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TESE DE DOUTORADO

Purificação e caracterização parcial de uma lectina presente no soro do peixe tilápia (*Oreochromis niloticus*): atividade imunomodulatória em esplenócitos de camundongos

CYNARHA DAYSY CARDOSO DA SILVA

Recife-PE

Julho / 2012

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco como pré-requisito para a obtenção do título em Doutor em Ciências Biológicas.

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Dedico este trabalho aos meus pais,
Paulo Fernando e Iolanda Cardoso,
pelo infinito apoio e amor
a mim concebidos.

“É preciso que eu suporte duas ou três lagartas,
se eu quiser conhecer as borboletas”

Antoine de Saint-Exupéry

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SUMÁRIO

RESUMO

As lectinas são um grupo de proteínas que se ligam especificamente a carboidratos e aglutina diferentes células através da ligação a glicoconjugados da superfície celular. A propriedade de ligação a carboidratos determina a classe da lectina que é promovida pelo domínio de reconhecimento de carboidratos (CRD). O estudo com lectinas de peixes propriedade, funções e eventos biológicos foi apresentado em forma de artigo de revisão. A lectina do soro de tilápia do Nilo (*Oreochromis niloticus*), OniL, foi parcialmente purificada e caracterizada. A pré-purificação foi realizada por precipitação com sulfato de amônio (fração 20-40%), F2, seguida de diálise, atividade hemaglutinante e inibição da atividade hemaglutinante. A fração foi cromatografada em matriz de afinidade (Con A-Sepharose 4B) e eluída com metil-α-D-mannopyranosideo (200 mM) em TBS seguida de diálise para avaliar suas características bioquímicas como: especificidade a caboidratos, teste de temperatura e íons, SDS-PAGE e PAGE. Nos ensaios imunológicos, *in vitro*, foram analisados índices de citotoxidade e proliferação, produção de citocinas, viabilidade celular e tipo de resposta imune em culturas de esplenócitos de camundongos BALB/c. No ensaio de citotoxidade, seis concentrações (100, 50, 25, 10, 5 e 1 µg/mL) de OniL foram analisadas por 24 h; saponina foi empregada como um controle positivo; para avaliar a atividade proliferativa, células foram tratadas por 72 h com OniL (2,5, 5 e 10 µg/mL) ou Con A (2,5 µg/mL) comparando com o controle; a citotoxidade e proliferação dos compostos foi determinada comparando o percentual de incorporação da [^{3}H]-timidina como indicador de viabilidade celular usando radiação beta counter. A produção de citocinas (IL-2, IL-6, IL-10 e IFN-γ) foi determinada por ELISA; sobrenadantes de cultura não tratadas foram considerados controles; as culturas foram estimuladas durante 24, 48, 72 h e 6 dias com concentrações de 10 µg/mL de OniL e 2,5 µg/mL de Con A, este último tratamento considerado o controle positivo. A morte celular, analisada por citometria de fluxo, foi verificada com marcadores Anexina V-FITC e Iodeto de Propídeo. Os esplenócitos foram estimulados por 24 e 48 h com OniL (10 µg/mL) e Con A (2,5 µg/mL); células marcadas com Anexina-FITC (negativa) e Iodeto de propídeo (positivo) foram consideradas necróticas; Anexina-FITC (positiva) e Iodeto de Propídeo (negativa), apoptóticas. O método colorimétrico de Griess avaliou a concentração de nitritos dos sobrenadantes de culturas tratadas com OniL (10 µg/mL) e Con A (2,5 µg/mL) nos tempos de 24, 48, 72 h e 6 dias de incubação. F2 e OniL apresentou atividade hemaglutinante específica de 330,3 e 94,7, respectivamente. A lectina purificada apresentou um rendimento de 31,1%; OniL, aglutina eritrócitos de coelho (título, 64^{-1}) e eritrócitos humanos A, B, AB e O (título, 8^{-1}, 64^{-1}, 4^{-1} e 32^{-1}, respectivamente); a atividade hemaglutinate foi detectada numa faixa de pH entre 7,0 e 11,0 e foi completamente preservada após o aquecimento de 10 min a 25, 30, 40, 50 e 60 °C, porém a atividade foi completamente abolidas após 70 °C. A adição de um agente quelante de Ca^{2+}, EDTA, diminuiu a atividade revelando que OniL é uma lectina cálcio-dependente. O peso molecular foi de 17 kDa na SDS-PAGE em condições não redutoras; e 11 e 6,6 kDa na presença de uma agente redutor a proteína apresentou duas subnidades ligadas por pontes disulfeto; OniL apresentou-se, através de PAGE, como uma proteína ácida com banda única de polipeptídeo. Os ensaios imunológicos revelaram que OniL não é citotóxica para esplenócitos de camundongos e induziu alta produção de citocinas em relação ao controle: IFN-γ (24 h), IL-2 e IL-6 em todos os tempos analisados. Porém, a produção de IL-10 e concentrações de nitritos foram baixas quando comparadas com o controle; OniL apresentou atividade proliferativa em relação ao controle para todas as concentrações e não induz morte celular significativa nos tempos analisados. A lectina purificada do soro de tilápia (*Oreochromis niloticus*) é manose-específica, não apresenta citotoxidade para esplenócitos, com atividade imunomoduladora induzindo produção de citocinas para diferenciação e proliferação de células preferencialmente Th1 CD4+, assim, esta proteína pode ser utilizada como um potente agente mitogênico em mamíferos.

