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CLÉBIA CLERIANE PEREIRA RABÊLO

ISOLAMENTO E CARACTERIZAÇÃO PARCIAL DE LECTINAS EM SEMENTES DE *Capparis yco*

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Orientadora: Profa. Dra. Patrícia Maria Guedes Paiva

Co-orientadora: Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho

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Orientador:

Dra. Patrícia Maria Guedes Paiva

(UNIVERSIDADE FEDERAL DE PERNAMBUCO)

Examinadores:

1 Examinador

Sandra Rodrigues de Souza

2 Examinador

Maria das Graças Carneiro da Cunha

3 Examinador

Maria Tereza dos Santos Correia

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“ O coração do homem planeja o seu caminho, mas o Senhor lhe dirige os passos.”

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LISTA DE ABREVIATURAS

AH	Atividade hemaglutinante
AHE	Atividade hemaglutinante específica
CM	Carboximetil
Con A	Concanavalina A (lectina de *Canavalia ensiformis*)
CySeL	Lectina de semente de *Capparis yco*
DEAE	Dietilaminoetil
EDTA	Ácido etilenodiaminotetracético
PAGE	Eletroforese em gel de poliacrilamida
PHA	Hemaglutinina de *Phaseolus vulgaris*
RIP-2	Proteína inativadora de ribossomo tipo 2
SDS	Dodecilsulfato de sódio
WGA	Aglutinina de gérmen de trigo

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RESUMO

Purificação de formas moleculares múltiplas da lectina de sementes de *Capparis yco* (CySeL) foi desenvolvida através de fracionamento com sulfato de amônio e cromatografia das frações (F) em CM-Celulose. O protocolo foi efetivo para obter, a partir das F20-40 e F40-80, $CySeL_I$ (12 mg) e $CySeL_2$ (9 mg), respectivamente; adicionalmente revelou diferenças nas propriedades de cargas das isoformas. Cromatografia em coluna de Hitrap SP, não foi eficiente na separação das isoformas. $CySeL_I$ e $CySeL_2$ foram inespecíficas para eritrócitos humanos e elevadas atividades hemaglutinantes específicas (AHE) foram detectadas com células de coelho. AH de $CySeL_I$ foi inibida com monossacarídeos e glicoproteínas, enquanto $CySeL_2$ reconheceu somente glicoproteínas. Cromatografia de afinidade em coluna de fetuína-agarose mostrou que a AH da F20-40 foi mais retida do que a AH da F40-80. O valor de pH e a presença de íons interferiram diferentemente na AH das isoformas; somente $CySeL_2$ permaneceu ativa em pH 10 e foi estimulada por 40 mM de Ca^{2+} e Mg^{2+}. $CySeL_I$ e $CySeL_2$ apresentaram o mesmo padrão eletroforético. Em PAGE sob condições nativas, três bandas protéicas que foram extraídas do gel mostraram AH; SDS-PAGE na presença de β-mercaptoetanol revelou dois polipeptídeos de Mr 15 e 14 kDa. Coloração do gel para carboidratos mostrou a natureza glicoprotéica da subunidade de 14 kDa. Quantidades miligramas de isoformas da lectina de sementes de C. *yco* foram obtidas. As diferenças estruturais de $CySeL_I$ e $CySeL_2$ detectadas na cromatografia em CM-Celulose, inibição da AH com monossacarídeos e efeito do pH e ions na AH associado ao elevado rendimento

da purificação das proteínas, indicam a potencial utilização de $CySeL_1$ e $CySeL_2$ em investigações estruturais e de aplicação biotecnológica.

SUMMARY

Purification of multiple molecular forms from *Capparis yco* seed lectin (CySeL) was achieved by ammonium sulphate fractionation of extract, followed by ion exchange chromatography of fractions (F) on CM-Cellulose column. The protocol was efficient to obtain from F20-40 and F40-80, $CySeL_1$ (12 mg) and $CySeL_2$ (9 mg), respectively; additionally revealed differences in isoforms charge properties. Chromatography on cation exchange Hitrap SP was not efficient to separete isoforms. The lectins were non-specific to human erythrocytes and high specific hemagglutinating activities (SHA) were detected with rabbit cells. HA of $CySeL_1$ was inhibited with monosaccharides and glycoproteins, while $CySeL_2$ recognized only glycoproteins. Affinity chromatography on fetuin-agarose column showed that F20-40 HA was more retained on column than F40-80 HA. The pH value and presence of ions interfered differently on isoforms HA; only $CySeL_2$ remained active at pH 10 and was stimulated by 40 mM Ca^{2+} and Mg^{2+}. $CySeL_1$ and $CySeL_2$ had the same electrophoretic patterns. Upon native PAGE, three protein bands that were extracted from gel showed HA; SDS-PAGE with β-mercaptoethanol revealed two polypeptides of Mr 15 and 14 kDa. Carbohydrate staining of gel showed the glycoprotein nature of 14 kDa subunit. The protocol used was able to isolate, in milligram quantities, lectin isoforms from *C. yco* seeds. The $CySeL_1$ and $CySeL_2$ structural differences detected in CM-Cellulose chromatography, inhibition of HA with monosaccharide and effect of pH and ions on HA associated to high yield

purification of proteins indicated their potencial use in structural and biotechnological applications.

INTRODUÇÃO

Lectinas

Por volta do final do século 19, evidências começavam a se acumular com relação à presença na natureza de proteínas que possuíam a habilidade de aglutinar eritrócitos. Tais proteínas eram referidas como hemaglutininas ou fitoaglutininas, pois foram originalmente encontradas em extratos de plantas. A primeira descrição de uma hemaglutinina foi feita por Peter Hermann Stillmark em 1888. Esta possuía alta toxicidade e foi isolada de sementes de feijão castor (*Ricinus communis*), chamada assim de ricina. Subseqüentemente, H. Hellin demonstrou a presença de uma hemaglutinina, igualmente tóxica, abrina, em extratos de feijão jequiriti (*Abrus precatorius*). Ricina e abrina logo estavam disponíveis comercialmente, e Paul Ehrlich as empregou como modelos de antígenos para estudos imunológicos.

Em 1919, James B. Sumner isolou de *Canavalia ensiformis* uma proteína que chamou de concanavalina A (Con A), sendo obtida pela primeira vez uma hemaglutinina pura. Sumner e Howell em 1936 relataram que a Con A aglutinava células como eritrócitos e leveduras e também precipitava o glicogênio em solução. Ainda demonstraram que a hemaglutinação proporcionada pela Con A era inibida por sacarose, sendo observada pela primeira vez a especificidade a carboidratos das lectinas. Com isso sugeriram que a hemaglutinação induzida pela Con A poderia ser uma conseqüência da reação da proteína com os carboidratos da superfície das células vermelhas. Estas observações foram corroboradas por Karl Landsteiner, o

descobridor dos grupos sanguíneos humanos A, B e O em 1900. Aproximadamente uma década depois ele relatou que a atividade hemaglutinante de vários extratos de sementes era diferente quando testada com eritrócitos de diferentes animais. A especificidade a diferentes tipos sangüíneos das hemaglutininas desempenhou papel crucial nas primeiras investigações a cerca das bases estruturais da especificidade de antígenos associados com os grupos sangüíneos do sistema ABO.

A habilidade das hemaglutininas de plantas em distinguir eritrócitos de diferentes tipos sanguíneos levou Boyd e Shapleigh, em 1954, proporem o nome lectinas, do latim *legere*, que significa escolher, selecionar. Este termo foi então generalizado por Sharon e Lis (1972) para abranger toda aglutinina carboidrato-específica de origem não imune, independentemente de sua fonte e especificidade a tipo sanguíneo.

Duas grandes descobertas feitas nas proximidades dos anos 60 contribuíram enormemente para trazer as lectinas ao centro das atenções. A primeira delas foi feita por Peter C. Nowell que descobriu que a lectina de *Phaseolus vulgaris* conhecida como fitohemaglutinina (PHA) era mitogênica, isto é, que possuía a habilidade de estimular linfócitos a sofrer mitose. Em um curto espaço de tempo, outras lectinas vieram ser comprovadas como mitogênicas, inclusive a Con A que, diferentemente de PHA tinha sua atividade mitogênica inibida com baixas concentrações de monossacarídeos, por exemplo, manose. Esta descoberta forneceu provas que a estimulação mitogênica era resultado da ligação das lectinas aos carboidratos existentes na superfície dos linfócitos (SHARON e LIS, 1987). Lectinas mitogênicas logo se tornaram ferramentas para estudos da transmissão de sinais intracelulares e

análise de eventos bioquímicos que ocorrem durante a estimulação linfocitária *in vitro* (SHARON e LIS, 2004). A segunda descoberta foi feita por Joseph C. Aub em 1965. Ele descobriu que a aglutinina de gérmen de trigo (WGA) tinha a habilidade de aglutinar preferencialmente células malignas. Investigações desta natureza forneceram evidências de que mudanças em carboidratos da superfície celular estavam associadas com o desenvolvimento do câncer (REMMELINK *et al*., 1999).

