e Complexo, em que a lectina tem especificidade por um oligossacarídeo complexo em vez de um monossacarídeo (AUDETTE *et al.*, 2000).

Íons metálicos carregados positivamente fazem parte da unidade de lectina. Um íon de cálcio e um íon de metálico de transição, geralmente de magnésio, estão presentes e adjacentes ao sítio de ligação. A presença destes íons é considerada para ajudar a manter a estrutura terciária da lectina em uma conformação favorável para a ligação de carboidrato (AUDETTE *et al.*, 2000). A figura 2 representa um modelo 3D da lectina de *Canavalia*

ensiformis (Concavalina A) em complexo com di-manose demonstrando os íons metálicos próximos do sítio de ligação.

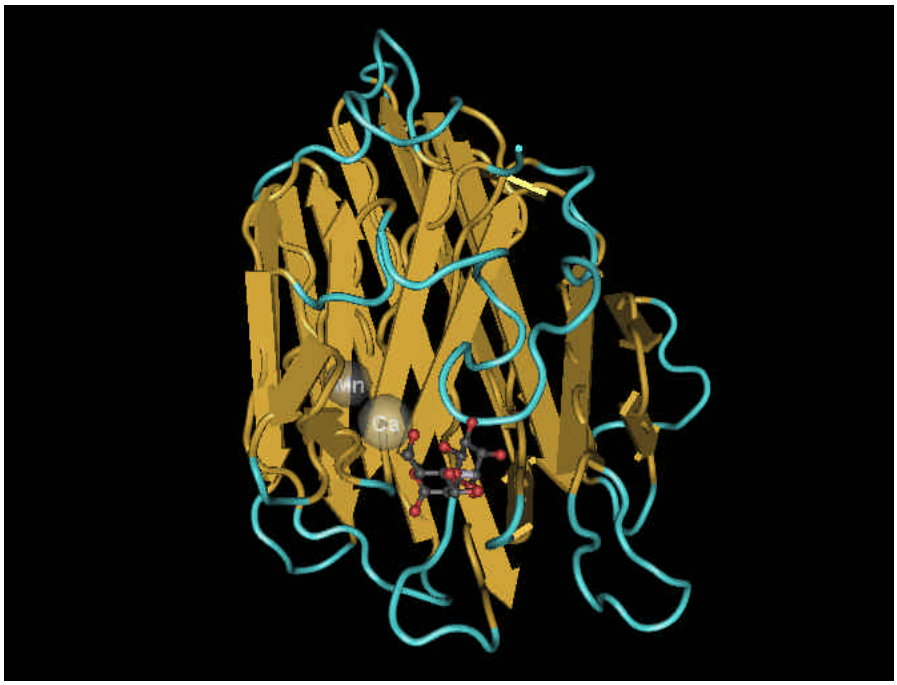

Figura 2. Um modelo 3D da lectina de *Canavalia ensiformis* ligando di-manose. Os íons de cálcio (Ca) e magnésio (Mg) estabilizem a ligação.

Fonte: http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?form=6&db=t&Dopt=s&uid=16923

Embora as estruturas monoméricas são conservadas em lectinas, existe uma grande variedade de estruturas quaternárias. Esta variedade tem implicações funcionais para a ligação de ligantes multivalentes de açúcar. As propriedades simétricas de oligômero protéico e o ligante multivalente afetam ambos a estrutura homogeneamente gradeada de ligações cruzadas, gerando uma maior especificidade que pode ser encontrada em monômeros individuais (BREWER, 1996).

Lectinas têm afinidades menores por carboidratos (10^2 - 10^6 M^{-1}) do que os anticorpos específicos (10^4 – 10^8 M^{-1}), isso lhes confere uma grande utilidade para desenvolver estratégias para a purificação de glicoconjugados. Se anticorpos foram usados como ligantes de afinidade, as condições para poder eluir as proteínas que foram adsorvidas na matriz durante a purificação devem ser muito rigorosas, além disso, a retenção de

atividade biológica das proteínas purificadas não é garantida nestas circunstâncias. Lectinas, ao contrário, quando usadas como ligantes de afinidade, precisam de condições brandas para eluir a proteína de interesse (SATISH; SUROLIA, 2001).

1.3 As lectinas de *Bauhinia*

A lectina de *B. purpurea* foi descrita a primeira vez por Uhlenbruck & Dahr (1971), reportando sua especificidade por N-acetil-D-galactosamina. Sua atividade hemaglutinante, especificidade e propriedades de ligação foram amplamente estudadas (IRIMURA; OSAWA, 1972; DAHR *et al.*, 1975; OSAWA *et al.*, 1978; ALLEN *et al.*, 1980; WU, 1984). O extrato de *B. carronii* demonstrou hemaglutinação e atividade mitogênica; ambas as atividades foram inibidas por lactose (FLOWER *et al.*, 1984). Atividades hemaglutinantes foram descritas em *B. racemosa*, *B. vahlii* (RAJARAM; JANARDHANAN, 1991) e *B. malabarica* (VIDJADAKUMARI *et al.*, 1993). Lectinas de *B. monandra* foram detectadas em sementes (ABREU *et al.*, 1990) e purificadas de folhas (COELHO; SILVA, 2000).

O gene da lectina de *B. purpurea* foi obtido através de uma biblioteca de cDNA das sementes germinadas detectando-o com sondas obtidas da parte N-terminal da proteína e das partes digeridas com Asp-N ou tripsina seqüenciadas (KUSUI *et al.*, 1991). A parte de ligação a carboidratos da lectina de *B. candicans* também foi seqüenciada (BAYMIEV *et al.*, 1999). Uma comparação de seqüência do cDNA de *B. purpurea* e o fragmento do DNA de *B. candicans* está ilustrada na figura 3.

Substituindo no cDNA da parte de ligação a carboidrato, codificando os aminoácidos DTWPNTEWS da lectina de *B. purpurea* por DTFYNAAW da lectina de *Lens culinaris* resultou uma lectina quimérica com uma especificidade por manose e galactose (YAMAMOTO *et al.*, 1992). A seqüência de carboidrato Manα1-6(Manα1-3)Manα1-6Man é essencial para uma forte ligação desta lectina quimérica (YAMAMOTO *et al.*, 2000a). Induzindo mutações ao acaso no cDNA codificando o nonapeptídeo DTWPNTEWS da lectina recombinante de *B. purpurea*, conservando Aps-135 (N), Asn-139 (N) e Trp-142 (W), e expressando no superfície do fago λfoo como proteínas de fusão

resultou em alguns fagos com diferentes afinidades de açúcar. Cinco novas combinações foram obtidas dentro deste nonapeptídeo, nenhuma foi similar à outra ou às outras seqüências das lectinas que ligam manose, tais como a lectina de *Canavalia ensiformes* Concanavalin A (Con A) e lectinas de *Lens culinaris*, *Pisum sativum*, *Vicia faba* e *Latirus ochrus* (YAMAMOTO *et al*, 2000b).

Figura 3. Um alinhamento de seqüências entre o cDNA da lectina de *B. purpurea* (Query) e o fragmento de DNA da região de ligação ao carboidrato de *B. candicans* (Sbjct). Entre os 210 de 1122 nucleotídeos do cDNA e o fragmento encontra-se uma homologia de 76%. Em baixo do alinhamento estão os aminoácidos que correspondem a tradução do fragmento alinhado (lectin). Fonte: http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi?

A lectina de folha de *B. monandra*, BmoLL, foi purificada através de fracionamento com sulfato de amônio (0-60%) seguido por cromatografia de afinidade em coluna de gel de guar, um polissacarídeo de manose com substituições de α 1-6 galactose (COELHO; SILVA, 2000). BmoLL, demonstrou em eletroforese em gel de poliacrilamida contendo sulfato sódico de dodecila (SDS-PAGE) uma banda principal de 33 kDa (glicosilada) e uma fraca banda menor de 26 kDa (não-glicosilada).

1.4 Desenvolvimento, caracterização imunológica e aplicação de anticorpos

Poucos trabalhos foram publicados centralizando a produção e caracterização de anticorpos policlonais contra lectinas de plantas. Ashford *et al.* (1982) produziram anti-soro de coelho contra a lectina de batata (*Solanum tuberosum*). Com o anti-soro houve reações cruzadas com lectinas de *Datura stramonium* e do tomate (*Lycopersicon esculentum*) resultando dos anticorpos que reagiram contra os carboidratos da lectina de batata. Foi também constatado inibição de atividade hemaglutinante após incubação com o anti-soro. Hankins e cols. (1979) também obtiveram reações cruzadas do anti-soro gerado contra a lectina de *B. purpurea* contra outras lectinas, em imunodifusões. Carlini *et al.* (1987) demonstraram também em imunodifusões com as IgG geradas contra a lectina e a toxina de *C. ensiformis* que existe nas 16 espécies testadas uma maior homologia entre as toxinas que entre as lectinas. Um esquema das precipitações vistas na imunodifusão está illustrada na figura 4. Correia e Coelho (1995) produziram anti-soro de coelho contra a isoforma 1 (Iso 1) da lectina de sementes de *Cratylia mollis* e purificaram IgG policlonal por cromatografia de afinidade em coluna de Proteína A Sepharose CL-4B.

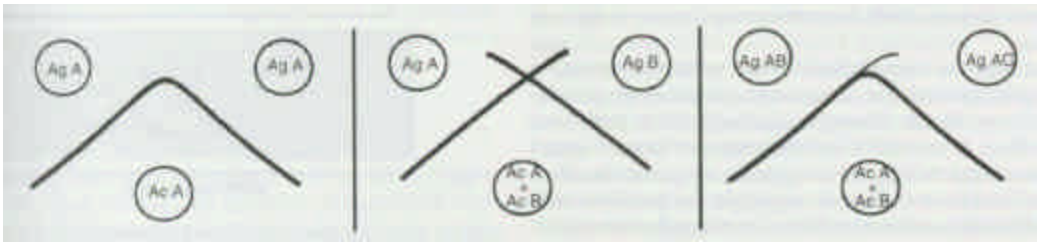

Figura 4. As precipitações vista na imunodifusão: (a) Os antígenos (Ag A) reconhecidos pelo anticorpo (Ac A) são idênticos. (b) Os antígenos (Ag A e Ag B) reconhecidos pelo anticorpo (Ac A + Ac B) não são relacionados. (c) Os antígenos (Ag AB e Ag AC) reconhecidos pelo anticorpo (Ac A + Ac B) são parcialmente relacionados. Fonte: Ferreira & Ávila (1996).

Para quantificar a concentração de lectina na hemolinfa de lagostim de água doce *Macrobrachium rosenbergii* foi utilizada um ensaio imunoadsorvente ligado a enzima (do inglês, *Enzyme Linked Immunosorbent* Assay, ELISA) com anticorpos monoclonais, podendo detectar quantidades a partir de 30 ng/ml (AGUNDIS *et al.*, 2000). As concentrações de lectina na hemolinfa foram mais altas no estágio juvenil que em adultos,

mas a atividade de hemaglutinação da lectina foi quatro vezes maior em adultos que no estágio juvenil.

Um ensaio de lectina ligado a enzima (do inglês, *Enzyme Linked Lectin Assay*, ELLA) foi utilizado para caracterizar sub-classes de IgG humana pela glicolização (KEUSCH *et al.*, 1996). As lectinas biotiniladas de *Sambucus nigra*, *Ricinus communis* I e *Bandeiraea simplicifolia* II foram utilizadas neste ensaio para detectar terminações de ácido siálico, galactose e *N*-acetilglicosamine.

Kelly *et al.* (1998) demonstraram que IgG de elefante africano *Loxodonta africana* teve reações cruzadas com 3 IgGs monoclonais anti-humana e IgGs policlonais anti-IgG de cão, boi, camelo, cavalo, foca e gato.

Dearman *et al.* (2001) caracterizaram a resposta de anticorpos em roedores após a ingestão de aglutinina de amendoim (*Arachis hypogea*), ovalbumina e extrato cru de batata inglesa (*Solanum tuberosum*) aumentando os niveis de IgG e IgE. Acre *et al.* (2002) produziram 6 IgGs monoclonais de anti-IgG de cão e caracterizaram o reconhecimento de sub-classes de IgG1 e 2, e isotipos de IgA, IgG, IgM canina. Reconhecimento de IgG de coelho e ovelha também foi reportado neste trabalho.

Rawson *et al.* (2002) produziram IgG policlonal contra a proteína recombinante codificando a região constante de corrente pesada de IgA (Cα) do marsupial *Trichorurus vulpecula*. Nos ensaios imunológicos ELISA, *dot*- e *Western blot* foram detectadas IgA no soro e nas secreções. Reações cruzadas foram encontradas com IgA de coala *Phascolarctos cireeus* e de cangaru *Macropus giganteus*, entre outros.

Town *et al.* (2001) imunizaram camundongos transgênicos PDAPP (modelo para doença de Alzheimer) com o peptídeo humano amiloido $A\beta_{1-42}$ e caracterizaram a resposta imunológica. Após 12 semanas de imunização foram detectadas na maioria IgG1, depois IgG2a e depois IgG2b (IgG1 > IgG2a > IgG2b). Também foi reportado que uma preparação de antígeno pré-agregado induziu uma forte resposta, enquanto mais diluída preparação não induziu resposta.

Watts *et al.* (2001) caracterizaram IgG de atum *Thunnus maccoyii* imunizado ou não com IgG de coelho, obtendo formas monoméricas e tetraméricas. Houve um maior rendimento na purificação com Proteína-A, seguida chromatografia de afinidade com IgG de coelho e a purificação com uma lectina específica por manana (do inglês, *Mannan*

Binding Protein, MBP). A atum anti-coelho IgG reagiu cruzadamente com IgG de outros mamíferos (não reduzidas), mas não reconheceu nenhuma IgG reduzida com β-mercaptoetanol.

1.5 Imobilização e caracterização eletroquímica de anticorpos

Biossensores são sistemas analíticos que incorporam o material biologicamente ativo em contato íntimo com um elemento apropriado de transdução, com o objetivo de detectar seletivamente a concentração ou atividade do produto de interesse (KOOYMAN & LECHUGA, 1997).

Dong e Chen (2002) descreveram algumas novidades nos biossensores, tratando materiais de imobilização, por exemplo: o uso de membranas de lipídeos bicamadas, a detecção de analíticos em solventes orgânicos ou em ambientes extremos de baixo pH.

Imunossensores eletroquímicos são baseados em conectar reações imunológicas com transdução eletroquímica. O componente imunogênico é imobilizado no eletrodo transdutor. A detecção é estabelecida através de gravação de processo elétrico resultante de reação antígeno-anticorpo (FERNÁNDEZ-SÁNCHEZ & COSTA-GÁRCIA, 1999).

Biossensores de Ressonância Plasmon de Superfície (do inglês, *Surface Plasmon Resonance*, SPR) medem a associação de moléculas livres (analíticos) com um ligante imobilizado. Em princípio, a ligação do analítico resulta em um aumento de índice refrativo que é monitorado em tempo real. A resposta é gravada como um sensorgrama representando o sinal de SPR medido em unidades de ressonância (do inglês, *Resonance Units*, RU) com o tempo (LIEDBERG *et al*., 1993).

Com o SPR, Usami *et al*. (2002) desenvolveram um ensaio para a detecção de estradiol. Neste ensaio foi verificada a ligação de receptor de estrógeno (RE) ao estradiol imobilizado sendo inibida por estradiol livre ou por outros componentes de teste com afinidade para o RE.

Meckelenburg *et al*. (2002) desenvolveram um ensaio de SPR que diferencia glicoproteínas de soro em pessoas saudáveis e em pessoas que sofrem uma infecção bacteriana, utilizando um painel de oito lectinas. Na superfície de ouro foi depositada uma

camada de 1-carboxil-16-tiol-hexadecano e após tratamento com *N*-hidroxisuccinimida (NHS), 1-etil-3-metil-(3-dimetilaminopropil)carbodiimida (EDAC) e amino-biotina, as ligações de streptavidina (AS), lectinas biotinilidas e o soro foram medidas em tempo real. Todas as leituras (a diferença antes e depois da incubação com o soro) foram em duplicata e analisadas em um sistema de reconhecimento de padrão (do inglês, *Pattern Recognition System*, PRS), um programa para combinar os oito parâmetros. O gráfico bi-dimensional de PRS demonstrou que os oito soros de pacientes são mais próximos que os vinte controles.