Palavras-chave: *Oreochromis niloticus*, lectin, purificação, atividade imunomodulatória, citocina, proliferação

ABSTRACT

Lectins are a group of sugar-binding proteins that specifically recognize carbohydrate structures and agglutinate various cells through binding to cell surface glycoconjugates. The carbohydrate binding property used to determine lectin class is promoted by the carbohydrate recognition domain (CRD). The study of fish lectin property, functions and biological events was presented in a review article. The lectin from Nile tilapia (*Oreochromis niloticus*) serum, OniL, was purified and characterized partially. The pré-purification was by ammonium sulfate precipitation (fraction 20-40%), F2, followed by dialysis, hemagglutinating activity and hemagglutinating activity inhibition. The fraction was chromatographed on affinity matrix (Con A-Sepharose 4B) and eluted with methyl-α-D-mannopyranoside (200 mM) in TBS followed by dialysis to evaluate their biochemical characteristics as: cabohydrates specificity, temperature and ions test, SDS-PAGE and PAGE. Immunological assays, *in vitro*, cytotoxicity and proliferation levels were analyzed, cytokine production, cell viability and immune response type in splenocytes cultures from mice BALB/c. In cytotoxicity assay, six concentrations (100, 50, 25, 10, 5 and 1 µg/mL) were evaluates in 24 h; saponin was used as a positive control; to assess proliferative activity, cells were treated for 72 h with OniL (2.5, 5 and 10 µg/mL) or Con A (2.5 µg/mL) comparing with control; proliferation and cytotoxicity of the compounds were determined by comparing the percentege of [^{3}H]-thymidine incorporation as an indicator of cell viability using beta-counter radiation. Cytokine production (IL-2, IL-6, IL-10 and IFN-γ) was measured by ELISA; untreated culture supernatants were controls considered; cultures were stimulated for 24, 48, 72 h and 6 days at OniL concentrations (10 µg/mL) and Con A 2.5 µg/mL, the latter treatment considered positive control. Cell death analyzed by flow cytometry, was evaluated with Annexin V-FITC and propidium iodide markers. Splenocytes were stimulated for 24 and 48 h with OniL (10 µg/mL) and Con A (2.5 µg/mL); cells FITC-Annexin (negative) and propidium iodide (positive) labeled were considered necrotic; Annexin-FITC (positive) and propidium iodide (negative), apoptotic. Griess colorimetric method evaluated nitrites concentration of the supernatants of treated cultures with OniL (10 µg/mL) and Con A (2.5 µg/mL) at 24, 48, 72 h and 6 days of incubation. F2 and OniL specific hemagglutinating activity showed 330.3 and 94.7, respectively. The purified lectin showed a yield of 31.1%; Onil, agglutinates rabbit erythrocytes (title, 64^{-1}) and human erythrocytes A, B, AB and O (title, 8-1, 64^{-1}, 4^{-1} and 32^{-1}, respectively); hemaglutinate activity was detected between a pH range of 7.0 and 11.0 and was fully preserved after heating for 10 min at 25, 30, 40, 50 and 60 °C, however it was completely abolished after 70 °C. Addition chelating agent EDTA diminished activity revealing that OniL is a calcium-dependent lectin. The molecular weight was 17kDa by SDS-PAGE under nonreducing conditions; and 11 kDa and 6.6 in the presence of a reducing agent, showing two subunits linked by disulfide bonds; PAGE showed that OniL is acidic protein with a polypeptide unique band. The immunological assays revealed that OniL was not cytotoxic mice splenocytes and induced cytokines production high compared to control: IFN-γ (24 h), IL-2 and IL-6 at all times analyzed. However, the IL-10 production and nitrite concentrations were low in comparison with control; OniL showed proliferative activity compared to control at all concentrations and does not induce significant cell death in the times evaluated. Lectin purified tilapia (*O. niloticus*) serum is mannose-specific, showed no cytotoxicity to mice splenocytes, with immunomodulatory activity inducing cytokines production for differentiation and proliferation cell Th1 CD4+ preferentially, thus, this protein can be used as a mitogen agent potent and active in mammals.