Até a década de 70, a presença de lectinas tinha sido referida em inúmeros organismos, primariamente em plantas, mas poucas haviam sido purificadas por técnicas convencionais. A velocidade de isolamento de lectinas aumentou dramaticamente com a introdução, por Irwin J. Goldstein e Bipin B. L. Agrawal, da cromatografia de afinidade em procedimentos de purificação de lectinas. O interesse em lectinas advém de sua utilização como ferramentas para a detecção, isolamento e caracterização de glicoconjugados, para estudos histoquímicos de células e tecidos e para analisar mudanças que ocorrem na superfície celular durante processos fisiológicos e patológicos (SHARON e LIS, 2004).

A presença de lectinas, em material biológico, é principalmente detectada através de ensaio de hemaglutinação, no qual uma diluição em série da lectina é realizada antes da incubação com eritrócitos humanos ou de outros animais (BLADIER, 1989). Estas proteínas são amplamente distribuídas na natureza, sendo encontradas em seres unicelulares (KAWAGISHI *et al*., 2001) e pluricelulares animais (DUNPHY *et al*., 2002) e vegetais (BRANCO *et al*., 2004). Nas plantas, são purificadas principalmente de sementes, chegando a constituir até 10% da proteína total (SHARON e LIS, 1990). A localização intracelular da maioria das lectinas nos

órgãos de estoque ocorre em organelas celulares originadas de vacúolos, como no caso das sementes de leguminosas (BABA *et al*., 1991). São também encontradas, contudo em menor proporção em folhas, frutos, casca de árvores e raízes (PEUMANS e VAN DAMME, 1995).

Goldstein *et al*. (1980) definiram lectinas como proteínas ou glicoproteínas de origem não imunológica que interagem com carboidratos através de pelo menos dois sítios de ligação, aglutinando células vegetais e/ou animais e precipitando polissacarídeo, glicoproteínas e glicolipídios. Esta definição baseia-se na propriedade biológica das lectinas de ligarem-se específica e reversivelmente a carboidratos.

Peumans e Van Damme (1995) redefiniram lectinas considerando que o único pré-requisito para uma proteína ser uma lectina é a presença de pelo menos um domínio não catalítico que se liga reversivelmente a um carboidrato específico. Conseqüentemente, lectinas de plantas podem ser definidas como toda proteína de planta que possui pelo menos um domínio não catalítico que se liga específica e reversivelmente a mono ou oligossacarídeos. Esta nova definição inclui proteínas que têm comportamentos distintos com relação à aglutinação e precipitação de glicoconjugados, tendo como base as características estruturais e não sua capacidade de aglutinação celular.

Baseado em sua estrutura global, três tipos principais de lectinas são distinguidos: merolectinas, hololectinas e quimerolectinas (PEUMANS e VAN DAMME, 1995). Merolectinas são proteínas que possuem exclusivamente um único sítio de ligação a carboidratos. São pequenas (único polipeptídio) e por sua natureza monovalente são incapazes de precipitar glicoconjugados e aglutinar células.

Heveína, proteína obtida do látex da seringueira e as proteínas monoméricas ligadoras de manose de orquídeas são exemplos deste grupo. Hololectinas também possuem exclusivamente sítio de ligação a carboidratos, contudo contém dois ou mais destes sítios que são idênticos ou muito homólogos. Este grupo compreende todas as lectinas que possuem múltiplos sítios de ligação e são, portanto capazes de aglutinar células e/ou precipitar glicoconjugados. A maioria das lectinas de plantas conhecidas são hololectinas, por causa do seu comportamento como hemaglutininas.

Quimerolectinas são proteínas que possuem um ou mais sítios de ligação a carboidratos, juntamente com um sítio com atividade catalítica ou outra atividade biológica que atua independentemente dos sítios de ligação a carboidratos. Dependendo do número de sítios de ligação a carboidratos as quimerolectinas comportam-se como merolectinas ou hololectinas. Por exemplo, proteínas ativadoras de ribossomos tipo 2 (RIP 2) possuem dois sítios de ligação em sua cadeia B (ricina) e aglutinam células, enquanto quitinases de plantas classe I, com um único sítio de ligação à quitina não aglutinam células.

Peumans e Van Damme (1998) sugeriram ainda a introdução de mais um grupo de lectinas, as superlectinas. Estas seriam proteínas que consistem de pelo menos dois sítios de ligação para carboidratos diferentes entre si. A lectina de tulipa, com dois sítios, um específico para manose e outro específico para N-acetilgalactosamina, é um exemplo de superlectina (PEUMANS *et al*., 2001).

Os sítios de ligação para carboidratos das lectinas tendem a ser na superfície da molécula protéica (SHARON e LIS, 1990; GOLDSTEIN, 2002), e a seletividade da

ligação é obtida através de pontes de hidrogênio e interações de van der Walls entre o açúcar e a proteína (SUROLIA *et al.*, 1996; WEIS e DRICKAMER, 1996).

Da mesma forma que ocorrem formas moleculares múltiplas de várias proteínas (HORIGUCHI *et al*., 2005), a presença de lectinas sob formas moleculares múltiplas também tem sido observada em tecidos vegetais (MISHRA *et al*., 2004) e animais (BULGAKOV *et al*., 2004). Entlicher *et al.*, em 1971, sugeriram o termo isofitohemaglutininas para lectinas vegetais de uma mesma fonte, que apresentem praticamente o mesmo peso molecular e propriedades biológicas idênticas, diferindo em suas propriedades iônicas. No mesmo ano, Howard *et al.*, admitiram que as formas moleculares múltiplas da lectina de *Lens culinaris* podiam ser decorrentes de clivagem proteolítica específica de uma forma produzindo a outra, assim como da expressão de genes distintos. Sharon e Lis (1972) sugeriram que isolectinas poderiam ocorrer como produtos de genes intimamente relacionados ou seriam formadas antes ou durante o processo de purificação, como resultado de modificações da cadeia lateral, como hidrólise de grupamentos amino de resíduos de glutamina e asparagina.

Moss (1982) apresenta o termo isoenzimas como formas moleculares múltiplas de uma enzima que ocorrem em uma mesma espécie, como resultado da presença de mais de um gene estrutural. Segundo Lis e Sharon (1986) isolectinas são definidas como um grupo de proteínas intimamente relacionadas que são originadas a partir da expressão de diferentes genes. O termo isoforma é atribuído para formas moleculares de lectinas presentes em uma mesma espécie, cuja heterogeneidade genética não foi definida (PAIVA e COELHO, 1992).

Na purificação de lectinas estão envolvidas técnicas comuns a protocolos de isolamento de proteínas. Em geral, a etapa inicial do isolamento de lectinas de plantas é a preparação de extratos em solução salina (KONOZY *et al*., 2003) ou utilizando tampões (OLIVEIRA *et al*., 2002). Posteriormente para purificação parcial, utiliza-se, na maioria das vezes, a precipitação protéica por tratamento do extrato com sulfato de amônio; as proteínas precipitadas em frações distintas mantêm a sua conformação nativa (COELHO e SILVA, 2000).

As lectinas parcialmente purificadas pelo fracionamento salino são normalmente submetidas a processos cromatográficos convencionais, tais como, cromatografia de troca iônica ou cromatografia de gel filtração. São inúmeras as matrizes disponíveis comercialmente (Sigma, USA) sendo a escolha do tipo de cromatografia e a seleção do suporte cromatográfico em função das propriedades inerentes a lectina. Os critérios de separação correspondem à carga líquida da proteína, em cromatografia de troca iônica e ao seu tamanho molecular, no caso da cromatografia de gel filtração.

Em cromatografia de troca iônica, proteínas são adsorvidas à matriz, devido principalmente a interações eletrostáticas que ocorrem com os grupos carregados imobilizados. Como principais grupamentos carregados ligados a matriz temos o DEAE (dietilaminoetil), grupo positivamente carregado e, portanto, um trocador aniônico e o CM (carboximetil), grupo negativamente carregado, sendo um trocador catiônico. Estes grupamentos estão ligados a uma matriz insolúvel composta geralmente por polímeros como agarose, acrilamida ou celulose (Sigma, USA). As proteínas com nenhuma ou pouca interação com o trocador de íons são excluídas

na etapa inicial de lavagem da coluna com o tampão de equilíbrio; as proteínas adsorvidas à matriz podem ser eluídas pelo aumento da força iônica ou pela mudança do pH da solução eluente. A eluição pode ocorrer pela utilização do gradiente em escada, com o aumento da concentração salina ou valor do pH em valores distintos de forma não contínua (MISHRA *et al*., 2004) ou pelo gradiente linear, onde a alteração do meio cromatográfico ocorre de maneira lenta e gradativa (REGO *et al*., 2002).

Método cromatográfico não convencional, a cromatografia de afinidade, desenvolvida inicialmente por Cuatrecasas *et al*. (1968), baseia-se na propriedade das lectinas de ligarem especificamente e reversivelmente a carboidratos. Sua aceitação em praticamente todos os esquemas de purificação de lectinas de especificidade definida deve-se a vantagens, tais como elevada recuperação e alta especificidade. Neste tipo de cromatografia ocorre a ligação da lectina ao suporte de afinidade e sua posterior dessorção, seja por competição bioespecífica pelo sítio protéico com uma solução contendo um competidor, seja por alteração do pH e da força iônica (KONOZY *et al*., 2003; BANERJEE *et al*., 2004).