Biossensores do tipo piezoelétrico são detectores sensíveis à massa baseados em um cristal de quartzo, que ressona numa própria freqüência (BABACAN *et al.*, 2000). Este cristal é coberto com materiais biológicos, tais como anticorpos ou enzimas; componentes que têm uma alta afinidade pela molécula de interesse. Quando o cristal carregado é exposto à substância de interesse, ocorre uma adsorção que muda a freqüência, que pode ser traduzida em uma quantidade de material adsorvido. Uma instalação utilizada para o ensaio piezoelétrico seco está ilustrada na figura 5.

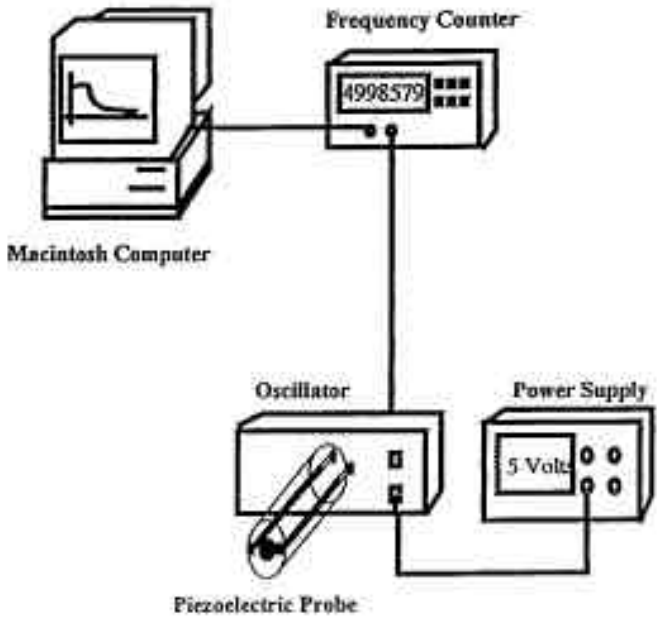

Figura 5. Sistema para o ensaio piezoelétrico seco com uma câmara para medir a mudança de freqüência do cristal de quartzo após a adição das camadas imobilizadas. Fonte: Babacan *et al.* (2000).

Piezo-eletricidade foi a primeira vez observada por Curie & Curie em 1880 (HARTENVELT *et al*., 1997). Compressão de um cristal de quartzo produziu um potencial elétrico. Depois, foi também observado o oposto: ondas podem ser geradas em materiais sólidos aplicando um potencial elétrico alternado no material piezelétrico. Em uma freqüência certa do potencial elétrico o cristal piezoelétrico vibra com uma perda de energia mínima (HARTENVELT *et al.*, 1997). Esta freqüência de ressonância depende de alguns fatores: a espessura (massa por unidade de área) do cristal e a viscosidade do líquido enquanto o cristal está sendo usado em solução. Quando o cristal é coberto com o material (bio)químico, a ligação do material analítico aumenta a espessura do cristal e modifica a freqüência de ressonância. Este princípio do sensor foi introduzido por King (1964).

Biossensores piezoelétricos têm algumas vantagens: a leitura ocorre no tempo real, diferente das demais técnicas imunológicas; sua resposta não depende de revelação com substratos tóxicos ou com radioisótopos e as quantidades de material biológico são bem menores que nas demais técnicas imunológicas. Entretanto, uma das limitações no uso deste sistema é a cobertura de cristal com o material biológico e as técnicas de imobilização que foram usadas para construir biossensores piezoelétricos. A superfície ativa de material biológico imobilizado deve ficar estável quimicamente durante a leitura, mantendo uma alta atividade, e a camada imobilizada deve ser fina e uniforme, tanto quanto possível. Estes cuidados são muito importantes, uma vez que uma alta sensibilidade somente pode ser obtida pelas camadas ativas, finas e rígidas (LUONG; GUIBAULT, 1991). Apesar de diferentes métodos de imobilização terem sido testados com biossensores piezoelétricos, nenhum ainda confere um ótimo rendimento de imobilização e alta estabilidade. Além disso, a complexidade e diversidade de componentes biológicos que podem ser usados com objetivos diferentes complicam ainda mais a obtenção de uma metodologia ideal. Para isso é necessário encontrar um método de imobilização adequado para cada material biológico, em uma aplicação particular.

Saber *et al.* (2002) desenvolveram um imunosensor para detectar albumina de soro humano (ASH), utilizando o biossensor piezeoelétrico. Os cristais de quartzo cobertos de prata foram tratados com diamina de etileno e os anticorpos anti-ASH foram imobilizados utilizando glutaraldeídeo. Foram testados a influência de concentração, pH e o tempo de incubação nas leituras obtidas durante a imobilização de anti-ASH e a incubação com ASH.

Wong *et al.* (2002) desenvolverem um imunoensaio para detectar *Salmonella*. Três anticorpos monoclonais MO2, 4 e 9, específicos por os isótipos A, B e D de *Salmonella* foram imobilizados no cristal de quartzo coberto de prata tratado com polietileneimina e glutaraldeídeo. Os três anticorpos foram testados com os três isótipos e com *Escherichia coli*, variando a concentração de anticorpo imobilizado, o número de células por mililitro e o tempo de incubação.

Abad *et al.* (1998) utilizaram o biossensor piezoelétrico para a detecção de anticorpos contra a proteína recombinante p12 do vírus da febre de javali africana que foi imobilizado no cristal por absorção. Para ampliar o sinal, foi utilizado um anticorpo anti-porco conjugado com peroxidase resultando que o 4-cloro-1-naftol oxidado aumente mais ainda o peso.

Chang *et al.* (1997) avaliaram no biossensor piezoelétrico a ligação de 6 proteínas ao lipopolisacarídeo (LPS) que foi imobilizado através de 4,4-ditiodi(ácido *n*-butírico). Entre eles, a polimixina B (PmB) demonstrou a maior redução de freqüência.

Hartenvelt *et al.* (1997) detectaram anticorpos contra a enterotoxina B de *Staphylococcus aureus* (SEB) utilizando um ensaio competitivo no biossensor piezoelétrico. A SEB foi imobilizada no cristal através de absorção simples aproveitando os pontos dissulfídricos. A imobilização do anti-SEB deu uma resposta baixa sugerindo a ausência destes pontos dissulfídricos na superfície dos anticorpos.

O fenômeno de interação em um sistema biológico pode ser descrito através de sinais eletroquímicos obtidos na interface das biomoléculas livres ou imobilizadas, quando expostas a um campo elétrico (FRICQUELMONT-LOÏZOS *et al*., 1997; COHEN *et al*., 1999).

Para construção de uma célula eletroquímica há necessidade de um eletrodo de referência, podendo ser utilizado o eletrodo de calomelano saturado, Hg/HgCl (ECS) ou o eletrodo de prata/ cloreto de prata, Ag/AgCl. Os eletrodos de trabalho freqüentemente utilizados são de prata (NIAURA *et al*., 1996), ouro (MATSUMOTO, *et al.*, 1998) e platina (SOUZA *et al*., 2001).

Sistemas analíticos eletroquímicos, que incorporam materiais biologicamente ativos em contato intimo com um elemento apropriado de transdução, tem o objetivo de detectar seletivamente a concentração ou atividade do produto de interesse (KOOYMAN;

LECHUGA, 1997). Imunossensores eletroquímicos são baseados em conectar reações imunológicas com transdução eletroquímica. O componente imunogênico é imobilizado no eletrodo transdutor. A detecção é estabelecida através de gravação de processo elétrico resultante de reação antígeno-anticorpo (FERNÁNDEZ-SÁNCHEZ; COSTA-GÁRCIA, 1999).

1.6 Objetivos

Geral

Desenvolver e purificar IgG contra a lectina de folha de *B. monandra* (IgG anti-BmoLL), caraterizando-a através de métodos imunológicos, ópticos e eletroquímico.

Específicos

- Desenvolver anti-soro de coelho contra a lectina de folha de *B. monandra* (BmoLL).
- Purificar anti-BmoLL IgG utilizando cromatografia de afinidade em Proteína A-Sepharose.
- Caraterizar anti-BmoLL IgG através de imunodifusão e ELISA; verificar a presença de lectina em outros tecidos de *B. monandra* e reações cruzadas com outras lectinas.
- Imobilizar anti-BmoLL IgG em superfície de ouro.
- Avaliar a imobilização de anti-BmoLL IgG através de método colorimétrico e biossensor piezoelétrico.
- Monitorar as propriedades eletroquímicas de interação de anti-BmoLL IgG e BmoLL; verificar se a interação tem efeito na ligação de BmoLL com galactose.

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Capítulo 1

Isolamento, purificação e caracterização parcial de IgG anti-lectina de folha de *Bauhinia monandra* (anti-BmoLL IgG)

Congresso da Sociedade Brasileira de Biotecnologia, 1., 2001
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Desenvolvimento, Purificação e caracterização de IgG contra a lectina de folha de *Bauhinia monandra*

Para ser submetido ao periódico:
Bioresource Technology

ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF ANTI-*Bauhinia monandra* LEAF LECTIN IgG (anti-BmoLL IgG)

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ABSTRACT

The *Bauhinia monandra* leaf lectin (BmoLL) was previously purified by ammonium sulphate fractionation (F 0-60%) followed by guar gel affinity chromatography[1]. To study BmoLL a polyclonal antibody was raised in rabbits against the lectin and anti-BmoLL IgG was purified on a Protein A – Sepharose column. Cross-reactions of the anti-BmoLL IgG with other lectins were also evaluated by immunodiffusion tests. Precipitations of anti-BmoLL IgG with extracts of other *B. monandra* tissues and ammonium sulphate fractions derived therefrom revealed the presence of other lectin molecular forms.

KEYWORDS

Leaf lectin – Antiserum characterization – IgG purification – Cross-reactions.

INTRODUCTION

Lectins are proteins or glycoproteins that specifically recognize and reversibly bind carbohydrates without modifying them. Although many plant lectins have been sequenced and their protein structures are known there are still uncertainties about their biological functions. There are indications that plant lectins bind to foreign carbohydrate moieties with the purpose of establishing symbioses or for plant defense[2]. One way to get more insight of the lectin and some biological properties without knowing the sequence could be achieved by raising an antibody against the lectin. With the antibody other plant tissues can be speculated about the presence of the lectin. Cross-reactions of lectin antibodies with other plant lectins can indicate protein homology[3] or similar carbohydrate moieties[4]. A purified anti-lectin IgG could be more important in identifying recombinant lectins by immunoscreening than identifying positive clones with cDNA of other lectins; protein homology of lectins is more conserved than the homology between lectin genes.

The genus *Bauhinia* (Fabaceae) is well distributed in Brazilian cities and within the genus hemagglutinating activity was detected in the globulin fraction from seeds of *B. Malabarica*[5], *B. purpurea*, *B. racemosa* and *B. Vahlii*[6]. Lectins have already been extracted from seeds of *B. monandra*[7]. The cDNA of the *B. purpurea* lectin[8] and the carbohydrate binding region of the *B. candicans* lectin[9] have been sequenced. Hypoglycemic activities have been reported in the species *B. divarticata*[10] and *B. candicans*[11]. However, no evidence has been published so far that a lectin could be responsible for the hypoglycemic activity. A leaf lectin was purified from *B. monandra* (BmoLL)[1]. In this work an antibody was raised against BmoLL and the anti-BmoLL IgG was purified on a Protein A – Sepharose CL-4B column. Cross-reactions of anti-BmoLL IgG with other lectins were evaluated by immunodiffusion tests.

MATERIALS AND METHODS

Preparation of anti-BmoLL serum and purification of anti-BmoLL IgG – BmoLL (150 μg of protein) in 1 mL of 0.1 M citrate phosphate buffer pH 6.8, containing 0.15 M NaCl was emulsified with 1 mL of Freund's complete adjuvant (first inoculation) or 1 mL Freund's incomplete adjuvant (four following inoculations) and intradermally injected into three males, New Zealand white rabbits, in a monthly interval[12]. Immediately before each inoculation, 10 mL of blood were collected from the ear central artery. Every mL of blood was left to coagulate in a glass tube at the angle of 45° at room temperature (*rt*) for 1 h and placed in the same angle at 4°C, overnight. The sera obtained were three times centrifuged at 1300 × *g*, for 5 min, at *rt*. Aliquots were stored at -20°C. Anti-BmoLL serum (3 mL) was chromatographed in a column (6.5 × 1.0 cm) containing 4.5 mL of Protein A - Sepharose CL-4B (Sigma). Unbound proteins were washed off with 0.1 M sodium phosphate buffer, pH 8.0, containing 0.15 M NaCl, until absorbance 280 < 0.01. Then, anti-BmoLL IgG was eluted with 0.1 M glycine, pH 2.8. The fractions with absorbance 280 > 1 were brought to pH 7 – 8 with 1 N NaOH and stored in aliquots at -20°C.

Immunodiffusion tests – Double immunodiffusion was carried out as previously described [4]. Gels of 1% (w/v) agarose containing 0.15 M NaCl were cast on squares of 2.5 cm x 2.5 cm with 3 mm thickness. In each gel 5 wells of 3 mm diameter were cut. Diffusion was allowed to take place for 24-36 h at 4°C in humidity chambers. The gels were washed with

0.15 M NaCl and distilled water, dried and stained with 0.1% (w/v) Coomassie Brilliant Blue R-250% in 45% (v/v) ethanol containing 10% (v/v) acetic acid.

RESULTS AND DISCUSSION

Double immunodiffusion tests showed that after the third series of inoculations positive reactions were achieved. The positive sera were selected for purification on Protein A column. Control sera (before inoculation) were all negative in immunodiffusions, with or without 0.1 M D-galactose, indicating that there is no recognition of carbohydrates from the antibodies by BmoLL (data not shown).

Cross-reactions were found between anti-BmoLL IgG and the lectins from *B. purpurea* and *Ulex europeus* I. The lectins from *Dolichos biflorus* and *Triticum vulgaris* showed a faint precipitation when incubated with anti-BmoLL serum but did not interact with anti-BmoLL IgG (Figure 1A). The lectins might recognize carbohydrate structures present in the antisera which were eliminated during the purification of anti-BmoLL IgG. Hankins *et al*. obtained cross-reactions in eight of fifteen lectins with antiserum raised against *B. purpurea*[3]. Three of them were lectins from *Bandeiraea simplicifolia, Dolichos biflorus* and *Ulex europeus*. *Lens culinaris* lectin showed no precipitation (as seen in Figure 1A). It should be noted that non-specific reactions might be caused when a high dose of lectin is inoculated. In this work 150 µg of lectin were inoculated each time; Ashford *et al*. used doses of 0.5 mg[4]. Non-specific reactions can appear when high quantities of lectin were used in immunodiffusion tests. Hankins *et al*. detected cross-reactions with quantities of 10 to 200 µg[3]; in this work quantities of 2.5 µg were used.

When precipitated with purified BmoLL, the antisera and purified IgG fractions showed no double precipitation bands in immunodiffusion tests. However, leaf extracts and leaf fractions showed two bands when precipitated with anti-BmoLL IgG or anti-BmoLL serum (Figure 1B and C). Asford *et al*. reported double precipitation bands as a result of two populations of antibodies: one specific for the lectin protein and the other specific for the carbohydrate chains of the lectin[4]. Purified fractions of BmoLL showed both glycosylated (33 kDa) and non-glycosylated polypeptides (26 kDa), the latter in a minor concentration[1]; a distinct pattern was seen in *B. purpurea* lectin[8]. To clarify whether anti-BmoLL IgG

recognizes the non-glycosylated and/or the glycsylated polypeptide chains of the lectin further tests should be performed.

BmoLL was highly immunogenic and the purified anti-BmoLL IgG permitted to detect the presence of lectins in other tissues of *B. monandra* and to evaluate the homology between them. Anti-BmoLL IgG have already been conjugated with peroxidase and applied in a lectin immunosensor.