As lectinas diferem entre si pela composição e seqüência de aminoácidos na cadeia polipeptídica, quanto ao número de subunidades na estrutura protéica, quanto à necessidade de presença de metais para atividade biológica, bem como na especificidade do sítio de ligação para mono ou oligossacarídeos (KENNEDY *et al*., 1995).

A caracterização de lectinas envolve métodos diversos como: inibição por carboidratos e/ou glicoconjugados, atividade hemaglutinante em presença de íons,

estabilidade da atividade lectínica em função de diferentes valores de pH, bem como técnicas eletroforéticas para indicar basicidade ou acidez da lectina, determinar peso molecular e caracterizá-la como glicoproteína (COELHO e SILVA, 2000).

Testes de inibição da atividade hemaglutinante têm como objetivo conhecer a especificidade de ligação a mono, di ou oligossacarídeos, desde que a especificidade é um critério para classificar lectinas de plantas em seis grupos de reconhecimento a carboidratos: grupo fucose, grupo galactose/N-acetilgalactosamina, grupo N-acetilglicosamina, grupo manose, grupo ácido siálico e grupo glicanos complexos. As lectinas de plantas exibem ampla especificidade para carboidratos, tendo algumas maior afinidade para oligossacarídeos do que para açúcares simples ou possuírem especificidade direcionada a glicanos estranhos (não presentes na própria planta). Lectinas estruturalmente diferentes podem ainda reconhecer o mesmo carboidrato (PEUMANS e VAN DAMME, 1998).

A ligação a carboidratos de algumas lectinas de plantas depende da presença de íons; a presença simultânea do cálcio e um íon metálico de transição foi primeiramente descrito para a Con A (LORIS *et al*., 1998). O ensaio de hemaglutinação em presença de íons, objetiva avaliar a dependência ou não da lectina por íons metálicos para promover a sua atividade biológica; lectina de *Erythrina speciosa* possui Ca^{+2} e Mn^{+2}, e quando tratada com EDTA sua AH é abolida, sendo a mesma recuperada após a adição dos respectivos íons (KONOZY *et al*., 2003). De outra forma, a lectina de *Helianthus tuberosus* não tem sua atividade suprimida quando tratada com EDTA nem necessita de íons metálicos como Ca^{+2}, Mn^{+2} e Mg^{+2} para a mesma (SUSEELAN *et al*., 2002).

A avaliação da AH de lectinas em diferentes condições de pH é de grande valia para a determinação da estabilidade com relação a sua capacidade de ligação a carboidratos e glicoconjugados. Lectinas podem ter sua atividade afetada (KONOZY *et al*., 2003) ou não (WITITSUWANNAKUL *et al.,* 1998) em diferentes faixas de pH.

Eletroforese em gel de poliacrilamida e dodecil sulfato de sódio (SDS-PAGE) é um método rápido, reprodutível e de baixo custo para comparação e caracterização de proteínas. Este método é baseado primariamente no peso molecular destas (LAEMMLI, 1970). O SDS liga-se as porções hidrofóbicas da proteína e o comprimento do complexo SDS-proteína é proporcional ao peso molecular da mesma. É uma importante técnica analítica podendo ser utilizada na análise do grau de pureza de uma proteína, determinação da massa molecular, detecção de proteólise, composição de subunidades, entre outras aplicações. A eletroforese em condições nativas caracteriza a proteína em relação à carga líquida. Lectinas ácidas (SANTOS *et al*., 2005) e básicas (SULTAN *et al*., 2004) foram reveladas por esta técnica. Para coloração dos géis de poliacrilamida, podem ser utilizados os corantes azul de Coomassie ou negro de amido, que revelam bandas polipeptídicas, ou com colorações específicas para glicoproteínas (GUZMAN-PARTIDA *et al*., 2004).

Ainda em métodos de caracterização temos a determinação do ponto isoelétrico (eletrofocalização), eletroforese bidimensional, análise da composição e seqüência de aminoácidos, espectroscopia de massa, dicroísmo circular, dentre outros.

O reconhecimento entre proteínas e carboidratos é de grande importância em vários processos biológicos como infecções virais, bacterianas, fúngicas e parasíticas; endereçamento de células e componentes solúveis; fertilização; metástase, crescimento e diferenciação celular. As lectinas de plantas têm sido escolhidas para estudar as bases moleculares destes eventos de reconhecimento, pois não são apenas mais facilmente purificadas em grande quantidade, como exibem uma larga variedade de especificidade a carboidratos (LORIS *et al*., 1998).

As lectinas constituem ferramentas em estudos citoquímicos e histoquímicos (DANGUY *et al*., 1998), fracionamento de células (OHBA *et al*., 2002), como moléculas bioadesivas no endereçamento de drogas (BIES *et al*., 2004) e na obtenção de oligossacarídeos e glicoconjugados quando imobilizadas em suportes insolúveis (FRANCO-FRAGUAS *et al*., 2003; BANERJEE *et al*., 2004). Podem ainda ser utilizadas para análise de mudanças que ocorrem na superfície celular durante processos fisiológicos e patológicos, desde células normais a tecidos transformados, como ocorre no câncer (SHARON e LIS, 2001).

Uma variedade de propriedades biológicas pode resultar da ligação de lectinas às células, incluindo: aglutinação de eritrócitos e identificação de grupos sanguíneos (PAIVA e COELHO, 1992; MATSUI *et al*., 2001); atividades antiinflamatória (ASSEREUY *et al*., 1999), antitumoral (ELSASSER-BEILE *et al.,* 2001; ZHAO *et al*., 2003), antimicrobiana (FREIRE *et al*., 2002) e hipoglicemiante (KAVALALI *et al*., 2003); estimulação mitogênica de linfócitos T humanos (PAJIC *et al*., 2002; MACIEL *et al*., 2004); liberação de óxido nítrico por macrófagos

(ANDRADE *et al.*, 1999) e indução de apoptose em tumores de células humanas (HAJTÓ *et al*., 2003).

Capparis yco

Capparis yco, conhecida vulgarmente como icó-branco (Figura 1), é uma planta pertencente à família Capparaceae, que se distribui nas Regiões Tropical e Subtropical, especialmente da África e das Américas. O gênero *Capparis* tem aproximadamente 250 espécies sendo 15 delas encontradas no Brasil principalmente nas vegetações de Caatingas e Restingas, sendo bem representado na flora de Pernambuco. As espécies são utilizadas na produção de madeira, alimentação e na medicina popular.

O icozeiro não vai, em média, além dos 2 a 3 metros. Seus ramos são longos, eretos, de casca castanho claro amarelado. Folhas opostas, algo pendentes, um pouco mais adensadas na porção superior dos ramos. Fruto baga, coriácea a sublenhosa, irregurlamente ovóide, com 8 a 10 cm de comprimento e 5 a 6 cm de diâmetro, denso pubescente, castanho claro amarelado. Sementes várias (14-20) subcocleadas, castanho claras, lustrosas, mergulhadas em massa esponjoso-farinácea, de cor creme claro, que passa a castanho claro ao secar. Eventualmente, seu fruto é referido ser comestível (CORRÊA, 1978).

Pesquisas existentes com espécies do gênero *Capparis* revelam diversas propriedades biológicas, mostrando com isso ser um material de estudo bastante promissor. Sementes de *C. masaikai* foram utilizadas no isolamento e caracterização

da proteína com propriedades adoçantes mabinlina II (LIU *et al*., 1993; NIRASAWA *et al*., 1994; KOHMURA e ARIYOSHI, 1998; GUAN *et al*., 2000); aglutinação e morte do parasito *Leishmania* em sua forma promastigota foram detectadas quando o vetor *Phlebotomus papatasi* foi alimentado com extrato de *C. spinosa*, sendo indicado ser isto devido à presença de várias lectinas no extrato (SCHLEIN e JACOBSON, 1994; JACOBSON e SCHLEIN, 1999); avaliação dos frutos de *C. decidua* (Pinju) como alternativa na dieta em regiões da Índia (DUHAN *et al*., 1992) e estudo do seu valor nutricional, principalmente como fonte de vitamina C e beta-caroteno (CHATUVERDI e NAGAR, 2001); identificação e isolamento do princípio ativo da *C. spinosa*, responsável por sua ação antiinflamatória (AL-SAID *et al*., 1988); determinação de atividade antihepatotóxica (GADGOLI e MISHRA, 1999) e antifúngica contra dermatófilos de *C. spinosa* (ALI-SHTAYET e ABU GHDEIB, 1999); estudo da ação hipoglicemiante de *C. decidua* (YADAV *et al*., 1997a e 1997b) e *C. spinosa* (YANIV *et al*., 1987); avaliação da toxicidade de *C. tomentosa* (AHMED *et al*., 1981; AHMED *et al*., 1993); determinação da atividade antioxidante e constituintes químicos de *C. spinosa* (GERMANO *et al*., 2002; BONINA *et al*., 2002 e KHANFAR *et al*., 2003).

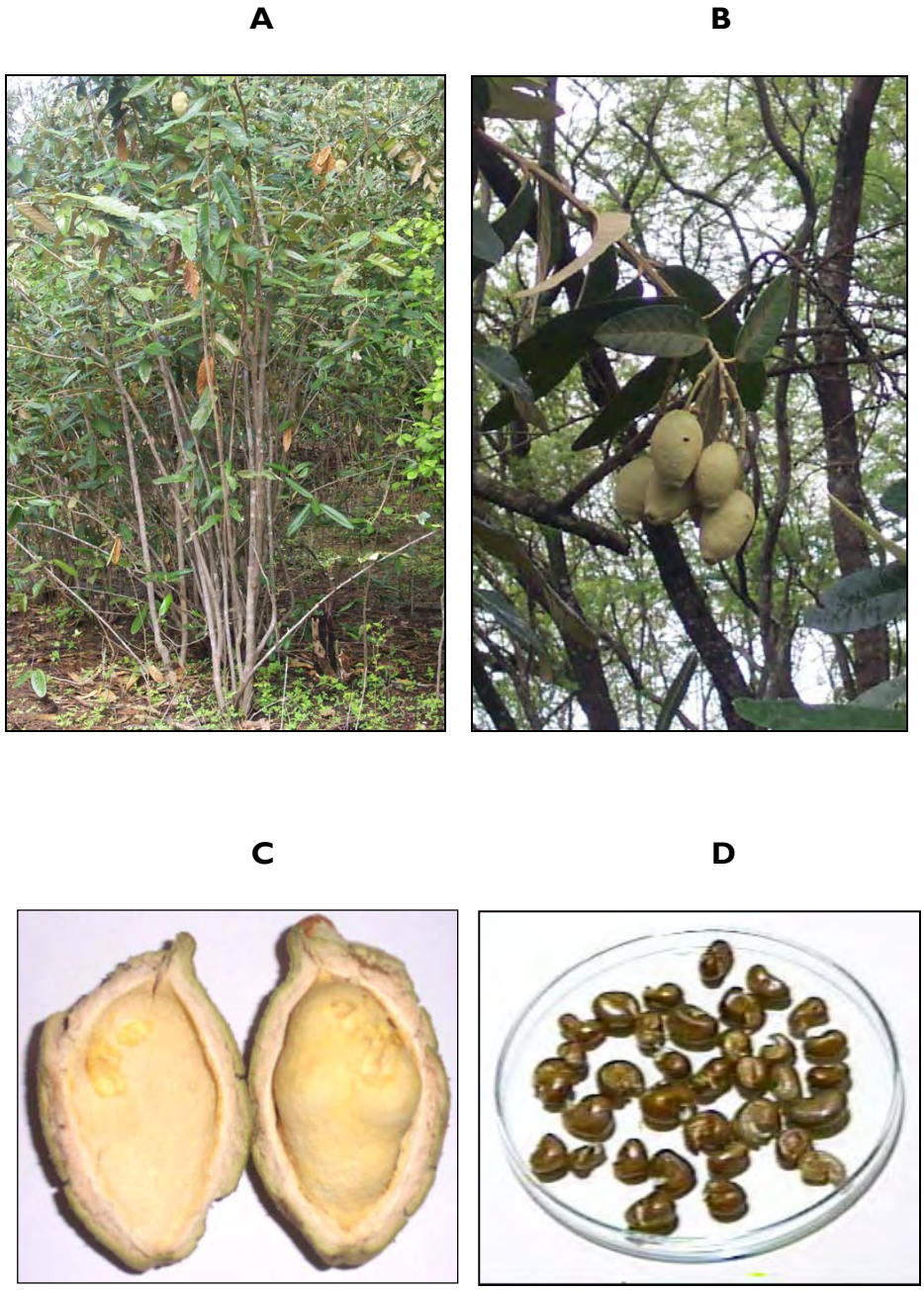

Figura 1- Aspectos de *Capparis yco*; árvore (A), folhas e frutos (B), interior do fruto (C) e sementes (D).

RELEVÂNCIA DO TRABALHO

As lectinas vêm sendo amplamente utilizadas em pesquisas biológicas devido ao seu grande potencial médico e biotecnológico. Estas moléculas são freqüentemente purificadas de sementes devido ao elevado teor protéico deste tecido vegetal. Desde 1983 o Laboratório de Glicoproteínas do Departamento de Bioquímica da Universidade Federal de Pernambuco vem contribuindo com o estudo de lectinas ao isolar, caracterizar, definir propriedades biológicas e aplicações biotecnológicas para essas moléculas, em diferentes fontes.

Pesquisas existentes com espécies do gênero *Capparis* revelam diversas propriedades biológicas, mostrando com isso ser um material de estudo bastante promissor. Sementes de *C. masaikai* foram utilizadas no isolamento e caracterização da proteína com propriedades adoçantes mabinlina II (LIU *et al*., 1993; NIRASAWA *et al*., 1994; KOHMURA e ARIYOSHI, 1998; GUAN *et al*., 2000); aglutinação e morte do parasito *Leishmania* em sua forma promastigota foram detectadas quando o vetor *Phlebotomus papatasi* foi alimentado com extrato de *C. spinosa*, sendo indicado ser isto devido à presença de várias lectinas no extrato (SCHLEIN e JACOBSON, 1994; JACOBSON e SCHLEIN, 1999).

OBJETIVOS

OBJETIVO GERAL

Isolar e caracterizar parcialmente lectina(s) em sementes de *Capparis yco*.

OBJETIVOS ESPECÍFICOS

Isolar lectina(s) em sementes de *C. yco* através de fracionamento com sulfato de amônio e métodos cromatográficos;

Determinar a atividade hemaglutinante em preparações de *C. yco* utilizando diferentes eritrócitos;

Determinar a especificidade de ligação da(s) lectina(s) a carboidratos e glicoproteínas;

Avaliar efeito de íons e pH sobre a atividade hemaglutinante;

Determinar, através de métodos eletroforéticos, a massa molecular e o grau de pureza das lectinas isoladas;

Avaliar a natureza glicoprotéica da(s) lectina(s) através de eletroforese em gel de poliacrilamida e coloração específica para glicoproteínas.

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ARTIGO A SER SUBMETIDO AO PERIÓDICO JOURNAL OF CHROMATOGRAPHY B

CHROMATOGRAPHIC SEPARATION OF *Capparis yco* LECTIN ISOFORMS

CHROMATOGRAPHIC SEPARATION OF *Capparis yco* LECTIN ISOFORMS

Clébia C. P. Rabêlo; Cintia M. G. Brito; Luana C. B. B. Coelho & Patrícia M. G. Paiva[1]

Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Avenida Moraes Rego, S/N**,** Cidade Universitária, Recife-PE, 50670-420, Brazil.

[1] Corresponding author: E-mail adress: ppaiva63@yahoo.com.br (P.M.G. Paiva). Tel.-fax: +55-081-21268354.

Abstract

Purification of two molecular forms from *Capparis yco* seed lectin (CySeL) was achieved by ammonium sulphate fractionation of extract, followed by ion exchange chromatography of fractions (F) on CM-Cellulose column. The protocol was effective to obtain $CySeL_1$ (12 mg) and $CySeL_2$ (9 mg); additionally revealed differences in isoform charge properties. The binding of isoforms was also evaluated on Hitrap SP, fetuin-agarose and guar gum columns. The lectins were non-specific to human erythrocytes and high specific hemagglutinating activities (SHA) were detected with rabbit cells. HA of $CySeL_1$ was inhibited with monosaccharides and glycoproteins, while $CySeL_2$ recognized only glycoproteins. The pH values and divalent ions interfered differently on isoforms HA. Upon native PAGE, $CySeL_1$ and $CySeL_2$ had three protein bands which extracted from gel showed HA; SDS-PAGE with β-mercaptoethanol revealed, two polypeptides of Mr 15 and 14 kDa. Carbohydrate staining of gel showed the glycoprotein nature of 14 kDa subunit. The protocol used was able to isolate, in milligram quantities, $CySeL_1$ and $CySeL_{2;}$ structural differences of isoforms associated to high yield purification of proteins indicated their potencial use in structural and biotechnological applications.

Keywords: *Capparis yco,* ion exchange chromatography, lectin isoform purification, protein charge properties.

1. Introduction

Seeds constitute a rich source of lectins, (glyco)proteins of non immune origin with carbohydrate binding properties occurring through cooperative hydrogen bonds, metal coordination, van der Waals and hydrophobic interactions [1]. The presence of, at least, two carbohydrate binding sites in the lectin molecule makes possible their simultaneous interaction with more than one erythrocyte cell surface producing hemagglutination [2].

Interest in lectins from microorganisms, animals and plants has greatly increased due to their applications as, for example, purification of glycosilated trypsin inhibitor by affinity chromatography on lectin immobilized column [3], evaluation of cell surface using lectin conjugated with horse radish peroxidase [4] and as an antiproliferative agent on lymphoma cells [5].

Multiple lectin molecular forms, isoforms or isolectins [6], are common in seeds and differ, for example, in relation to erythrocyte agglutination pattern, carbohydrate specificity, net charge and glycoprotein nature [6], thermal and pH stabilities [7]; HA ion dependence [8], molecular mass and subunit composition [9]. The heterogeneity can confer different biological properties [10] and may be explored to isolate isoforms. The carbohydrate affinity drive the isoform adsorption process on carbohydrate or glycoprotein immobilized matrices; the lectin separation can be obtained increasing carbohydrate inhibitor concentration in elution buffer [11]. On the other hand, if carbohydrate binding of isoforms differ in Ca^{2+} dependence, selective

desorption can occur by quelant agent addition in elution buffer [12]. Charge heterogeneity makes the ion exchange chromatography efficient to separate isoforms in distinct peaks during elution with NaCl linear gradient [13].