Acknowledgements

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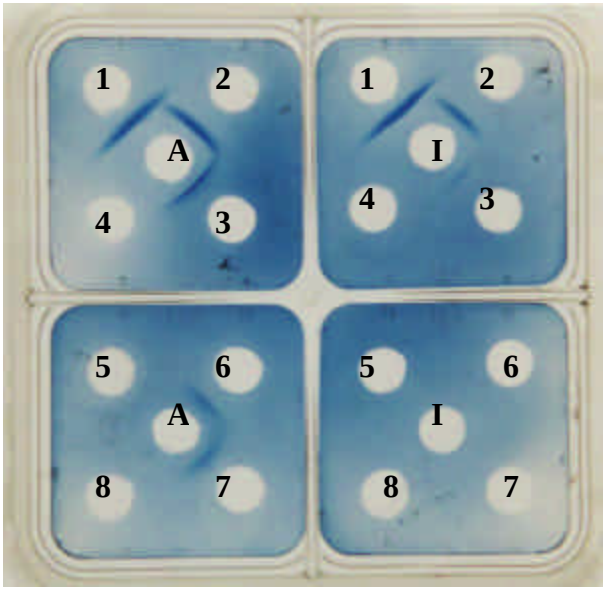

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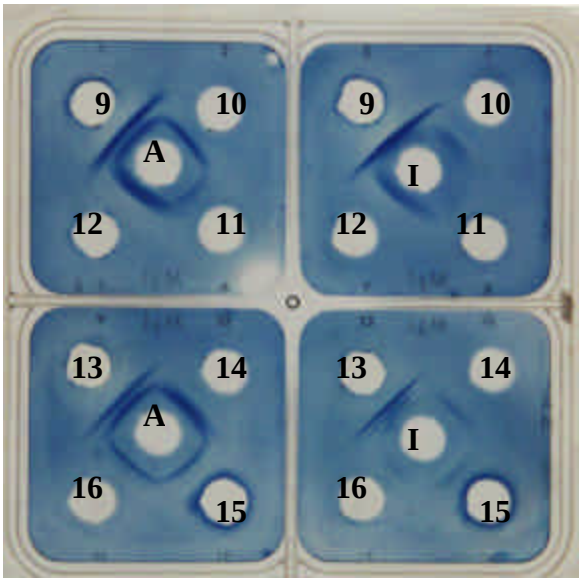

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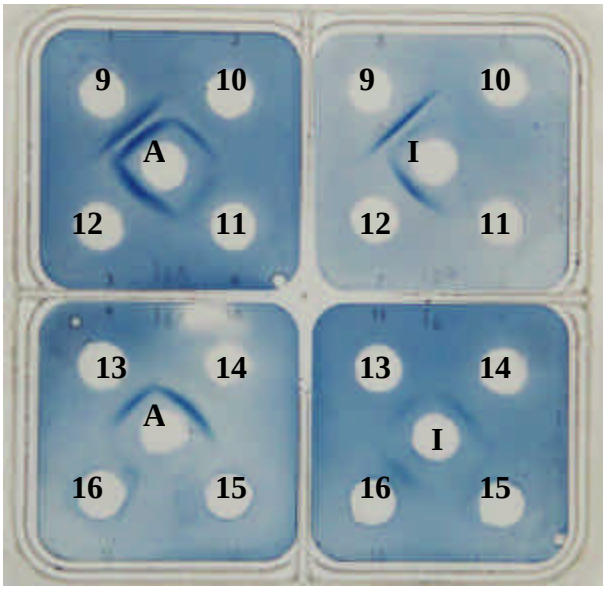

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Figure 1 – Double immunodiffusion tests on agarose gels. Cross-reactions of anti-BmoLL serum or anti-BmoLL IgG with other lectins (a), determination of the presence of a lectin in various tissue extracts from *Bauhinia monandra* (b) and in the 0-60% ammonium sulphate fractions derived therefrom (c).
Central wells: A = anti-BmoLL serum, I = anti-BmoLL IgG.
Lateral wells: 1 = BmoLL, 2 = *B. purpurea* lectin, 3 = *Ulex europeus* lectin I, 4 = *Ulex europeus* lectin II, 5 = *Bandeiraea simplicifolia* lectin II, 6 = *Dolichos biflorus* lectin, 7 = *Triticum vulgaris* lectin, 8 = *Lens culinaris* lectin, 9 = leaves, 10 = flowers, 11 = stems, 12 = roots, 13 = petioles, 14 = seeds, 15 = buds, 16 = branches of stem.

DEVELOPMENT, PURIFICATION AND PARTIAL CHARACTERIZATION OF IgG AGAINST *Bauhinia monandra* LEAF LECTIN

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Abstract

Bauhinia monandra leaf lectin (BmoLL) was previously purified by ammonium sulphate fractionation (F 0-60%) followed by guar gel affinity chromatography. Polyclonal antibodies against BmoLL were raised in rabbits and IgG was purified on a Protein A-Sepharose column (anti-BmoLL IgG). Cross-reactions of anti-BmoLL IgG with other lectins were evaluated by immunodiffusion tests. Precipitations of anti-BmoLL IgG with extracts and ammonium sulphate fractions from *B. monandra* leaves, flowers, stems, roots, petioles, seeds, buds and branches of stem revealed the presence of other lectin molecular forms in the medicinal low-growing tree. Enzyme linked immunosorbent assays confirmed lectin presence and cross-reactions.

Keywords: Leaf lectin; IgG purification; antiserum characterization; *Bauhinia monandra*.

1. Introduction

Lectins are proteins or glycoproteins of non-immune origin that selectively recognize and reversibly bind carbohydrates, agglutinate cells and precipitate polysaccharides or glycoconjugates (Goldstein *et al.*, 1980). Just a few plant lectins have been sequenced and detailed in their protein structures; biological functions of most lectins remain uncertain. There are indications that plant lectins bind to foreign carbohydrate moieties to establish symbioses or plant defense (Peumans & Van Damme, 1995).

An antibody raised against a lectin constitutes a tool for lectin quantification (Agundis *et al.*, 2000), to explore lectin homology (Hankins *et al.*, 1979) or similar

carbohydrate moieties (Ashford *et al.*, 1982). Pure IgG against the lectin certainly would be of use to amplify lectin binding to different tissues (Brooks *et al.*, 1997). With the antibody other plant tissues can be speculated about the presence of the lectin. Anti-lectin IgG could be more important in identifying recombinant lectins by immunoscreening than identifying positive clones with cDNA of other lectins since protein homology of lectins is more conserved than the homology between lectin genes (this detail was revealed when DNA and protein sequence of the *Bauhinia purpurea* lectin were compared with 35 other lectin sequences using BLAST (Altschul *et al.*, 1990), data not shown).

A galactose specific leaf lectin was previously purified from *Bauhinia monandra*, BmoLL (Coelho & Silva, 2000). In this work an antibody was raised against BmoLL and the anti-BmoLL IgG was purified on a Protein A-Sepharose CL-4B column. The presence of lectins in *B. monandra* tissues and cross-reactions of anti-BmoLL IgG with other lectins were evaluated by immunodiffusion tests. Anti-BmoLL IgG was conjugated with peroxidase and the conjugate (anti-BmoLL IgG Per) was used in enzyme linked immunosorbent assays to confirm lectin presence in tissues and cross-reactions.

2. Methods

2.1. Preparation of anti-BmoLL serum and purification of anti-BmoLL IgG

BmoLL (150 µg of protein) in 1 ml of 10 mM citrate phosphate buffer pH 6.8, containing 0.15 M NaCl was emulsified with 1 ml of Freund's complete adjuvant (first inoculation) or 1 ml Freund's incomplete adjuvant (four following inoculations) and intradermally injected into three males, New Zealand white rabbits, in a monthly interval according to Correia & Coelho (1995). Immediately before each inoculation, 10 ml of blood were collected from the ear central artery. Every ml of blood was left to coagulate in a glass tube at the angle of 45 ° at room temperature for 1 h and placed in the same angle at 4 °C, overnight. The sera obtained were three times centrifuged at 1300 × *g*, for 5 min, at room temperature. Aliquots were stored at -20 °C. Anti-BmoLL serum (3 mL) was chromatographed in a column (6.5 × 1.0 cm) containing 4.5 ml of Protein A-Sepharose CL-4B (Sigma). Unbound proteins were washed off with 0.1 M sodium phosphate buffer, pH

8.0, containing 0.15 M NaCl, until absorbance 280 < 0.01. Then, anti-BmoLL IgG was eluted with 0.1 M glycine, pH 2.8. The fractions with absorbance 280 > 1 were brought to pH 7-8 with 1 N NaOH and stored in aliquots at -20 °C. Protein concentrations were measured according to Lowry *et al.* (1951).

2.2 Immunodiffusion tests

Double immunodiffusion was carried out according to Ashford *et al.* (1982). Gels of 1 % (w/v) agarose containing 0.15 M NaCl were cast on squares of 2.5 cm x 2.5 cm with 3 mm thickness. In each gel 5 wells of 3 mm diameter were cut. Diffusion was allowed to take place for 24-36 h at 4 °C in humidity chambers. The gels were washed with 0.15 M NaCl and distilled water, dried and stained with 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 45 % (v/v) ethanol containing 10 % (v/v) acetic acid.

2.3 Conjugation of anti-BmoLL IgG and its evaluation in an Enzyme Linked Immunosorbent Assay (ELISA)

Anti-BmoLL IgG (3 mg/ml) dialyzed against 0.1 M sodium phosphate buffer, pH 6.8 was conjugated to peroxidase (Sigma) based on Weir (1973). An Enzyme Linked Immunosorbent Assay (ELISA) was performed as described previously (Correia & Coelho, 1995). Lectin (10 µg/ml) in 0.1 M $NaHCO_3$ containing 0.5 M NaCl and was absorbed in a microtiter plate for 16 h at 4 °C. The plate was washed three times with PBS containing 0.1 % Tween 20 (Merck), PBST. Incubation (overnight, 4 °C) with 50 µl of anti-BmoLL IgG Per, diluted (50^{-1}-6400^{-1}) in PBST containing 0.5 % casein was followed by three washes with PBST. Then, a fresh solution of 0.08 % (w/v) dihydrochloride *o*-phenylenediamine (37 mg/50 ml, Sigma) in 10 mM citrate phosphate buffer, pH 6.0, containing 0.03 % (v/v) H_2O_2 (15 µl/50 ml), was added. The reaction was kept in the dark for 30 min, stopped with 100 µl 0.5 N citric acid and read at 490 nm in a Biorad Microplate Reader, model 3550.

3. Results and discussion

To study the immunological identity of BmoLL comparing it with lectins present in other tissues of *B. monandra* and its homology with other lectins polyclonal antisera against BmoLL raised in tree rabbits. Double immunodiffusion tests showed that after the third inoculation positive reactions were achieved. The positive sera selected for purification on Protein A column are indicated in table 1. Control sera (before inoculation) were all negative in immunodiffusions. Identical results were obtained with or without 0.1 M $_{D}$-galactose added to gels, indicating that there is no recognition of carbohydrates from the antibodies by BmoLL (data not shown).

Table 1. Precipitations of rabbit antisera with BmoLL in immunodiffusion tests before and after inoculation with BmoLL

Inoculation	Rabbits		
	I	II	III[c]
0[a]	-	-	-
1	±	±	ND
2	±	±	ND
3	+	+	ND
4[b]	+		

a. Pre-inoculation sera.

b. Only to rabbit I were applied four inoculations.

c. Third inoculation selected for IgG purification.

(-) no precipitation, (±) some precipitation, (+) precipitation, (ND) not determined.

Selected antisera were chromatographed on a protein A column. Affinity purification of IgG is shown in figure 1. Elutions were performed with 0.1 M glycine, pH 2.8. Yields of 44 mg IgG (1.1 mg/ml) were achieved. The pH, measured in all fractions, dropped after the second peak in the protein A chromatography (data not shown).

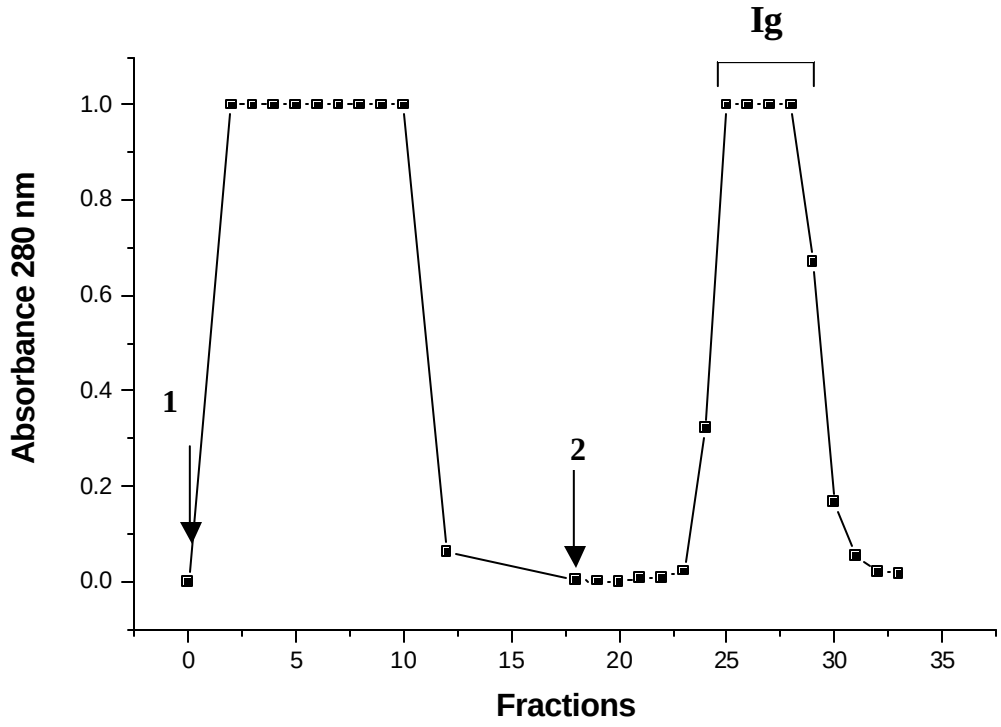

Figure 1. Protein A column afinity chomatogram of IgG preparation. Antiserum (3 ml) was applied to a 4.5 ml column and fractions (1 ml) were collected at 33 ml/h. Unbound proteins were washed off with 0.1 M sodium phosphate buffer pH 8.0 (event 1), followed by the elution of the IgG with 0.1 M glycin pH 2.8 (event 2). Eluted fractions of A_{280} [3] 1 were pooled and stored at –20 °C.

Cross-reactions were found between anti-BmoLL IgG and the lectins from *B. purpurea* and *Ulex europaeus* I. *Dolichos biflorus* and *Triticum vulgaris* lectins showed a faint precipitation band when incubated with anti-BmoLL serum but did not interact with anti-BmoLL IgG (Figure 2a). The lectins might recognize carbohydrate structures present in the antisera which were eliminated during the purification of anti-BmoLL IgG. Hankins *et al.* (1979) obtained cross-reactions in eight of fifteen lectins with antiserum raised against *B. purpurea*. Three of them were lectins from *Bandeiraea simplicifolia*, *Dolichos biflorus* and *Ulex europaeus*. *Lens culinaris* lectin showed no precipitation band (Figure 2a). Non-specific reactions might be caused when a high dose of lectin is inoculated. In this work 150 µg of lectin were inoculated each time; Ashford *et al.* (1982) used doses of 0.5 mg. Non-specific reactions can appear when high quantities of lectin were used in

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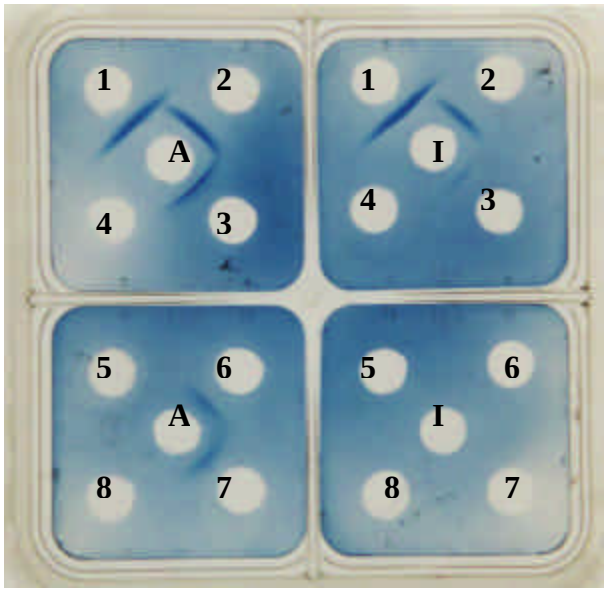

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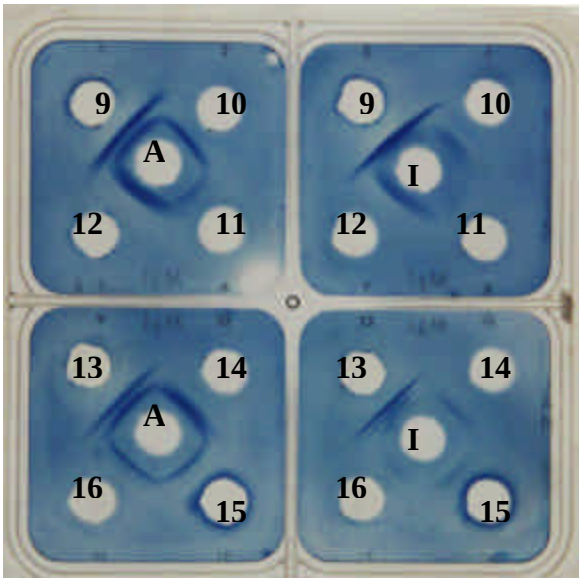

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Figure 2 – Double immunodiffusion tests on agarose gels. Cross-reactions of anti-BmoLL serum or anti-BmoLL IgG with other lectins (a), determination of the presence of a lectin in various tissue extracts from *Bauhinia monandra* (b) and in 0-60% ammonium sulphate fractions derived therefrom (c).