Several isoforms have been determined as protein products of different genes. Two *Vigna mungo* seed lectins were expressed in distinct days after flowering and co-translational or post-translational modification was not detected [9] if two related *Oryza sativa* isoforms corresponded to protein products of salt-stress-induced genes [14]. Post-translational modifications have been also implicated in production of isoforms [15]. Proteolytic processing can result in different physicochemical and biological properties. Comparison between native Jacalin (njacalin), the lectin of *Artocarpus integrifolia*, and its recombinant form (rjacalin) revealed that proteolytic processing that occur only in njacalin, was not a prerequisite for rjacalin sugar binding, however decreased its carbohydrate affinity and reduced its stability under extreme pH range [16].

Here, a simplified procedure for the purification of two lectin isoforms from *Capparys yco* seeds, $CySeL_1$ and $CySeL_2$, has been developed using cation exchange chromatography on CM-Cellulose. The protocol was able to provide milligram quantities of isoforms of high specific hemmagglutinating activity (SHA). $CySeL_1$ and $CySeL_2$ were partially characterized by evaluation of its activities at different HA assay conditions, carbohydrate and glycoprotein inhibition pattern, as well as by electrophoresis in polyacrylamide gel (PAGE).

2. Materials and methods

2.1. Seed extract and salt fractionation

Powder (20 g) of *Capparis yco* mature seeds was added to 50 mM sodium phosphate buffer/0.15 M NaCl, pH 6.5 (100 ml); after magnetic agitation for 4 h at 26ºC, the mixture was centrifuged (5,000 g, 15 min) and the supernatant (extract) was used as the starting material to isolate isoforms. The extract was then treated with ammonium sulphate and obtained fractions (F) 0-20, 20-40 and 40-80% were dialyzed against 50 mM sodium phosphate buffer/0.15 M NaCl, pH 6.5.

2.2. Protein determination

Protein concentration was estimated according to Lowry *et al.* [17] using bovine serum albumin (31 to 500 μg ml^{-1}) as standard and by absorbance at 280 nm.

2.3. Hemagglutinating activity (HA) and inhibition of HA

Lectin activity of serially diluted samples in 0.15 M NaCl was assayed with glutaraldehyde (Merck, Germany) treated rabbit and human erythrocytes according to Paiva and Coelho [6] using microtiter plates (Kartell S.P.A.,

Milan). The HA (titer) was defined as the reciprocal of the highest dilution giving positive hemagglutination. Specific HA (SHA) means titer/mg of protein. Lectin inhibition assay was made with 0.2 M monosaccharide (D(+)fructose, D(+)glucose, D(+)galactose, D(+)mannose; from Sigma, USA) or 500 µg/ml glycoprotein (fetuin, ovalbumin and thyroglobulin; from Sigma, USA) solutions.

The pH effect on HA was evaluated with the lectin diluted in 0.1 M Tris-HCl buffer (6.5 to 8.0) and 0.1 M sodium carbonate-bicarbonate (pH 10). The ion assay was performed with the lectin diluted in 10, 20 and 40 mM Ca^{2+} or Mg^{2+} solutions containing 0.15 M NaCl.

2.4. Isolation of lectin isoforms by ion exchange chromatography on CM-Cellulose

F20-40 or F40-80 (20 mg of protein) were chromatographed on a CM-Cellulose (Sigma, USA) column (1.5 x 7.0 cm) equilibrated with 50 mM sodium phosphate buffer/0.15 M NaCl, pH 6.5. After sample application, the column was washed with the equilibrium buffer until 0.05 of absorbance at 280 nm; following, the column was eluted with 0-1.0 M NaCl linear gradient. Active obtained fractions were assayed for HA and the pooled.

2.5. Evaluation of HA binding on Hitrap SP, fetuin-agarose and guar gum columns

F20-40 and F40-80 (20 mg) were applied to Hitrap SP column (0.7 x 2.5 cm; Pharmacia Fine Chemicals,Sweden) equilibrated (flow rate of 1 ml/min; ÄKTA FPLC System) with 50 mM sodium phosphate buffer, pH 6.5. Affinity columns were prepared with fetuin-agarose (Sigma, USA; 0.8 x 4.0 cm) or cross-linking refined guar gum (Sigma, USA; 1.5 x 7.0 cm) equilibrated (flow rate of 20 ml/h) with 50 mM sodium phosphate buffer/0.15 M NaCl, pH 6.5. All columns were washed with respective equilibration solutions until A280 nm < 0.05. The HA was then eluted with 0-1 M NaCl linear gradient (Hitrap SP) or with 0.5 M NaCl followed by 1.0 M NaCl (fetuin-agarose and guar gum).

2.6. Polyacrylamide gel electrophoresis (PAGE)

Non denaturating PAGE was performed according to Reisfeld et al. [18] and stained with amido black. Previously detected polypeptide bands were also extracted by cutting the gel following by maceration in 0.15 M NaCl and evaluation for HA [19]. SDS-PAGE was carried out in a 15% polyacrylamide gel at presence and absence of β-mercaptoethanol [20]. Polypeptide bands from samples were stained with Coomassie Brilliant Blue. The gel was also stained according to Pharmacia Fine Chemicals [21] using Schiff´s reagent (Merck, Germany).

3. Results and discussion

Purification procedure for CySeL isoforms has been developed using extract, protein salt fractionation and cation exchange chromatography. HA assay with rabbit erythrocytes showed two ammonium sulphate fractions, F20-40 and F40-80, of high SHA (Table 1) corresponding to, respectively, 50% and 33% of total extracted HA. Evaluation of fraction HA with human cells revealed F20-40 as the most active preparation (Table 2) suggesting distinct HA in the fractions. The presence of lectin multiple molecular forms has been documented in seeds. Isolectins (Iso) 1, 2 and 3 of *Cratylia mollis* shows different erythrocyte agglutination pattern; Iso 1 and 3, respectively, glucose/mannose and galactose lectins [6] occur in the same cellular compartment and it is suggested distinct biological roles [22].

HA of fractions were retained on CM-Cellulose column; 66% of F20-40 HA, named $CySeL_1$ was eluted with 0.5 M while 69% of F40-80 HA, $CySeL_2$, was eluted 0.3 M NaCl (Figure 1). However, when chromatography was performed on Hitrap SP column, a strong cation exchange, F20-40 and F40-80 HA were eluted with 1.0 M NaCl (Tabela 3). The different NaCl concentrations required to protein desorptions in CM-Cellulose reflected the strength of isoform adsorption processes. Probably, at pH 6.5, $CySeL_1$ had a more positive charge surface than $CySeL_2$ contributing for its selective adsorption. Multipoint interactions promoted by high binding capacity of Hitrap SP impaired the isoforms separation by its charge properties. In fact, Pessela

et al. [23] evaluating ionic exchangers determined that the β-galactosidase binding changed according to content of amino groups; when the lowly activated ion exchange was used, full desorption of the enzyme occurred with lower ionic strength.

HA inhibition assay showed that only $CySeL_1$ recognized monosaccharide and both isoforms were inhibited by glycoproteins (Table 4). Despite of similar glycoprotein inhibition data, different HA yields were obtained in the chromatography on fetuin-agarose and guar gel affinity columns (Table 3). F20-40 HA was mainly retained on fetuin-agarose while highest adsorption of F40-80 HA was achieved on guar gel affinity chromatography. Distinct structural characteristics of isoforms may be leaded to different binding to inhibitors as well as fetuin-agarose adsorption. In fact, the pattern of monosaccharide and oligosaccharide is determined by selectivity of lectin primary site and additional sub-sites or extended sites [1, 24]. Minimal changes in the primary sequence of mutated plant lectins resulted in different binding to erythrocyte surface carbohydrates [25].

Structural characteristics of isoforms were also indicated when HA was assayed with divalent ions and distinct pH values. The ion presence (Figure 2A) main difference was detected at 40 mM Ca^{2+} and Mg^{2+}; $CySeL_2$ HA was stimulated while $CySeL_1$ was inhibited (Ca^{2+}) or inactivated (Mg^{2+}). The inhibition or stimulation of HA probably reflected different conformational changes in isoform structures [1]. The pH assay (Figure 2B) revealed $CySeL_2$ more stable than $CySeL_1$, since only $CySeL_2$ showed HA at pH 10. In fact, alteration of protein structure has been associated to pH value; the secondary

structure of *Artocarpus hirsuta* lectin was not modified at 1-8 pH range, but was distorted above pH 9, altering its binding constant to methyl α-galactopyranoside [26].