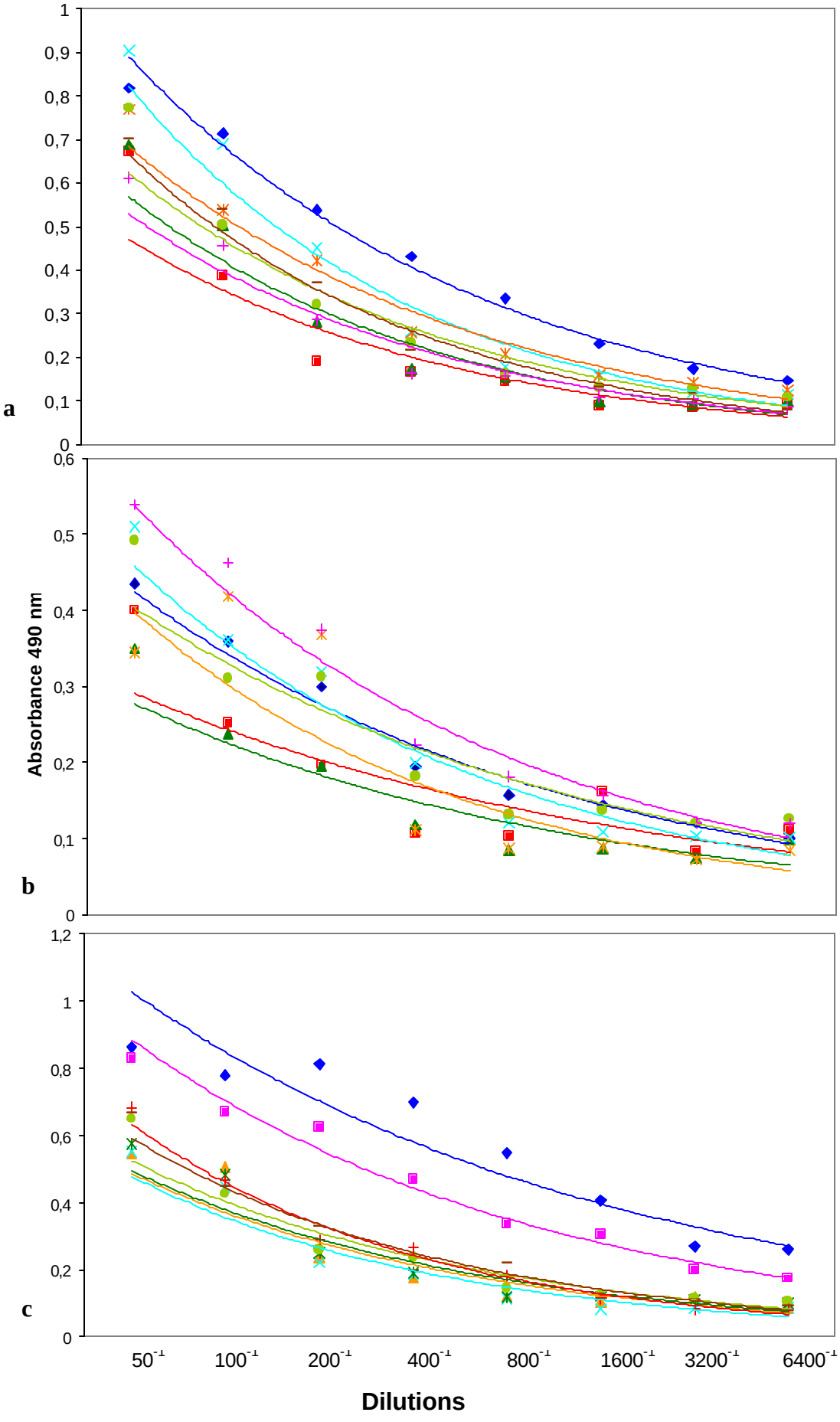
Central wells: A = anti-BmoLL serum, I = anti-BmoLL IgG.

Lateral wells: 1 = BmoLL, 2 = *B. purpurea* lectin, 3 = *Ulex europaeus* lectin I, 4 = *Ulex europaeus* lectin II, 5 = *Bandeiraea simplicifolia* lectin II, 6 = *Dolichos biflorus* lectin, 7 = *Triticum vulgaris* lectin, 8 = *Lens culinaris* lectin, 9 = leaves, 10 = flowers, 11 = stems, 12 = roots, 13 = petioles, 14 = seeds, 15 = buds, 16 = branches of stem.

When precipitated with purified BmoLL, the antisera and purified IgG fractions showed no double precipitation bands in immunodiffusion tests (2a). However, leaf extracts and leaf fractions showed two bands when precipitated with anti-BmoLL IgG or anti-BmoLL serum (Figure 2b and c). Asford *et al*. (1982) reported double precipitation bands as a result of two populations of antibodies: one specific for the lectin protein and the other specific for the carbohydrate chains of the lectin. Purified fractions of BmoLL showed both glycosylated (33 kDa) and non-glycosylated polypeptides (26 kDa), the latter in a minor concentration (Coelho & Silva, 2000); a distinct pattern was seen in *B. purpurea* lectin (Kusui *et al.*, 1991). According to Coelho & Silva (2000) at least two lectins can be purified from *B. monandra* leaves, with distinct electrophoretic migration. The isolectins were clearly detected by the raised antiserum.

Enzyme linked immunsorbent assays with anti-BmoLL IgG Per confirmed the presence of lectins in various tissues of *B. monandra* (figure 3a and 3b). Extracts from leaves and roots have major lectin expressions. Fractions of 0-60% showed minor absorbances than the corresponding extracts. Homology was seen between *B. monandra* and *B. purpurea* (figure 3c).

Figure 3 (next page). Enzyme linked immunosorbent assay with anti-BmoLL IgG Per, serially diluted, incubated with 10% extracts from *B. monandra* (a) or 0-60% ammonium sulphate fractions derived therefrom (b): (¨) Leaves, (¡) Flowers, (¿) Stems, (×) Roots, (*) Petioles, (¿) Seeds, (+) Buds, (–) Branches of stem. With anti-BmoLL IgG Per the immunological identity of BmoLL is compared to other lectins (c): (¨) BmoLL, (¡) *Bauhinia purpurea*, (¿) *Ulex europaeus* I, (×) *Ulex europaeus* II, (*) *Dolichos biflorus*, (¿) *Bandeiraea simplicifolia* II, (+) *Triticum vulgaris*, (–) *Lens culinaris*.


a
b
c
Absorbance 490 nm
1
0,9
0,8
0,7
0,6
0,5
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0,3
0,2
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1,2
1
0,8
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0,4
0,2
0
50-1
100-1
200-1
400-1
800-1
1600-1
3200-1
6400-1
Dilutions

BmoLL was highly immunogenic and the purified anti-BmoLL IgG permitted to detect the presence of lectins in other tissues of *B. monandra* and to evaluate the homology between them. Anti-BmoLL IgG have already been applied in a lectin immunosensor and is in use to test the lectin yield in *Agrobacter* transformed roots of *B. monandra*.

Acknowledgements

This work was supported by the following Brazilian institutions: The National Council for Scientific and Technological Development (CNPq), The Foundation for Scientific and Technological Support from the State of Pernambuco (FACEPE) and by The National Plan of Science and Technology of Petroleum and Natural Gas Sector (CTPETRO).

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Capítulo 2

Avaliação de imobilização de anticorpo na camada de dextrana modificada com cianeto por aplicação no biossensor piezoelétrico

Para ser submetido ao periódico:
Analytica Chimica Acta

Evaluation of antibody immobilization on a cyanide modified dextran layer for piezoelectric biosensor application

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Abstract

A hydrogel of dextran treated with cyanide was used to immobilize polyclonal anti-*Bauhinia monandra* Leaf Lectin IgG (anti-BmoLL IgG) on the gold surface of a Quartz Cristal Microbalance (QCM) crystal to be used in a piezoelectric biosensor. High yields of immobilization were achieved with 1.1 mg/ml (46%) and 2.2 mg/ml (33%) anti-BmoLL IgG. Flow injection analysis (FIA) with BmoLL (91 mg/ml) resulted a frequency decrease (15 kHz) with anti-BmoLL IgG (1.1 mg/ml). According to its high yield, stability and sensitivity the QCM assay is also a potent tool for detection and analysis of lectins.

***Keywords*: Immobilization, IgG, *Bauhinia monandra* Leaf Lectin, Quartz Cristal Microbalance.**

1. Introduction

Piezoelectric biosensors are mass-sensitive detectors based on an oscillating piezoelectric quartz crystal that resonates at a fundamental frequency. The general approach to take advantage of the piezoelectric effect is to coat a piezoelectric crystal with biological

materials such as antibodies or enzymes; these compounds have high selectivity for a target molecule. When the coated crystal is exposed to a particular substance of interest, adsorption occurs, causing a frequency change that can be used to determine the amount of material absorbed [1].

As an alternative to common immunological techniques the use of piezoelectric biosensors for diagnosis is of great interest with time and costs reduced. Therefore, a number of antigen or antibody immobilization protocols are developed and evaluated using physical adsorption [2-4], self-assembled monolayers of thiocompounds [5,6], latex [7,8], polyethyleneimine (PEI)/glutaraldehyde (GA) [1,2,9] or protein A [1,10].

In our work polyclonal IgG raised against *Bauhinia monandra* leaf lectin (BmoLL), galactose-specific [11] was immobilized using a protocol originally developed for Surface Plasmon Resonance [12] combining a self-assembling monolayer of a thiocompound with a modified dextran layer. Some modifications were made to reduce the immobilized mass. The immobilization yield of anti-BmoLL IgG and its binding activity to BmoLL were evaluated using a Quartz Crystal Microbalance (QCM) assay.

2. Experimental

2.1 Reagents and apparatus

Higly purified BmoLL was obtained as previously described [11]. A polyclonal antibody against BmoLL was raised in rabbits and anti-BmoLL IgG was purified on a protein A column [13]. Dextran, ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAC) and N-hydroxysuccinimide were purchased from Sigma and were used as received. All other chemicals were of analytical grade and were used as received. Deionized water from a Milli-Q system was used to prepare all solutions.

The crystals (AT-cut, quartz plate ∅ 14 mm, gold electrode ∅ 8 mm, 10 MHz frequency) were purchased from Universal Sensors (New Orleans, USA). A Flow Injection Analysis (FIA) system was used, consisting of one peristaltic pump and an oscillator circuit coupled to a frequency counter (GFC-8131, Good Will). A crystal was incorporated in two Plexiglas blocks, clamped together with screws. Each block has a chamber of 210 mm^3, but

only one side gets exposed to liquid. Two O-rings, one at each side of the gold electrode were used to provide a watertight seal and two more to avoid the risk of break and to minimize direct pressure on crystal. Each reading was manually recorded after ten seconds.

A Biorad Microplate Reader, model 3550, was used to measure protein quantification absorbances.

2.2 *Immobilization of anti-BmoLL IgG and yield evaluation*

Prior to use, the gold QCM surfaces and gold plates of $4 \times 4 \times 0.5$ mm^3 were cleaned with a PIRANHA solution [14] (three parts of 30% H_2O_2 and seven parts of H_2SO_4. *Caution!* Reacts violently when prepared) for 10 min, followed by rinsing with Milli-Q water and drying with a hairdryer.

The immobilization process [12] used on QCM and on golden plates was the same, however in every step 20-25 µl of reagent were deposited on one side of crystal; for immobilization yield analysis the gold plates were deposited in PCR tubes with 100 µl reagent. Crystals and plates were treated with 4 mM mercaptoethanol in 80% ethanol for 16 h at room temperature. Then were treated with a solution of 0.2 mM HCl and 0.4 mM NaCl for 4 h at 25°C. After 5 wash steps with ethanol and milli-Q water alternately the surface was treated with a freshly prepared solution of 30% (w/v) dextran in NaOH 0.1 mM for 20 h at 25°C. The dextran layer was sensitized by treatment with a solution of 100 mM CNBr and NaOH 2 mM for 8 h at 25°C and then incubated with a solution of 0.1 mM hydrochlorated ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDAC) and N-hydroxysuccinimide 25 µM for 30 min at RT. Crystals and plates were incubated with 0, 1.1 or 2.2 mg/ml purified anti-BmoLL IgG for at least 3 h at room temperature. Then crystals were blocked with 3% casein (w/v) for another 3 h.

After immobilization each reaction (100 µl) was collected to measure the unbound IgG (U) and plates were washed three times with 100 µl of PBS (10 mM, pH 7.2) to measure the washed-off IgG (W1, W2 and W3). Protein concentrations were calculated by a standard protein quantification test [15]. Knowing the concentrations of IgG (I) used for immobilization the yield for each IgG concentration was calculated by the formula $(I - U - W1 - W2 - W3)/I \times 100\%$. Values of immobilization/area were also calculated (figure 1).

2.3 *Analysis of BmoLL binding to immobilized anti-BmoLL IgG with a wet piezoelectric assay system*

Using a wet piezoelectric assay system, composed of a power supply, osciloscope, probe, peristaltic pump and a frequency counter, the crystal with immobilized anti-BmoLL IgG was monitored on the binding of BmoLL to the system. BmoLL (454 μg/ml) was passed with a flow of 330 μl/min or in dilutions of 10^{-3}, 10^{-2}, 10^{-1} and 5^{-1} through the reaction chamber to get bound by the immobilized IgG.

3. Results and Discussion

Immobilization of an anti-lectin antibody instead of the lectin itself provides at least two vantages. Firstly, the presence of lectin can be detected in distinct plant tissues; secondly, direct immobilization of the lectin may reduce its activity by severe changes of the conformation during the immobilization process. Maintenance of the lectin activity was expected when bound by immobilized antibody. It was shown that by physical adsorption no antibody immobilization was detected [4]. Therefore, we chose an immobilization method originally developed for SPR analysis [12]. A reduction of immobilized mass was expected when β-mercaptoethanol was used instead of 16-mercaptohexadecan-1-ol.

Anti-BmoLL IgG was immobilized in quantities of 0, 110 and 220 μg. The concentrations of unbound IgG were measured with a protein quantification method [15] after immobilization and washes with PBS. A quantity of 110 μg resulted in a immobilization of 51 μg IgG (46%); 220 μg resulted in a immobilization of 73 μg (33%). The control, without IgG, showed some activity due to non-protein interference. Figure 1 reveals the immobilization/area. PBS wash-steps hardly release protein when a concentration of 1.1 mg/ml was used. So it was estimated that 1.28 μg/mm^2 of anti-BmoLL IgG was immobilized on gold. Babacan *et al.* [1] achieved immobilization yields of 42% and 32% using protein A and PEI/GA, respectively; applying 15 μl of 4-5 mg/ml IgG on an area of 20 mm^2, resulted in immobilizations of 1.41 and 1.08 μg/mm^2, respectively.

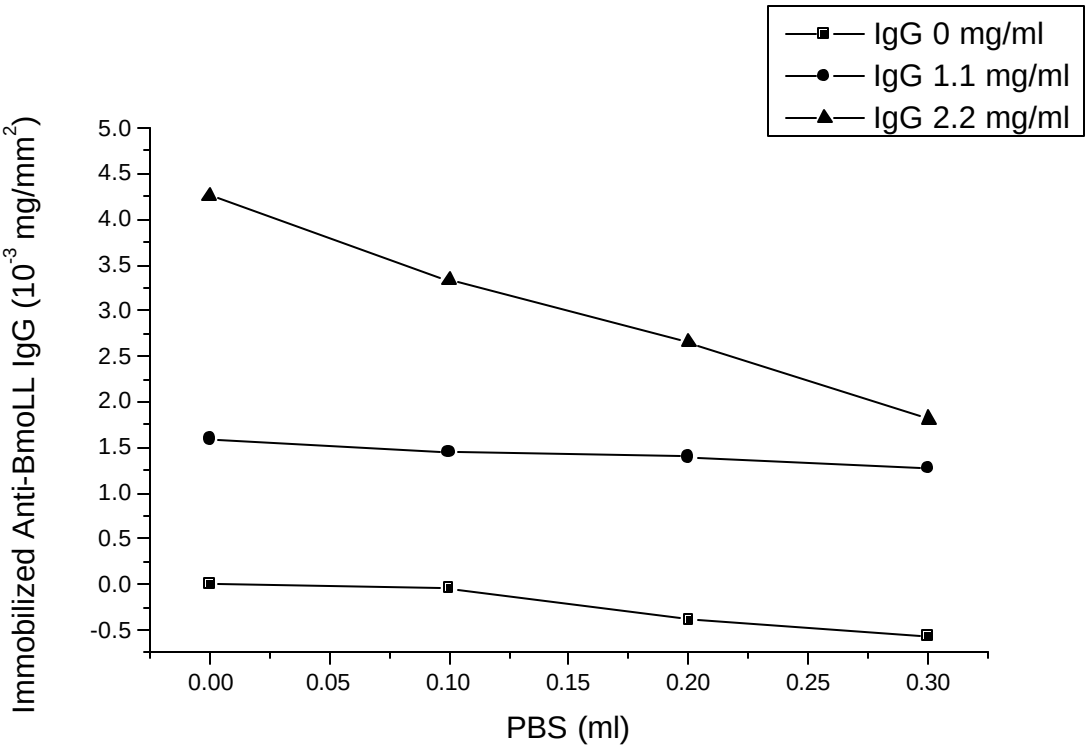

Figure 1. Immobilization/area on gold plates (4 ´ 4 ´ 0.5 mm^3) were evaluated using anti-BmoLL IgG in three concentrations. Unbound (0 ml PBS) and washed-off proteins (0.1, 0.2 and 0.3 ml PBS) were measured by protein quantification and calculated values of immobilized IgG are showed (see experimental for details).

When 1.1 mg/ml anti-BmoLL IgG was immobilized, an incubation with BmoLL (91 µg/ml) gave a reduction of the frequency with 5 kHz (22.5 kHz to 17.5 kHz) showing a strong recognition of the lectin by the immobilized IgG (figure 2). A delay in response of 3.5 min was observed and this was the time the sample needed to pass the FIA-system before entering the reaction chamber of QCM. No reduction of frequency was detected after 14.5 min (9 min incubation with BmoLL with 3.5 min delay) at this time 454 µg BmoLL had passed the reaction chamber. A second incubation with BmoLL showed no reactivity indicating that the system was already saturated. Interference was detected during the assay because no Farraday's cage was used. The control (19.5 kHz), without immobilized IgG, showed no response after incubation with BmoLL. A major immobilization was achieved with 2.2 mg/ml IgG (14 kHz when 19 kHz in control).

However, no reactivity was detected with BmoLL in dillutions 10^{-3}, 10^{-2}, 10^{-1} and pure indicating that the crystal was too loaded (data not shown).

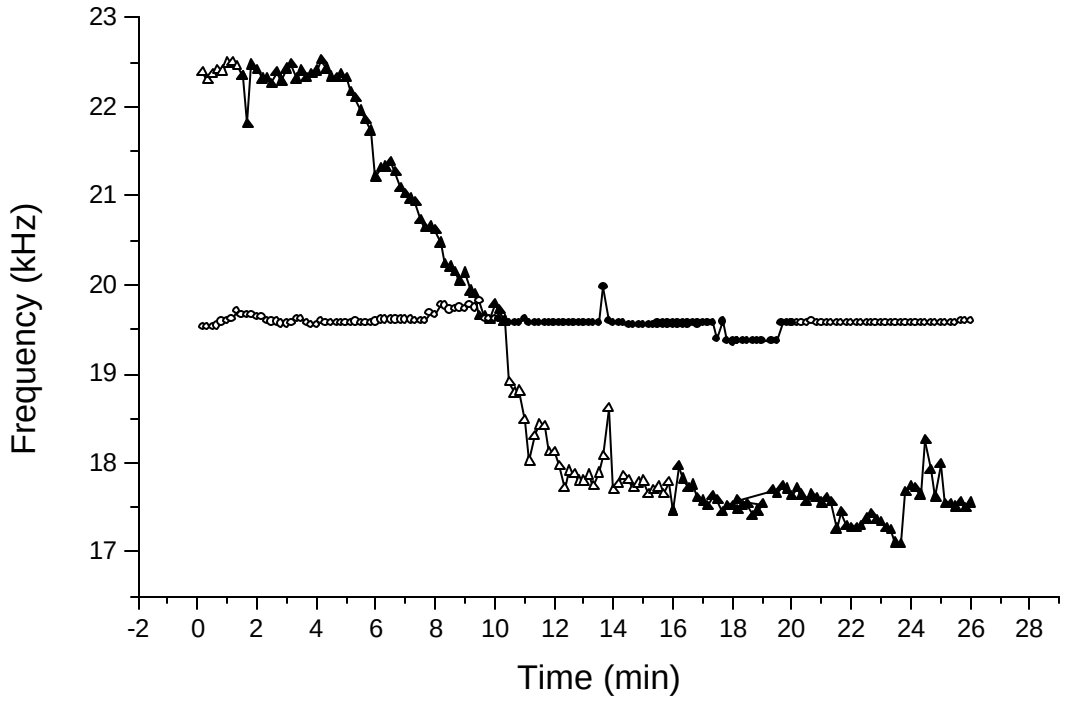

Figure 2. The piezoelectric assay showed a binding of BmoLL (91 mg/ml) to immobilized anti-BmoLL IgG, 1.1 mg/ml (?). The control without immobilized IgG showed no reaction when incubated with BmoLL (?). The system was also incubated with PBS (open symbols).

Generally a Sauerbrey's equation [16] was used to calculate the immobilization mass by the reduction of frequency. However it was discovered recently that in liquid phase the QCM does not behave as predicted by the equation [17, 18]. Using the FIA-system we didn't apply the equation to calculate the mass of bound BmoLL. The quantification of mass in a dry QCM assay during the immobilization process and after incubation with BmoLL is under further studies.

4. Conclusion

Measurement of the immobilization yield is important for estimating the maximal antigen binding capacities of the biosensor. The assay was effective to detect the immobilized anti-BmoLL IgG. Based on high stability and sensitivity, low quantities of antigen and antibody used, and real time measurement of response, the QCM showed to be a very potent immunological assay.

Acknowledgements

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Capítulo 3

Potencial eletroquímico da lectina de folha de *Bauhinia monandra*

Para ser submetido ao periódico:
Bioresource Technology

ELECTROCHEMICAL POTENTIAL OF *Bauhinia monandra* LEAF LECTIN

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Abstract

A galactose-specific *Bauhinia monandra* leaf lectin (BmoLL) has been highly purified through ammonium sulphate fractionation and guar gel affinity chromatography. An antiserum was raised in rabbits and anti-BmoLL IgG was purified on a protein A Sepharose column. The electrochemical potential of BmoLL was obtained using an electrochemical cell in relation to the binding of galactose and anti-BmoLL IgG. The applied system could be of use to evaluate BmoLL interactions at the carbohydrate binding sites.

Keywords: *Bauhinia monandra*, leaf lectin, electrochemical potential, galactose binding, potentiometric methods.

1. Introduction

Lectins are proteins or glycoproteins with at least two carbohydrate binding sites. These molecules bind selectively and reversibly carbohydrates; precipitate polysaccharides, glycoproteins and glycolipids; and induce cell agglutination (Singh *et al.*, 1999). Lectins are widely distributed in unicellular and multicellular organisms, however very little literature is available concerning lectins derived from leaf tissue (Coelho & Silva, 2000). The genus *Bauhinia*, member of the family Fabaceae, are widely distributed in the Brazilian cities. In the popular medicine the tea of the leaves of this plant is used by carriers

of diabetes with a probable hypoglycaemic activity. *B. monandra* leaf lectin (BmoLL), galactose-specific, has been highly purified through ammonium sulphate fractionation and guar gel affinity chromatography (Coelho & Silva, 2000).

There is a crescent interest to obtain electron direct transfer between electrodes and proteins, based on lectin-glycoprotein binding (Köneke *et al*., 1996). Capacity changes at the electrode/electrolyte interface are ideal model systems to explain protein-carbohydrate interactions. The description of phenomena involving the interaction of active sites of proteins can be evaluated through electrochemical signs which are entirely associated with changes of the double electrical layer occurring at the surface of the electrode. This approach using electrochemical methods is tightly related with conformational changes of a protein due to interaction with specific ligants (Cohen *et al.*, 1996; Ficquelmont-Loïzos *et al*., 1997; Yoshizumi *et al*., 1999; Marken *et al.*, 2002). Evaluation of changes at the electrode/electrolyte interface are ideal model systems to explain protein-carbohydrate interactions.

The aim of the study was to evaluate the electrochemical properties of interaction between anti-BmoLL IgG and BmoLL and to elucidate if these interactions have effect on the galactose binding activity of BmoLL.

2. Methods

The electrochemical potential was measured through two electrochemical systems, each containing a pair of electrodes: a platinum wire as work electrode with a reference electrode (Ag/AgCl), both were coupled to an impedance high multimeter.

The interaction electrochemical potential was measured by 0.5 mg/ml of BmoLL with or without anti-BmoLL IgG (1 mg/ml) acting with 0, 50, 100, 200, 300 and 400 mM galactose, respectively.

Measurements of turbidity were obtained in a turbidity meter (Orbeco-Hellige model 966) by interaction of BmoLL with different galactose concentrations (0, 50, 100, 200, 300 and 400 mM). The turbidity is the optical effect that occurs when a beam of light is dispersed by particles in suspension. The turbidity of the sample was calculated by

measuring the amount of light reflected at a 90° angle comparing it with a standard reference suspension.

3. Results and discussion

A simple method has previously been developed to purify milligram quantities of BmoLL (Coelho & Silva, 2000), which allowed the electrochemical characterization of the lectin.

The phenomenon of interaction in a biological system can be described through electrochemical signals in the interface of biomolecules (Fricquelmont-Loïzos et al., 1997; Cohen et al., 1996). Immunosensors based upon electrochemical systems have characteristic time responses between 40-50 min. A potentiometric immunosensor for anti-H IgG using the immobilization in Immobilon membrane for the antigen marked with glucoseoxidase showed responses of 50 min after 60 min incubation and a lifetime of days (Campanella *et al.*, 1999). Electrochemical analytical systems that incorporate biologically active materials on an appropriate transduction element have the purpose to measure the concentration or activity of the product of interest (Kooyman & Lechuga, 1997). Electrochemical immunosensors are based on immune interactions (antibody-antigen); the immunogenic component is immobilized in the electrode transducer and the detection is established through the recording of resultant electric process of antigen-antibody interaction (Fernández-Sánchez & Costa-Gárcia, 1999). The electrochemical potential of BmoLL (0.5 mg/ml) was high in relation to galactose concentrations (0, 50 and 100 mM, Figure 1). The mechanism was regulated by electron transference rate between lectin and different carbohydrate concentrations (Sugawara *et al*., 1998; Niki *et al*., 2001).

Biomolecules adsorbed on conductor surface can reveal a change of the structural properties (Zeder-Lutz *et al*., 1997; Kobayashi *et al*., 2001). Studies of the boundary between metal and molecules in solution are of substantial interest. They constitute an essential prerequisite for understanding the adsorption phenomena on this interface and for interpreting the kinetics or mechanism of electrode processes (Cohen *et al*., 1996).

The electrochemical potential of biomolecules depend upon the conformation of the bound molecule as well as from the applied electrochemical system properties (Bassi *et al*., 1999; Zhang *et al*., 2000).

Studies concerning the binding of molecules in solution to a metal surface are of substantial interest.

BmoLL showed biological activity in an electrochemical system, composed of a platinum plate and a saturated (Ag/AgCl) electrode, applied to study the electrochemical potential of interaction with different concentrations of galactose (Figure 1).

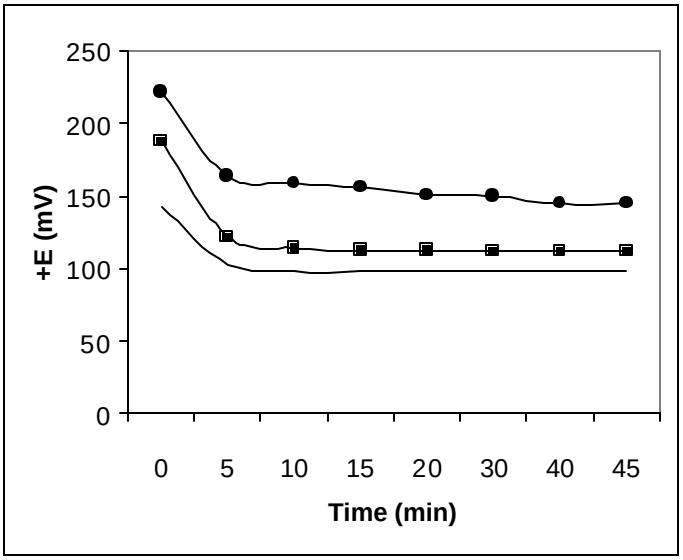

Figure 1. Interaction electrochemical potential between BmoLL (0.5 mg/ml) and galactose, 0 (●) , 50 (■), and 100 (—) mM, in function of time.

The change capacity at the interface of work electrode as a result of carbohydrate-lectin complex formation showed also high sensitivity, although the values of the electrochemical potentials were lower (Figure 2). It allowed the development of versatile systems, of great interest at basic or applied level of studies, which constitute a field to be exploited for several years.

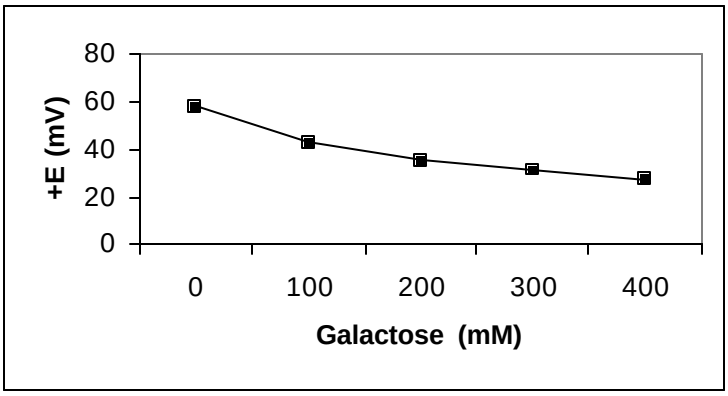

Figure 2. Electrochemical potential of BmoLL interacting with galactose in different concentrations (0, 100, 200, 300 and 400 mM) measured in relation with the working electrode (platinum wire) and the reference electrode (Ag/AgCl).

The recognition of galactose by BmoLL showed a turbidity in NTU (Nephelorimetric Turbidity Units) which was related to the concentration of galactose added (Figure 3).

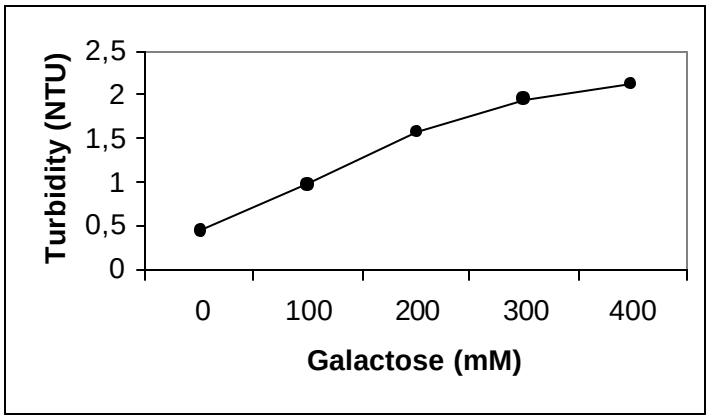

Figure 3. Measurements of turbidity obtained by interaction of BmoLL with different galactose concentrations (0, 100, 200, 300 and 400 mM).