Electrophoretic methods revealed isoform structural similarities. $CySeL_1$ and $CySeL_2$, in non-denaturating PAGE, yielded the same three bands (Figure 3) which after elution from gel showed HA (titer of 64^{-1}). Denaturating PAGE revealed that $CySeL_1$ and $CySeL_2$ were constituted by multiple subunits. Under SDS-PAGE, without β-mercaptoethanol, the isoforms migrated as a diffuse band with Mr of 28 to 14 kDa; in the presence of reducing agent, two subunits with Mr of 15 and 14 kDa were detected (Figure 4B). Carbohydrate staining of gel revealed that only 14 kDa band was glycosylated (Figure 4A). Interestingly, the diffuse bands observed in the absence of β-mercaptoethanol were not stained. The different electrophoretic pattern obtained at reducing and non-reducing conditions suggests that 14 CySeL subunit was linked by disulfide bonds, similarly to other seed lectins [27]. The detection of glycoprotein nature only after treatment of isoforms with reducing agent may indicate that the carbohydrate moiety is buried due to subunit associations. Crystal structure of *Erythrina corallodendron* lectin revealed that a glycan constituting the subunit interfaced in the dimmer [28]. SDS-PAGE results and detection of HA in the individual bands eluted from non-denaturating PAGE, indicated that *C. yco* seeds contain three differently charged lectin isoforms which can be constituted of each 15 and 14 subunits or corresponding to a molecular arrangement of these subunits, as determined for *Erythrina variegata* isolectins [29].

$CySeL_1$ and $CySeL_2$ structural differences detected in CM-Cellulose chromatography, inhibition of HA with monosaccharide and effect of pH and ions on HA associated to high yield purification of proteins, indicate their potencial use to structural and biotechnological applications.

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Table1

Summary of $CySeL_1$ and $CySeL_2$ isolation

Preparation	Total protein (mg)	SHA	Purification (fold)
Extract	6,534	113	1
F20-40	1,809	204	1.8
F40-80	1,296	190	1.7
$CySeL_1$	12	1,828	16
$CySeL_2$	9	2,560	23

To assay hemagglutinating activity (HA) *C. yco* preparations (50 μl) were serially two-fold diluted before addition of 2.5% (v/v) suspension of glutaraldehyde treated rabbit erythrocytes (50 μl). Specific HA means titer/protein (mg/ml); total protein of isoforms corresponded to protein (mg) obtained in each chromatographic experiment.

Table 2

Specific hemagglutinating activity (SHA) of ammonium sulphate fractions

Fraction	Erythrocyte				
	Rabbit	A	AB	B	O
F20-40	204	410	205	102	205
F40-80	190	46	23	23	23

Hemagglutinating activity (HA) was performed with 2.5% (v/v) suspension of glutaraldehyde treated erythrocytes. SHA, specific HA (titer/protein).

Table 3

Evaluation of F20-40 and F40-80 HA binding on Hitrap SP, fetuin-agarose and guar gel matrices

Matrix/ chromatographed sample	Yield (%)	SHA	Purification (fold)
Hitrap SP/F20-40	75	226	1.2
HitrapSP/F40-80	75	277	1.4
Fetuin-agarose/F20-40	15	800	4
Fetuin-agarose /F40-80	2.6	800	4
Guar gel/F20-40	19	800	4
Guar gel/F40-80	25	985	5

Yield was expressed as percentage of chromatographed HA recovered in the elution step.

Table 4

Inhibition of $CySeL_1$ and $CySeL_2$ HA

Inhibitor	$CySeL_1$ (titer^{-1})	$CySeL_2$ (titer^{-1})
Monosaccharide		
Fructose	32	NI
Galactose	64	NI
Glucose	NI	NI
Mannose	NI	NI
Glycoprotein		
Azocasein	128	128
Casein	8	8
Fetuin	16	16
Ovalbumin	256	128
Thyroglobulin	8	16

Hemagglutinating activity (HA) was made with 2.5% (v/v) suspension of glutaraldehyde treated rabbit erythrocytes. HA of $CySeL_1$ and $CySeL_2$ in 0.15 M NaCl, 512^{-1} and 1024^{-1}, respectively. NI means no lectin inhibition.

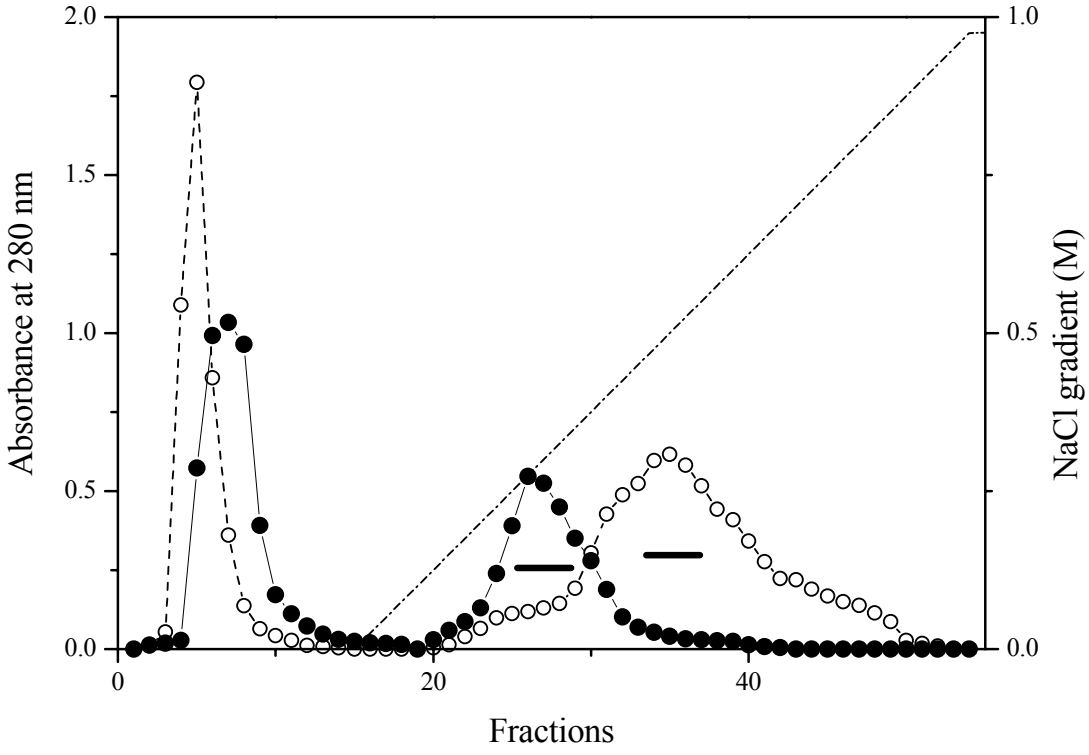
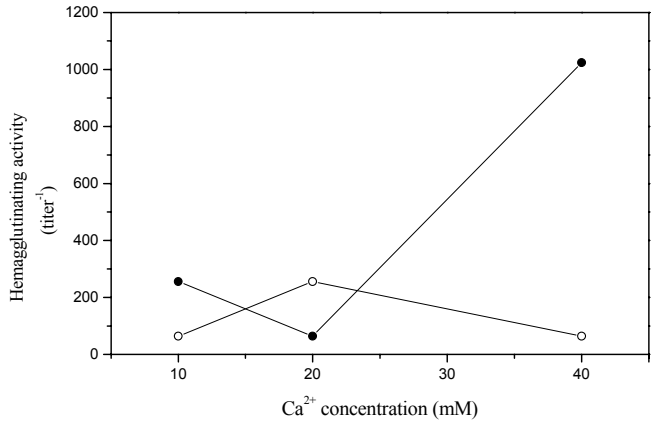
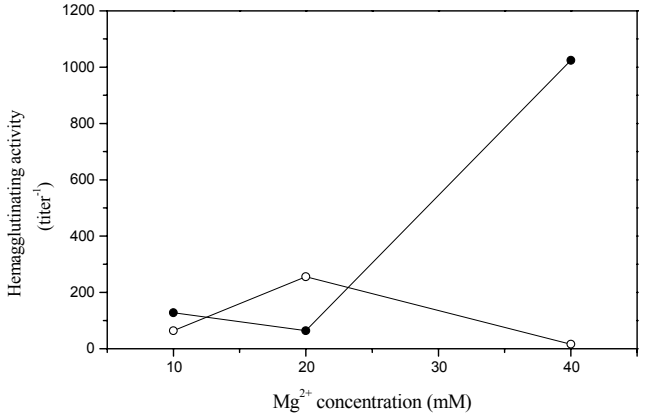


Figure 1. $CySeL_1$ (○) and $CySeL_2$ (●) isolation by ion exchange chromatography. Protein (20 mg) was applied to CM-Cellulose column (1.5 x 7.0 cm) equilibrated (20 ml/h) with 50 mM sodium phosphate buffer containing 0.15 M NaCl, pH 6.5. The column was eluted with 0 - 1.0 M NaCl linear gradient. Obtained fractions (2 ml) were assayed for HA (▬). F20-40 was the first sample chromatographed, followed by F40-80.