Yoshizumi *et al*. (1999) observed that the turbidity decreased in environment containing a solution of methacryloyloethyl-D-glucopyranoside polymer and concanavalin A (Con A) when the concentration of α-Methyl-Mannose increased, indicating a strong specificity of the carbohydrate.

Anti-BmoLL IgG showed no alteration in the applied electrochemical assays and in the turbidity assay (data not shown) indicating that there was no affinity of IgG to BmoLL carbohydrate binding sites.

Changes at the electrode/solution interface could result in high or low ability of proteins to participate in electron transference reactions (Souza *et al*., 2001). The results showed were in accordance with the inhibition assay described by Coelho and Silva (2000), could be attributed to a high specificity of BmoLL for galactose.

Acknowledgements

The authors are very grateful to the following Brazilian Institutions: The National Council for Technological and Scientific Development (CNPq), The Foundation for Scientific and Technological Support from the State of Pernambuco (FACEPE) and The National Plan Of Science and Technology of Petroleum and Natural Gas Sector (CTPETRO).

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3. CONCLUSÕES

- No desenvolvimento de anti-soro BmoLL foi imunogênica. Após a terceira inoculação houve alta sensibilidade e especificidade de anti-BmoLL soro por BmoLL. Não houve reconhecimento de imunoglobulinas por BmoLL.
- Alto rendimento foi obtido na purificação de anti-BmoLL IgG (44 mg). Estruturas de carboidratos presentes no soro foram eliminadas.
- Imunodifusão e ELISA tiveram resultados coerentes na caracterização de anti-BmoLL IgG. Maiores expressões de lectina em *Bauhinia monandra* foram encontradas nas folhas, raízes e peciolos. BmoLL e a lectina de *B. purpurea* são homólogas.
- Anti-BmoLL IgG foi imobilizada com alto rendimento. O método de imobilização demonstrou uma alta estabilidade no biossensor piezoelétrico. Leitura no tempo real BmoLL mostrou uma forte ligação a anti-BmoLL IgG imobilizada.
- Análise potenciostática e de turbidêz demonstraram que anti-BmoLL IgG não altera a ligação de galactose por BmoLL.

Anexos

19th INTERLEC, 25 – 30 March 2001, Fortaleza - Brazil

P08

Plant Lectins

ISOLATION, PURIFICATION AND CONJUGATION OF ANTI-*Bauhinia monandra* LEAF LECTIN IGG

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The *Bauhinia monandra* leaf lectin (BmoLL) was previously purified by ammonium sulphate fractionation (F 0-60 %) followed by guar gel affinity chromatography (Coelho & Silva, *Phytochem. Anal.* 11: 295-300, 2000). To study BmoLL a polyclonal antibody was raised in rabbits against the lectin. An emulsion of BmoLL, 150 µg in 1 ml of 0.1 M citrate phosphate buffer pH 6.5, with 1 ml of Freund's complete adjuvant was inoculated subcutaneously, followed by 3 inoculations of BmoLL plus Freund's incomplete adjuvant. Antibody production was monitored by immunodiffusion tests carried out in 1 % (w/v) agarose gel. Anti-BmoLL IgG was purified on a protein A column eluted with 0.1 M glycine, pH 2.9. The fractions containing IgG with OD_{280} > 1 were selected by spectrophotometry. For immunological tests 3 mg of anti-BmoLL IgG were crosslinked to 9 mg of peroxidase with 50 µL of a glutaraldehyde solution (1 %, v/v). Protein quantification showed a high antibody yield of anti-BmoLL IgG (44 mg). Immunodiffusion studies showed a high sensitivity when 2.7 µg of BmoLL were incubated with 18.8, 27.5, 55 and 110 µg of anti-BmoLL IgG. Incubation of anti-BmoLL IgG (110 µg) with 2.5 µg of the lectins from *Bandeiraea simplicifolia* (BSII), *Dolichos biflorus, Lens culinaris, Ulex europaeus* (UEA I and UEA II) and *Triticum vulgaris* (WGA) showed no reaction. Anti-BmoLL IgG was specific and pure; the conjugate was successfully obtained. Anti-BmoLL IgG has been applied in biosensors.

Financial Support: *CNPq and FACEPE.*

ISOLATION, PURIFICATION AND CONJUGATION OF ANTI- *Bauhinia monandra* LEAF LECTIN IgG

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INTRODUCTION

The Bauhinia monandra leaf lectin (BmoLL) was previously purified by ammonium sulphate fractionation (F 0-60%) followed by guar gel affinity chromatography (Coelho & Silva, 2000). To study BmoLL a polyclonal antibody was raised in rabbits against the lectin and the anti-BmoLL IgG was purified on a protein A column. Cross-reactions of the anti-BmoLL IgG with other lectins were tested by immunodiffusion tests. Anti-BmoLL IgG was also conjugated with peroxidase.

MATERIALS AND METHODS

Preparation of Anti-BmoLL Serum and Purification of IgG

Pure BmoLL (150 mg) in 1 ml of 0.1 M citrate phosphate buffer pH 6.8, containing 0.15 M NaCl, was emulsified with 1 ml of Freund's complete adjuvant (first inoculation) or 1 ml Freund's incomplete adjuvant (four following inoculations) and intradermally injected into three New Zealand white rabbits (males), in a monthly interval. Immediately before each inoculation, 10 ml of blood were collected from the ear central artery. Every millilitre of blood was left to coagulate in a glass tube at the angle of 45 at RT for 1 h and placed in the same angle at 4ºC overnight. The sera obtained were centrifuged three times at 1300 g, for 5 min, at RT. Aliquots were stored at -20ºC (Figure 1).

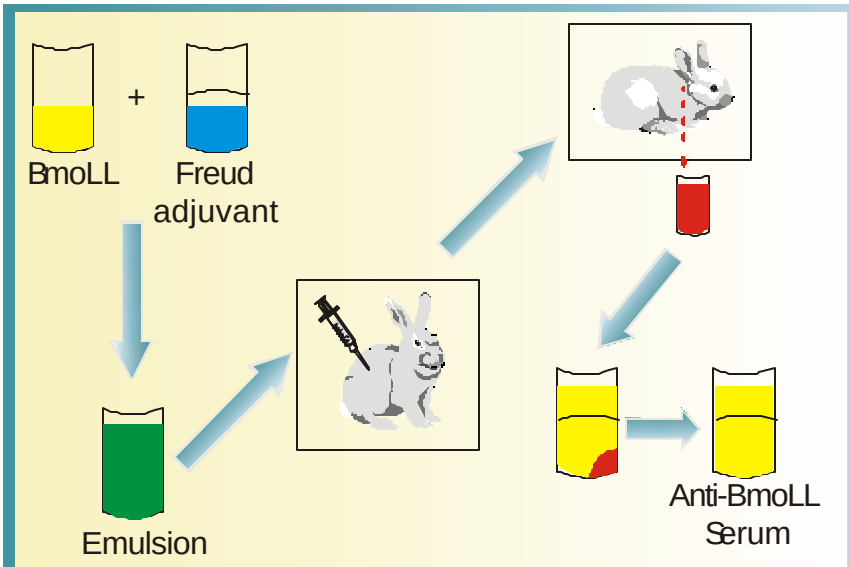

Figure 1. Preparation of anti-BmoLL Serum

The antilectin sera (3 ml) were chromatographed in a column (6.5 x 1.0 cm) containing 4.5 ml of protein A - Sepharose CL-4B (Sigma). Unbound proteins were washed off with 0.1 M sodium phosphate buffer pH 8.0, containing 0.15 M NaCl, until absorbance 280 < 0.01. Then, anti-BmoLL IgG was eluted with 0.1 M glycine pH 2.8. The fractions with absorbance 280 > 1 were brought to pH 7 8, if necessary, with 1 N NaOH and stored in aliquots at -20ºC (Figure 2).

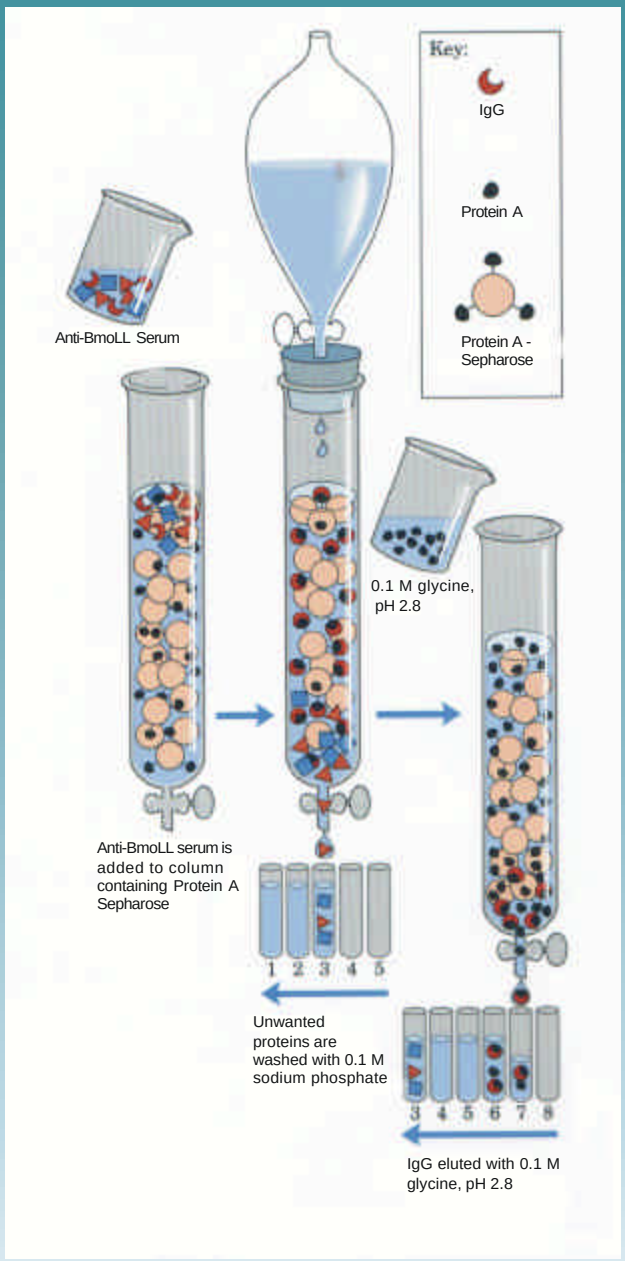

Figure 2.
Chromatography of anti-BmoLL serum in protein A Sepharose column

Immunodiffusion tests

Double immunodiffusion was carried out in 1% (w/v) agarose gels in 0.15 M NaCl according to Ashford et al. (1982). Anti-BmoLL IgG (11 mg/ml) and anti-BmoLL serum were serial diluted from 1, 21, 41 to 81. IgG, antiserum, BmoLL (0.27 mg/ml) and the lectins (0.25 mg/ml) of *Bandeiraea simplicifolia* (BSII), *Dolichos biflorus* (DB), *Lens culinaris*, *Ulex europaeus* (UEA I and UEA II) and *Triticum vulgaris* (WGA) were applied in volumes of 10 µl.

Conjugation of Anti-BmoLL IgG

Three milligrams of anti-BmoLL IgG, dialyzed against 0.1 M sodium phosphate buffer pH 6.8 overnight at 4ºC, were conjugated to 9 mg of peroxidase (Merck) for 2 h at RT and again dialyzed with 0.01 M sodium phosphate buffer pH 6.8, containing 0.15 M NaCl, according to Weir (1973).

Protein Quantification

Protein quantification was performed according to Lowry et al. (1951).

RESULTS

Double immunodiffusion tests showed that after the third serial inoculation strong positive reactions were achieved (Table 1). The sera with intense precipitations were purified on a protein A column. Control sera (before inoculation) were all negative in immunodiffusions with or without 0.1 M D-galactose indicating that there is no recognition of antibody carbohydrates by BmoLL. All purified IgG showed intense precipitation with BmoLL. The purification of anti-BmoLL IgG (Figure 3) resulted in a high yield (44 mg). Immunodiffusion assays showed a strong precipitation line when BmoLL was incubated with anti-BmoLL IgG and anti-BmoLL serum in serial dilutions (Figure 4A). Incubation of anti-BmoLL IgG with the lectins from *Bandeiraea simplicifolia* (BSII), *Dolichos biflorus*, *Lens culinaris*, *Ulex europaeus* (UEA I and UEA II) and *Triticum vulgaris* (WGA) showed no precipitation with anti-BmoLL IgG. However, some lectins revealed a faint precipitation when incubated with anti-BmoLL serum (Figure 4B).

Serum source	*Inoculation*	*Precipitation wiht BmoLL*
Rabbit 1	Control	
	1st	ND
	2nd	+
	3rd	++
	4th	++
Rabbit 2	Control	
	1st	ND
	2nd	+
	3rd	++
Rabbit 2	Control	
	1st	ND
	2nd	ND
	3	ND

Table 1. Precipitation of control sera and anti-BmoLL sera with BmoLL by immunodiffusion tests.

(-) negative
(+) some precipitation detected
(++) strong precipitation
(ND) not determined

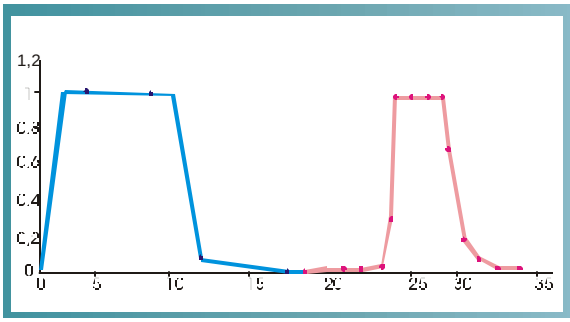

Figure 3. Purification of anti-BmoLL IgG on a Protein A Sepharose CL-4B column.

Anti-BmoLL sera (3ml), collected after the third and fourth inoculations, in 0.1 M sodium phosphate buffer pH 8.0 (7 ml) was applied to the column and twice recycled. Unbound proteins were washed off with sodium phosphate buffer (blue) until A 280 < 0.01. IgG was eluted with 0.1 M glycine pH 2.8 (pink). Eluted fractions of A 280 > 1 were pooled and stored in aliquots at -20C. Flow rate: 0.33 ml/min.

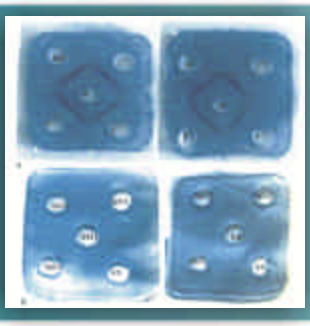

Figure 4. Immunodiffusion assay
(A) Anti-BmoLL IgG and anti-BmoLL serum in serial dilutions reacting with BmoLL.
(B) Anti-BmoLL IgG and anti-BmoLL serum tested on crossreactions with other lectins.
(IgG) Anti-BmoLL IgG; (AS) Anti-BmoLL serum; (B) BmoLL; (BSII) *Bandeiraea Simplicifolia* lectin II; (DB) *Dolichos biflorus* lectin.

CONCLUSIONS

Anti-BmoLL IgG was specific and pure; the conjugate was successfully obtained. Anti-BmoLL IgG has been applied in biosensors. Anti-BmoLL IgG or its conjugate will be of most value to characterize molecular forms of the lectin present in different tissues of the plant.

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H - 97

PROTEIN QUANTIFICATION TO ANALYSE THE YIELD OF ANTI-*Bauhinia monandra* LEAF LECTIN IgG (ANTI-BmoLL IgG) IMMOBILIZED ON A GOLD SURFACE COATED WITH A THIN MATRIX OF CARBOXYLATED DEXTRAN.

Haver, N.J.[1]; Souza, S.R.[2,4,5]; Dutra, R.F.[2,3]; da Silva, M.B.R.[1]; Cabral, L.T.B.[2]; Correia, M.T.S.[1]; Lima-Filho, J.L.[1,2]; Coelho, L.C.B.B.[4]

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Biosensors with immobilized anti-lectin antibodies can be used for lectin detection in plant tissues. In this work an antibody immobilization method was evaluated to be used in biosensors. A polyclonal antibody against *Bauhinia monandra* leaf lectin (BmoLL) was raised in rabbits and anti-BmoLL IgG was purified on a protein A column. Anti-BmoLL IgG was immobilized in quantities of 0, 110 and 220 μg. The concentrations of unbound IgG were measured with a modified Bradford protein quantification method after immobilization and washes with PBS. A quantity of 110 μg resulted in a immobilization of 51 μg IgG (46%); 220 μg resulted in a immobilization of 73 μg (33%). The control, without IgG, showed some activity due to non-protein interference generally detected in protein quantification methods. Measurement of the immobilization yield is also important for estimating the maximal antigen binding capacities of the biosensor. The assay was effective to detect the immobilized anti-BmoLL IgG.