A


Hemagglutinating activity
(titer^{-1})
1200
1000
800
600
400
200
0
10
20
30
40
Ca^{2+} concentration (mM)


Hemagglutinating activity
(titer^{-1})
1200
1000
800
600
400
200
0
10
20
30
40
Mg^{2+} concentration (mM)

B

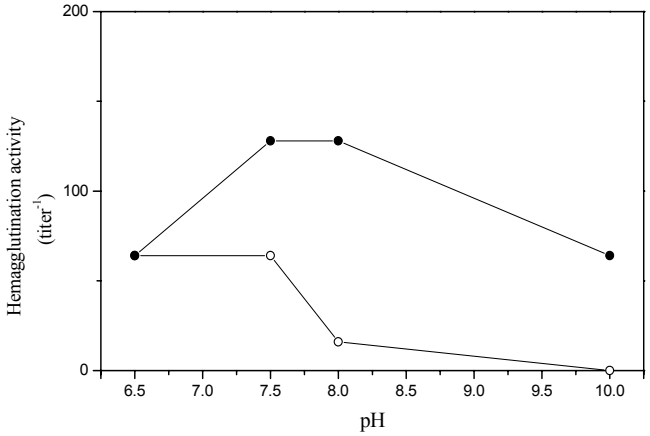

Hemagglutination activity
(titer^{-1})
200
100
0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

Figure 2. Effect of divalent ions (A) and pH (B) on HA of $CySeL_1$ (○) and $CySeL_2$ (●). The ion assay used lectin diluted in 10, 20 and 40 mM Ca^{2+} or Mg^{2+} solutions containing 0.15 M NaCl. The pH effect on HA was evaluated with the lectin diluted in 0.1 M Tris-HCl buffer (6.5 to 8.0) and 0.1 M sodium carbonate-bicarbonate (pH 10). After dilution, isoforms were incubated by 15 min before rabbit erythrocyte addition. $CySeL_1$ and $CySeL_2$ HA in 0.15 M NaCl without ion, were, respectively, 128^{-1} and 512^{-1}. $CySeL_1$ and $CySeL_2$ HA in 50 mM sodium phosphate buffer was 64^{-1}.

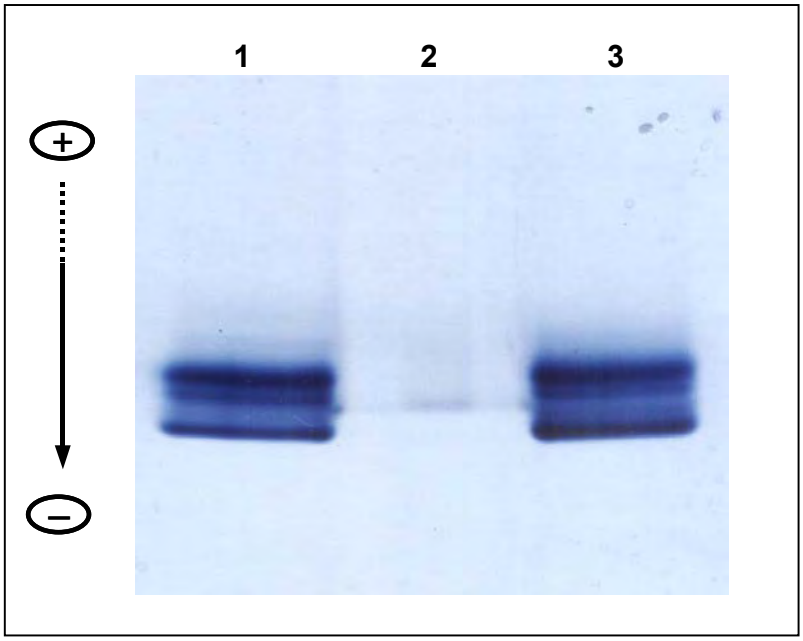


Figure 3. $CySeL_1$ and $CySel_2$ electrophoretic patterns upon native polyacrylamide gel. Samples (50 μg of protein) of $CySeL_1$ (1) and $CySel_2$ (3) as well as cytocrome C (10 μg; 2) were applied on 20 % polyacrylamide gel stained with amido black.

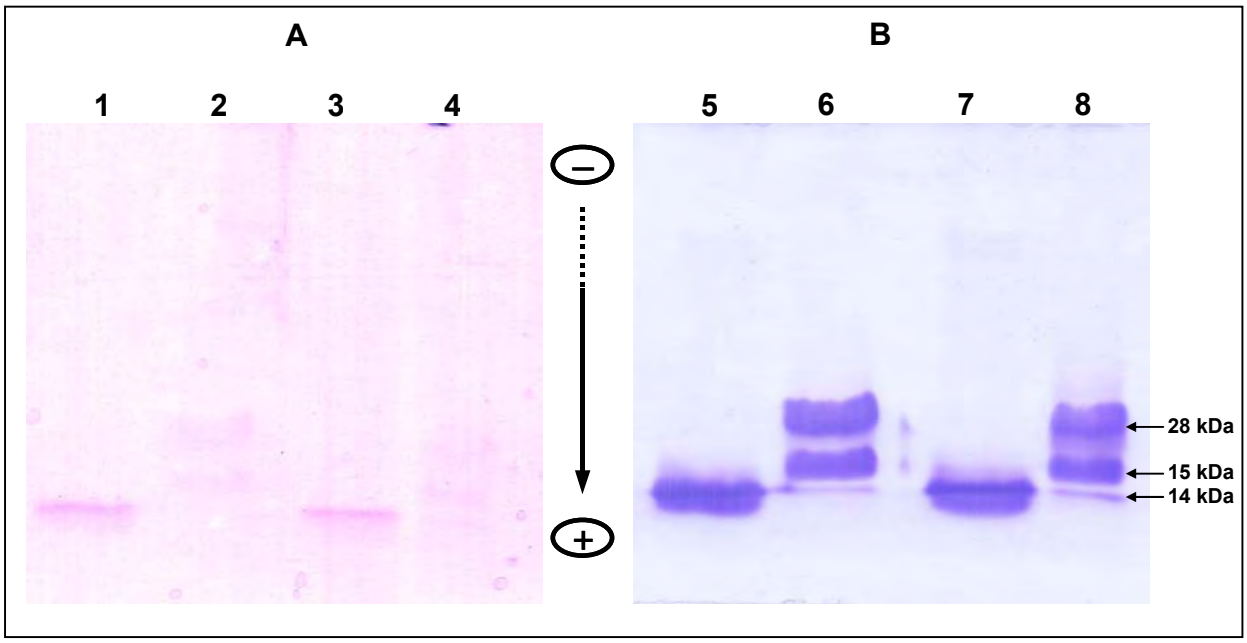


Figure 4. SDS-polyacrylamide gel electrophoresis (15 %) of $CySeL_1$ and $CySeL_2$. The β-mercaptoethanol treated $CySeL_1$ (1) and $CySeL_2$ (3) as well as non-reduced $CySeL_1$ (2) and $CySeL_2$ (4) were stained with Schiff´s reagent (A). The reduced $CySeL_1$ (5), $CySeL_2$ (7) and non-reduced $CySeL_1$ (6), $CySeL_2$ (8) were stained with Coomassie Brilliant Blue (A). Lanes contain 50 µg of isoforms.

CONCLUSÕES

Sementes de *Capparis yco* contém isoformas de lectina ($CySeL_1$ e $CySeL_2$).

$CySeL_1$ e $CySeL_2$ foram isoladas por cromatografia de troca iônica em coluna contendo CM-Celulose.

$CySeL_1$ e $CySeL_2$ aglutinaram eritrócitos de diferentes tipos sangüíneos.

$CySeL_1$ e $CySeL_2$ foram principalmente inibidas por glicoproteínas.

Ca^{2+}, Mg^{2+} e pH afetaram de forma diferente a AH de $CySeL_1$ e $CySeL_2$.

$CySeL_1$ e $CySeL_2$ são proteínas básicas, constituídas de duas subunidades diferentes.

$CySeL_1$ e $CySeL_2$ são glicoproteínas.

ANEXO

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ANALYTICAL TECHNOLOGIES IN THE BIOMEDICAL AND LIFE SCIENCES

Editorial Office *Journal of Chromatography B*
P.O. Box 681, 1000 AR Amsterdam, The Netherlands
Radarweg 29, 1043 NX Amsterdam, The Netherlands
Tel: +31-20-4852794
Fax: +31-20-4852304
e-mail: chrom-eo@elsevier.com

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JOURNAL OF CHROMATOGRAPHY

INSTRUCTIONS TO AUTHORS

● Scope

The *Journal of Chromatography B* addresses advancements in and applications of analytical methodologies related to drugs, other biologically active compounds, metabolites, biomarkers, as well as to bio-polymers such as proteins, peptides, nucleic acids, glycans. The areas considered include:

- clinical analysis, therapeutic drug monitoring, pharmaceutical analysis, toxicological analysis, bio-environmental analysis and novel approaches to sample preparation in analysis of biological matrices
- the qualitative and quantitative analysis of biopolymers including proteins, peptides and their post-translational modifications as well as nucleic acids and glycans
- the screening and profiling of body fluids, tissues, biological matrices and system related to monitoring the level of active substances, including metabolities, biomarkers and toxicants
- the comparative analysis of biological systems using proteomics, genomics, metabonomics and other 'omics' including novel ways of data handling and interpretation
- preparative aspects related to separation and isolation of bioactive compounds including biopolymers

Analytical techniques covered include the various facets of chromatography, electrophoresis and related methods, including mass spectrometry and affinity-based methodologies.

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[4] A. Veide, C. Hassinen, D. Hallen, M. Eiteman, B. Lassen, K. Holmbert, in R.D. Rogers, M.A. Eiteman (Eds.), Proceedings of the American Chemical Society Symposium on Aqueous Biophasic Separation. Plenum Publishers, New York, NY, 1995, p. 133.