Supported by CNPq and FACEPE.

PROTEIN QUANTIFICATION TO ANALYSE THE YIELD OF ANTI-*Bauhinia monandra* LEAF LECTIN IgG (ANTI-BmoLL IgG) IMMOBILIZED ON A GOLD SURFACE COATED WITH A THIN MATRIX OF CARBOXYLATED DEXTRAN

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INTRODUCTION

Biosensors with immobilized anti-lectin antibodies can be used for lectin detection in plant tissues. In this work an antibody immobilization method was evaluated to be used in biosensors.

METHODOLOGY

A polyclonal antibody against pure Bauhinia monandra leaf lectin (BmoLL, Coelho & Silva, 2000) was raised in rabbits and anti-BmoLL IgG was purified on a protein A column according to Correia & Coelho (1995). Anti-BmoLL IgG was immobilized in quantities of 0, 110 and 220 mg by a modified immobilization protocol originally developed for Surface Plasmon Ressonance analysis using carboxylated dextran (figure 1).

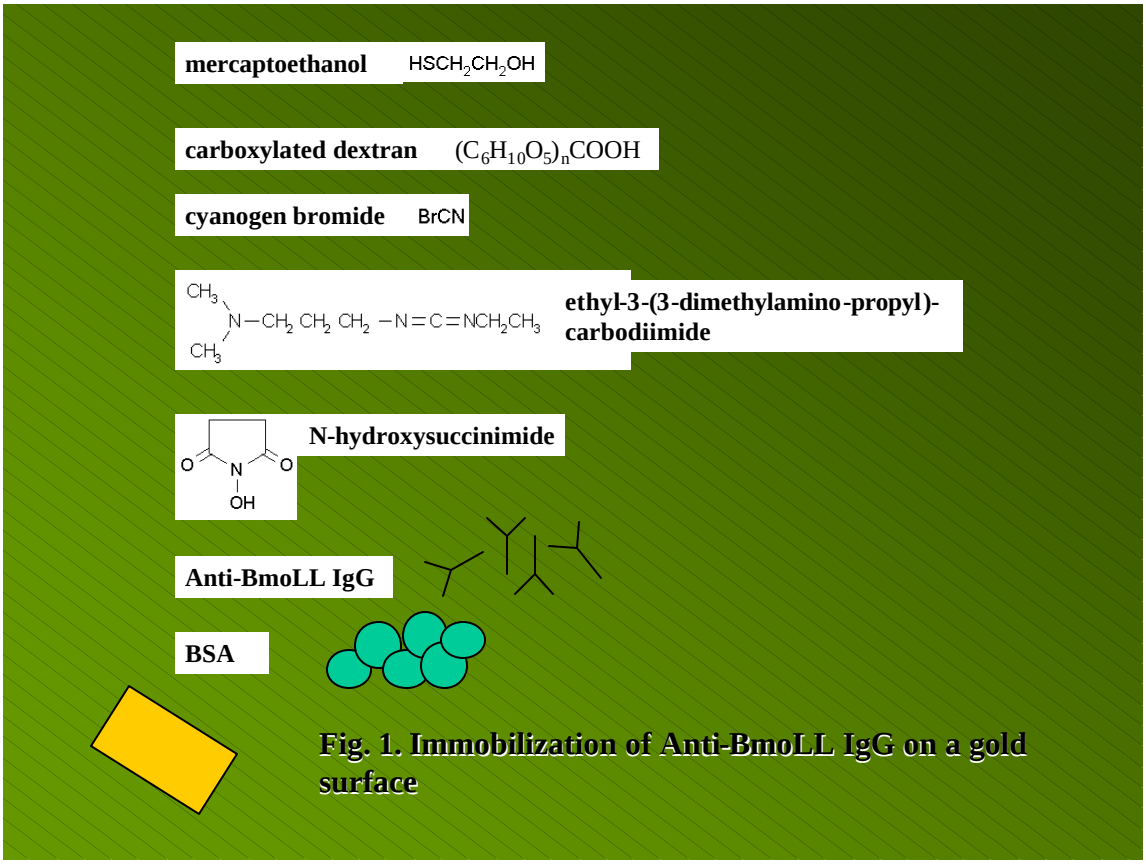

Fig. 1. Immobilization of Anti-BmoLL IgG on a gold surface

The concentrations of unbound IgG were measured with a modified Bradford protein quantification method after immobilization and washes with PBS. The percentage calculation of the immobilization yield was obtained by the formula:

$$\frac{I - (U + P1 + P2 + P3)}{I} \times 100$$

Incubated IgG = I

Unbound IgG = U

Protein (P) in PBS wash steps = P1, P2, P3

RESULTS

A quantity of anti-BmoLL IgG (110 mg) resulted in a immobilization yield of 46%; 220 mg resulted in a immobilization yield of 33%. The control, without IgG, showed some non-protein interference, generally detected in protein quantification methods (Figure 2).

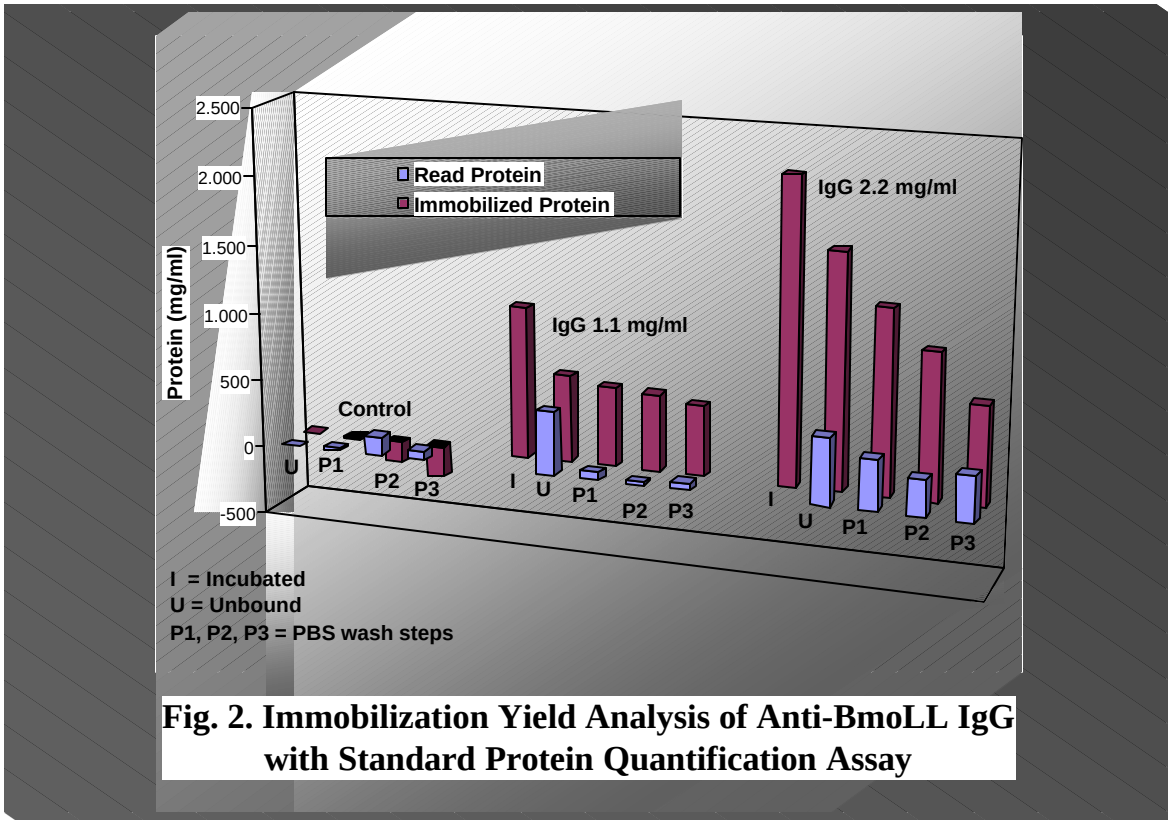

Fig. 2. Immobilization Yield Analysis of Anti-BmoLL IgG with Standard Protein Quantification Assay

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H - 99

A PIEZO-ELECTRIC BINDING ANALYSIS OF THE *Bauhinia monandra* LEAF LECTIN (BmoLL) WITH IMMOBILIZED ANTI-BmoLL IgG.

Haver, N.J.[1]; Souza, S.R.[2,4,5]; Dutra, R.F.[2,3]; da Silva, M.B.R.[1]; Maciel, J.C.C.[2]; Correia, M.T.S.[1]; Lima-Filho, J.L.[1,2]; Coelho, L.C.B.B.[1]

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Biosensors have been used in clinical and industrial chemistry for environmental monitoring and control, chemical measurements in agriculture, as well as in food and drug industries. During the last two decades attention has been focused on important aspects of biosensors in relation to the analytical potential of selective recognition of target compounds. To study the *Bauhinia monandra* leaf lectin (BmoLL) – a lectin with galactose specificity which was purified by 60% ammonium sulphate fractionation followed by guar gel affinity chromatography - a polyclonal antibody was raised against the lectin in rabbits; anti-BmoLL IgG was purified on a protein A column. Anti-BmoLL IgG (110 μg/ml) was left to immobilize on a Quartz Crystal Microbalance (QCB) in a thin carboxylated dextran matrix on a gold surface. In the performance of the PIEZO-electric assay, QCB was incubated with a BmoLL solution (454 μg/ml in phosphate-citrate buffer, pH 6.5) followed by a reduction of the frequency with 5 MHz showing a strong recognition of the lectin by the immobilized IgG. The control, without immobilized IgG, showed no response after incubation with BmoLL. For statistical analysis a Sauerbrey equation was used. Based on high stability and sensitivity, low quantities of antigen and antibody used and real time measurement of response, the QCB showed to be a very potent immunological assay.

Supported by CNPq and FACEPE

A PIEZO-Electric Binding Analysis of the *Bauhinia monandra* Leaf Lectin (BmoLL) with Immobilized anti-BmoLL IgG

Haver, N.J.[1]; Souza, S.R.[2,4,5]; Dutra, R.F.[2,3]; Silva, M.B.R.[1]; Maciel, J.C.C.[2]; Correia, M.T.S.[1]; Lima-Filho, J.L.[1,2]; Coelho, L.C.B.B.[1]

[1]Departamento de Bioquímica-UFPE, [2]Laboratório de Imunopatologia Keizo Asami, LIKA-UFPE; [3]Departamento de Patologia, Instituto de Ciências Biológicas-ICB-UPE; [4]Departamento de Química, UFRPE; [5]Faculdade Frassinetti do Recife, FAFIRE.

INTRODUCTION

Biosensors have been used in clinical and industrial chemistry for environmental monitoring and control, chemical measurements in agriculture, as well as in food and drug industries. During the last two decades attention has been focused on important aspects of biosensors in relation to the analytical potential of selective recognition of target compounds. In our approach to obtain a high antibody immobilization yield and to achieve high sensitivity and specificity in a PIEZO biosensor we adapted an immobilization technique which was originally developed for Surface Plasmon Resonance analysis. The *Bauhinia monandra* leaf lectin (BmoLL; Coelho & Silva, 2000) was used as antigen. Anti-BmoLL IgG was immobilized to study its binding activity to BmoLL.

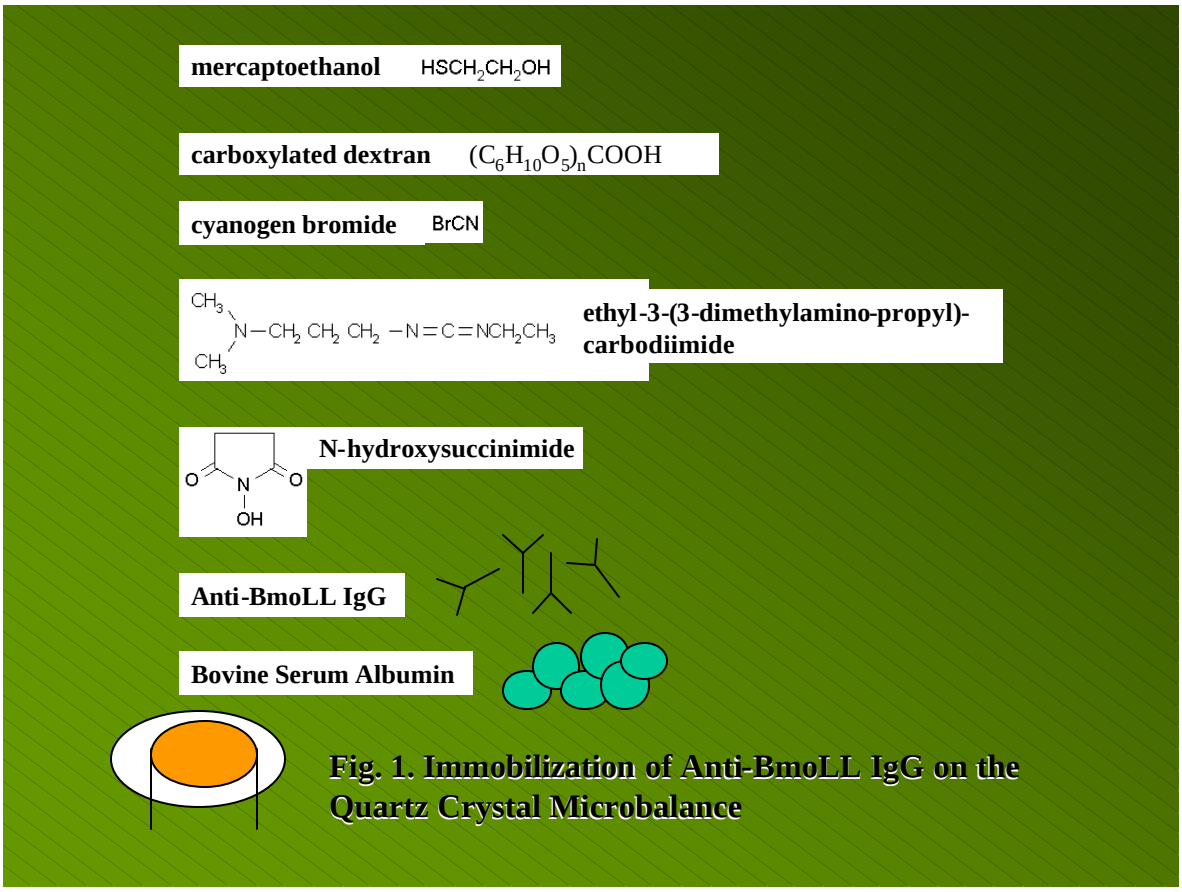

Fig. 1. Immobilization of Anti-BmoLL IgG on the Quartz Crystal Microbalance

METHODOLOGY

A polyclonal antibody against BmoLL was raised in rabbits and anti-BmoLL IgG was purified on a protein A column according to Correia & Coelho (1995). Anti-BmoLL IgG was immobilized by a modified carboxylated dextran method (figure 1). With a liquid PIEZO electric assay system, (figure 2), a crystal with immobilized anti-BmoLL IgG was monitored on the binding of BmoLL. Pure or diluted BmoLL was passed through the reaction chamber to get bound to the immobilized IgG.

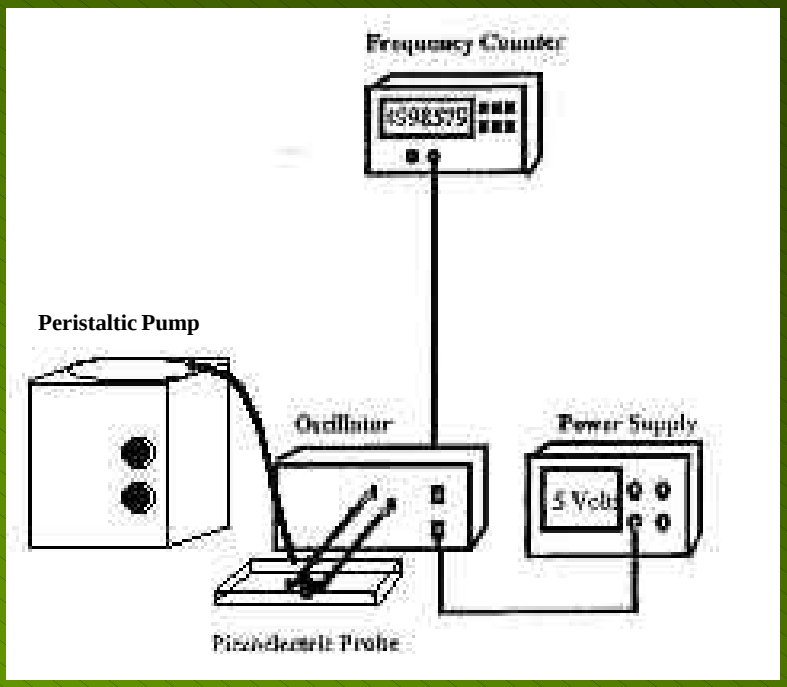

Fig. 2. Piezoelectric liquid assay analysis system with a piezoelectric probe for measuring the frequency change of the quartz crystal due to the binding of BmoLL to the immobilized anti-BmoLL IgG

RESULTS

When anti-BmoLL IgG (1.1 mg/ml) was immobilized, an incubation with pure BmoLL gave a reduction of the frequency with 5 kHz showing a strong recognition of the lectin by the immobilized IgG. The control without immobilized IgG, showed no response after incubation with BmoLL (figure 3).

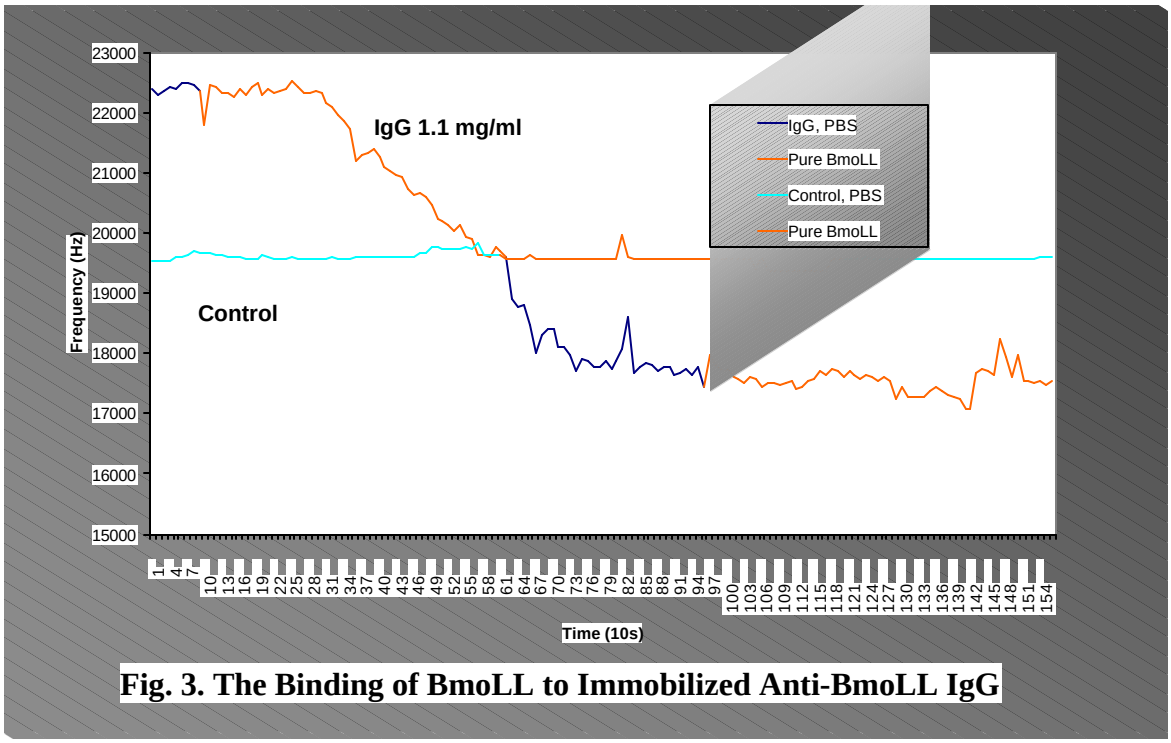

Fig. 3. The Binding of BmoLL to Immobilized Anti-BmoLL IgG

A major immobilization was achieved with double amount of anti-BmoLL IgG. No reactivity was detected with BmoLL indicating that the crystal was heavily loaded (figure 4).

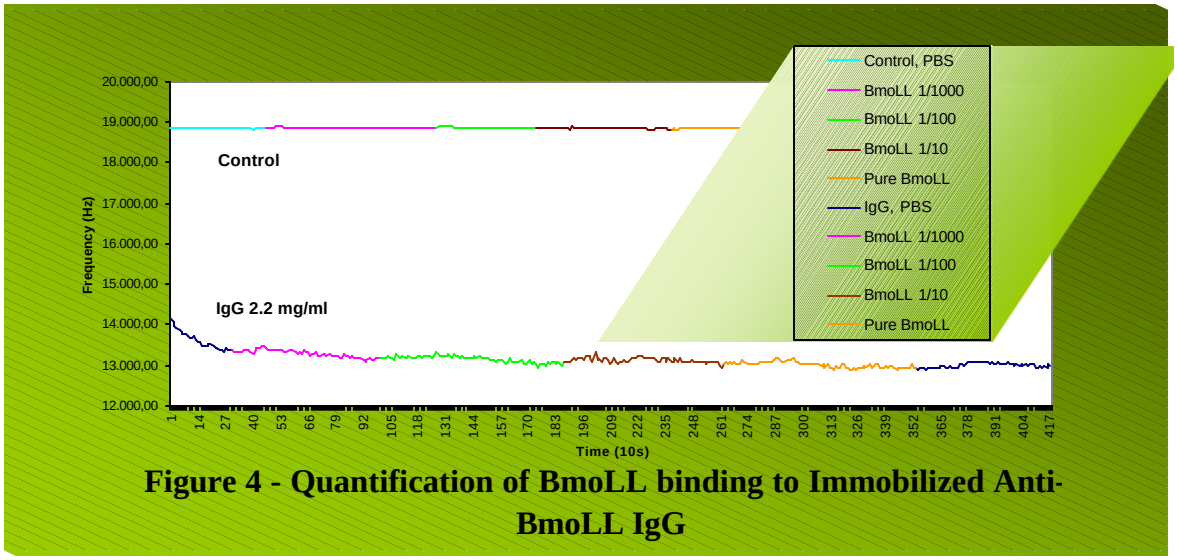

Figure 4 - Quantification of BmoLL binding to Immobilized Anti-BmoLL IgG

CONCLUSIONS

Based on high stability and sensitivity, low quantities of antigen and antibody used, and real time measurement of response, the PIEZO showed to be a very potent immunological assay. More improvement can be made by a reduction in time and quantities used during the immobilization protocol maintaining the high specific binding.

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ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF ANTI-*Bauhinia monandra* LEAF LECTIN IgG (anti-BmoLL IgG)

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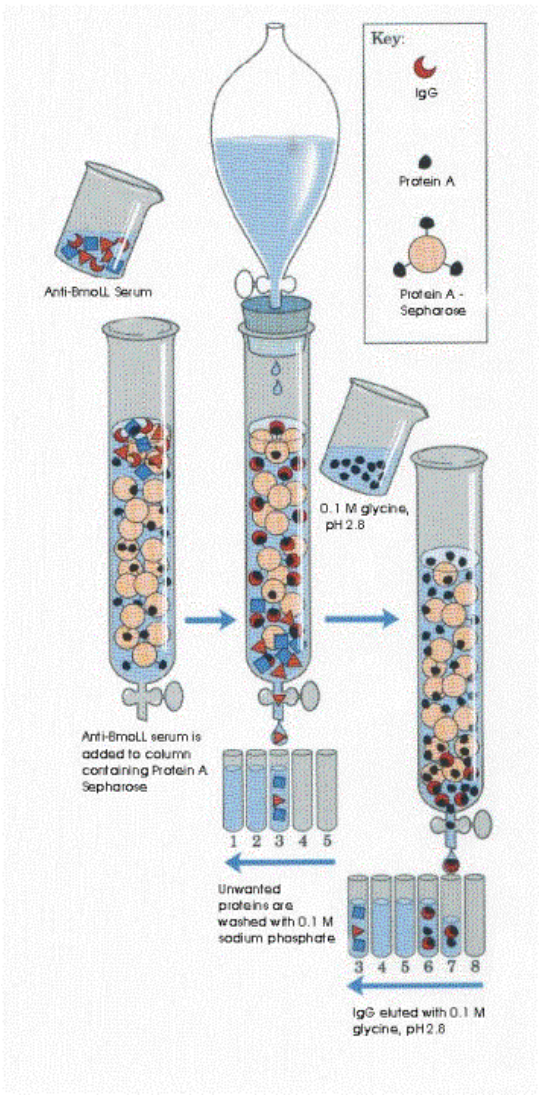
INTRODUCTION

Lectins are proteins or glycoproteins that specifically recognize and reversibly bind carbohydrates without modifying them. Although many plant lectins have been sequenced and their protein structures are known there are still uncertainties about their biological functions. There are indications that plant lectins bind to foreign carbohydrate moieties with the purpose of establishing symbioses or for plant defense[1]. Different plant tissues can be evaluated with a lectin antibody to search the presence of the protein. Cross-reactions of lectin antibodies with other plant lectins can indicate protein homology[2] or similar carbohydrate moieties[3]. The genus *Bauhinia* (Fabaceae) is well distributed in Brazilian cities. A leaf lectin was purified from *B. monandra* (BmoLL)[4]. In this work an antibody was raised against BmoLL and the anti-BmoLL IgG was purified on a Protein A – Sepharose CL-4B column. Cross-reactions of anti-BmoLL IgG with other lectins were evaluated by immunodiffusion tests.

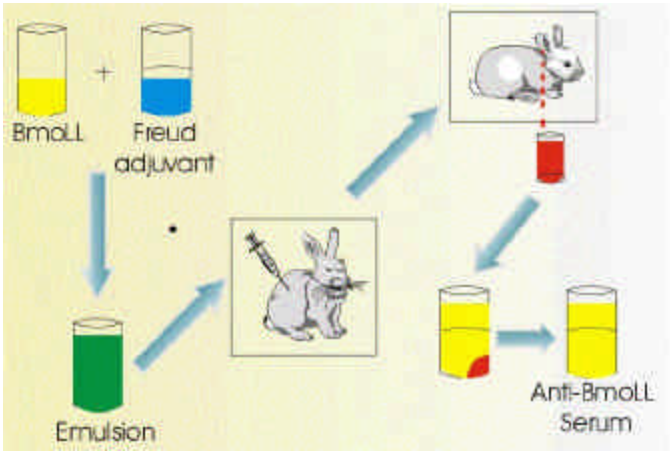
MATERIALS AND METHODS

Preparation of anti-BmoLL serum and purification of anti-BmoLL IgG. BmoLL (150 mg of protein) in 1 ml of 0.1 M citrate phosphate buffer pH 6.8, containing 0.15 M NaCl was emulsified with 1 ml of Freund's complete adjuvant (first inoculation) or 1 ml Freund's incomplete adjuvant (four following inoculations) and intradermally injected into three males, New Zealand white rabbits, in a monthly interval[5]. Immediately before each inoculation, 10 ml of blood were collected from the ear central artery. Every ml of blood was left to coagulate in a

glass tube at the angle of 45° at room temperature (*rt*) for 1 h and placed in the same angle at 4°C, overnight. The sera obtained were three times centrifuged at 1300 x *g*, for 5 min, at *rt*. Aliquots were stored at -20°C (Figure 1a). Anti-BmoLL serum (3 ml) was chromatographed in a column (6.5 x 1.0 cm) containing 4.5 ml of Protein A - Sepharose CL-4B (Sigma). Unbound proteins were washed off with 0.1 M sodium phosphate buffer, pH 8.0, containing 0.15 M NaCl, until absorbance 280 < 0.01. Then, anti-BmoLL IgG was eluted with 0.1 M glycine, pH 2.8. The fractions with absorbance 280 > 1 were brought to pH 7 – 8 with 1 N NaOH and stored in aliquots at -20°C (Figure 1b). *Immunodiffusion tests*. Double immunodiffusion was carried out as previously described[3].



1b



1a

Figure 1 – Preparation of anti-BmoLL serum and purification of anti-BmoLL IgG. Inoculation of BmoLL and collection of anti-BmoLL serum (a) and chomatography of anti-BmoLL IgG on

RESULTS AND DISCUSSION

Double immunodiffusion tests showed that after the third series of inoculations positive reactions were achieved. The positive sera were selected for purification on Protein A column. Control sera (before inoculation) were all negative in immunodiffusions, with or without 0.1 M D-galactose, indicating that there is no recognition of carbohydrates from the antibodies by BmoLL (data not shown). Cross-reactions were found between anti-BmoLL IgG and the lectins from *B. purpurea* and *Ulex europaeus* I. The lectins from *Dolichos biflorus* and *Triticum vulgaris* showed a faint precipitation when incubated with anti-BmoLL serum but did not interact with anti-BmoLL IgG (Figure 2A). The lectins might recognize carbohydrate structures present in the antisera which were eliminated during the purification of anti-BmoLL IgG. Non-specific reactions might be revealed when a high dose of lectin is inoculated or high quantities of lectin were used in immunodiffusion tests. The antisera and purified IgG fractions showed one precipitation band in immunodiffusion with purified BmoLL. However, leaf extracts and fractions showed two bands when precipitated with anti-BmoLL IgG or anti-BmoLL serum (Figure 2B and C). To clarify whether anti-BmoLL IgG recognizes the non-glycosylated and/or the glycosylated polypeptide chains of the lectin further tests should be performed. BmoLL was highly immunogenic and the purified anti-BmoLL IgG allowed to detect the presence of lectins in other tissues of *B. monandra* and to evaluate the homology between them. Anti-BmoLL IgG have already been conjugated with peroxidase and applied in a lectin immunosensor.

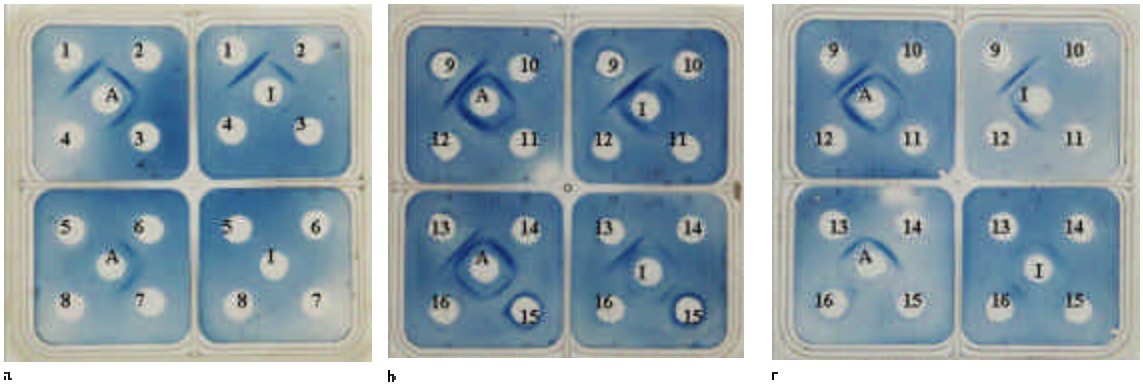


Figure 2 – Double immunodiffusion tests on agarose gels. Cross-reactions of anti-BmoLL serum or anti-BmoLL IgG with other lectins (a), precipitation in various tissue extracts from *Bauhinia monandra* (b), and in 0-60% ammonium sulphate fractions derived therefrom (c).

Central wells: A = anti-BmoLL serum, I = anti-BmoLL IgG. Lateral wells: 1 = BmoLL, 2 = *B. purpurea* lectin, 3 = *Ulex europaeus* lectin I, 4 = *Ulex europaeus* lectin II, 5 = *Bandeiraea simplicifolia* lectin II, 6 = *Dolichos biflorus* lectin, 7 = *Triticum vulgaris* lectin, 8 = *Lens culinaris* lectin, 9 = leaves, 10 = flowers, 11 = stems, 12 = roots, 13 = petioles, 14 = seeds, 15 = buds, 16 = branches of stem.

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