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Appendix 1: Experimental conditions to be specified

Experimental conditions should preferably be given on a separate sheet, headed "Conditions". These conditions will, if appropriate, be printed in a block, directly following the heading "Experimental".

General

Chemicals. Supplier (+ city/town, state, country) and degree of purity of all less common chemicals; EC number of enzymes; optical purity of enantiomers.

Equipment. Model and manufacturer (+ city/town, state, country) of commercial instruments (e.g. chromatographs and detectors). For instruments that are not commercially available, sufficient detail (or a reference) should be given to allow others to construct their own instrument. Detection parameters (e.g. type, wavelength, attenuation, linearity range, limit of detection at a specified signal-to-noise ratio).

Sample preparation. Application papers should contain full details (or a reference) of the method of sample preparation. For centrifugation steps, give details of g value and time. Injection device and volume and concentration of the injected sample should be specified.

Column liquid chromatography

Column. Column dimensions (length × internal diameter), manufacturer and location, packing material (for non-commercial columns or columns that are not widely used the chemical composition should be specified), particle diameter, pore diameter, column temperature.

Mobile phase. Complete and unambiguous description of the mobile phase composition or procedure for its preparation; pH; flow-rate; gradient programme.

k values. When reporting values, the method for determining the hold-up time (t_0) must be described.

Gas chromatography and supercritical fluid chromatography

Column. In addition to the parameters mentioned for column liquid chromatography, specify type of column (packed, capillary, etc.) support material, film thickness of the stationary phase, and surface modification, if applicable.

Carrier gas. Type, purity, flow-rate or inlet pressure (bar or MPa).

Temperature. All relevant temperatures (or temperature programmes) should be detailed.

Planar chromatography

Chamber. Internal dimensions, manufacturer and location, saturation, temperature, humidity.

Thin layer or paper. Manufacturer and location, material, dimensions, type (laboratory-prepared or commercially precoated) and thickness of layer, additives (fluorescent indicator, binder), position of starting line, development mode, method of activation.

Solvent. Composition of solvent, monophasic or upper or lower phase of two-phase mixture, total volume.

Sample. Application method, size of spot or streak, solvent and amount of solute and volume of solution applied.

Detection. Spray reagent, wavelength, details of colours, R_F values.

Electrophoresis

Matrix. For example, cellulose acetate, agarose, polyacrylamide; gel concentration; percentage cross-linker; dimensions and material of tube, sheet, etc., surface modification, length between column inlet and detector, temperature.

Buffers. Complete and unambiguous description of buffers used, pH and how the pH was set or adjusted.

Other. Injection method, voltage, current. In electropherograms, anode and cathode should be indicated.

Mass spectrometry

Inlet system. Direct on-line, off-line, postcolumn splitting, postcolumn buffer or matrix addition.

Source. Ionization energy, temperature, trap current, reagent gas. For LC interfaces, complete and unambiguous description of the same and their operating parameters (vaporizer and capillary temperature, buffers, nebulizing, auxiliary or ionizing gases, gas pressures, source and interface voltages, up-front CID voltages.

Mass analyzer. Accelerating voltage, scan mode, collision gas for tendem MS work, collision gas pressure, collision energy, resolution and mass range.

Detection. Electron multiplier voltage and/or electometer gain, ions monitored in SIM and dwell times.

Appendix 2: Conversion table for the non-SI units most frequently used

The use of some non-SI units has been accepted for practical reasons; to this category belong units for time (min, h), volume (l), pressure ($1\ \text{bar} = 10^5$ Pa), temperature (°C), energy ($1\ \text{eV} \approx 160\,219 \cdot 10^{-21}$ J), mass ($1\ \text{u} \approx 1.66053 \cdot 10^{-27}$ kg) and activity ($1\ \text{Ci} = 3.7 \cdot 10^{10}$ Bq). This journal also accepts Å ($= 0.1$ nm). Concentration should formally be expressed in mol dm^{-3} or mol l^{-1}, but the symbol M is accepted; normality (N) should not be used, however. The frequently used "daltons" are not compatible with the SI system — the relative molecular mass (M_r) should be given as a value only (dimensionless). Gravitational force must be expressed in g; rpm is not allowed for centrifugation (but it is, e.g., for vortex mixing). The table below summarizes some conversion factors; to obtain the value in SI units, the value in non-SI units should be multiplied by the factor.

Physical quantity	Type of conversion	Factor
Length	in. → cm	2.54
	ft. → cm	30.4801
Area	$in.^2 \rightarrow cm^2$	6.451626
Mass	lb. → kg	0.45359237
Volume	gallon (USA) → l	3.785332
	gallon (UK) → l	4.54609
Pressure	atm → Pa	101 325
	mmHg or Torr → Pa	133.322
	$mmH_2O \rightarrow Pa$	9.80665
	$kp\ cm^2 \rightarrow Pa$	98066.5
	$lbs.\ in.^{-2}$ or p.s.i. → Pa	6894.76

Other frequently used non-SI "units" are ppm, ppb and ppt. When used in this journal, the American billion (10^9) and trillion (10^{12}) are meant. The use of ppm, ppb and ppt is *only* permitted if they refer to mass/mass or volume/volume ratios; they should **not** be used for mass/volume ratios. The first time such a "unit" appears in an article, it should be indicated whether it refers to mass/mass or to volume/volume.

Appendix 3: Abbreviations and symbols that may be used without definition

Abbreviations and symbols should not be used in article titles. Please note that most abbreviations should only be used in combination with a value, or in structural formulae.

Abbreviations

A, C, G, T	adenine, cytidine, guanine, thymine
Ac, OAc	acetyl, acetate
A/D	analog-to-digital
ADP, AMP, ATP, and similar nucleoside phosphates	adenosine 5′-di-, -mono-, triphosphate, etc.
a.c.	alternating current
amino acids	standard 3- and 1-letter codes
AU	absorbance units
BET	Brunauer—Emmett—Teller
b.p.	boiling point
Bu	butyl
cpm	counts per minute
CE	capillary electrophoresis
d, m, p, r, t (in nucleosides/ nucleotides/nucleic acids)	deoxy, messenger, phosphate, recombinant/ ribosomal, transfer
d.c.	direct current
DDD, DDT, DDE	di-, trichloro-bis(chlorophenyl)ethane, -ethylene
DEAE	diethylaminoethyl
DNA, DNase	deoxyribonucleic acid, deoxyribonuclease
Dns, dansyl	5-dimethylaminonaphthalene-1-sulfonyl
DOPA	3,4-dihydroxyphenylalanine
dpm	desintegrations per minute
EC	enzyme commission numbering system
EDTA	ethylenediaminetetraacetate, -acetic acid
equiv.	equivalent
Et	ethyl
FS	full scale
FSOT	fused-silica open tubular
FT	Fourier transform
GC, GLC, GSC	gas chromatography, gas–liquid chromatography, gas–solid chromatography
HP...	high-performance...
I.D.	internal diameter
IgG	immunoglobulin G
i.m.	intramuscular
i.p.	intraperitoneal
IR	infrared
I.S.	internal standard
I.U.	international unit

i.v.	intravenous
LC	liquid chromatography
LD	lethal dose
Me	methyl
m.p.	melting point
MS	mass spectrometry
NAD, NADH (NADP, NADPH)	nicotinamide—adenine dinucleotide (phosphate)
NMR	nuclear magnetic resonance
O.D.	outer diameter
Ph	phenyl
Pr	propyl
PTFE	poly(tetrafluoroethylene)
RNA, RNase	ribonucleic acid, ribonuclease
RP....	reversed-phase....
rpm	revolutions per minute
RSD	relative standard deviation (preferred over coefficient of variation)
SD	standard deviation
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane
u	atomic mass units (reference to mass of ^{12}C; preferred over a.m.u./amu: reference to mass of ^{16}O)
UV	ultraviolet
vol., v/v	volume, volume/volume
Vis	visible
WCOT	wall-coated open tubular
wt., w/w, m/m	mass, mass/mass

Symbols

A	peak area or absorbance
α	separation factor
D	diffusion coefficient
d_f	film thickness
d_p	particle diameter
ε	interparticle porosity or molar adsorptivity
F	mobile phase flow-rate
ΔG^o	standard Gibbs free energy change
ΔH^o	standard enthalpy change
H	plate height
h	reduced plate height
J	coupling constant
K	equilibrium constant
k	retention factor
K_c	distribution constant (preferred over partition coefficient)
L	length
λ	wavelength
M_r	(relative) molecular mass
μ	electrophoretic mobility
N	number of plates
n	number of determinations
η	viscosity
p	pressure or probability
P	relative pressure
p...	negative logarithm of... (as in pH, pI, pK_a)
r	relative retention or correlation coefficient
R	molar gas constant
R_F	retardation factor
R_M	log $(1/R_F - 1)$
R_s	resolution
ρ	density
ΔS^o	standard entropy change
S/N	signal-to-noise ratio
T	temperature
t	time
t_0	retention time of unretained compound
t_R (t'_R)	(adjusted) retention time
u	mobile phase velocity
V_0	retention volume of unretained compound

V_R (V'_R)	(adjusted) retention volume
w_b	peak width at base
w_h	peak width at half height

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