Keywords: *Oreochromis niloticus*, lectins, purification, immunomodulatory activity, cytokine proliferation.

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Figure 5: **Schematic representation of immunomodulatory mechanism from OniL splenocyte proliferation.** OniL recognized glycans expressed on the splenocyte surface by Carbohydrates Recognition Domain (CRD) specific site and proliferative activity mediated by high levels of IL-2, IL-6, IFN-γ and low IL-10 production that are essential cytokines for differentiation of Th cell to Th1 induces effective immunomodulatory response. The mitogenic cytokines released by interaction between OniL and splenocytes did not promote cell damage. The figure was based on previous knowledge [19, 8, 6, 12] and present results

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LISTA DE ABREVIATURA

APCs – Células apresentadoras de antígeno

BlL - Lectina de Veneno de *Bothrops leucurus*

BmoLL – Lectina da Folha de *Bauhinia monandra*

BmoRoL – Lectina de Raízes secundárias de *Bauhinia monandra*

Ca^{2+} - Cálcio

ClaveLL – Lectina do Líquen de *Cladonia verticillaris*

CMoL – Lectina de Semente de *Moringa oleifera*

ComaSeL – Lectina do Soro de *Colossoma macropomum*

Con A - Concanavalina A

Cramoll 1,4 - Lectina de Sementes de *Cratilya mollis*

CrataBL – Lectina da Casca de *Crataeva tapia*

CRD - Domínio de Reconhecimento a Carboidrato

CTL – Lectina tipo C

CTLD – Domínio com a afinidade a lectina tipo C

EDTA - Ácido Etilenodiamino Tetra-acético

ELISA – *Enzyme-Linked Immunosorbent Assay*

EROs – Espécies Reativas de Oxogênio

F2 – Fração 2

FITC - Isotilcianato de Fluoresceína

HA – Atividade Hemaglutinante

HAI – Inibição da Atividade Hemaglutinante

I-BSI – Lectina de *Bandeiraea simplicifolia*

IFN-γ – Interferon gama

IgM – Imunoglobulina M

II-RCA - Lectina de *Helix pomatia e Ricinuscommunis*

IL-10 - Interleucina-10

IL-12p40 – Interleucina 12p40

IL-17 - Interleucina-17

IL-17F- Interleucina-17F

IL-2 – Interleucina-2

IL-22 - Interleucina-22

IL-4 - Interleucina-4

IL-5 - Interleucina-5

IL-6 – Interleucina-6

I-RCA I – Lectina de *Ricinus communis*

kDa - Kilodaltons

MASP – Serino Protease Associada à Membrana

MBL – Lectina que se liga a manose

MHC classe II – Complexo Principal de Histocompatilidade classe II

MuBL – Lectina da Casca de *Myracrodruon urundeuva*

MuHL – Lectina do Cerne de *Myracrodruon urundeuva*

MuLL – Lectina da Folha de *Myracrodruon urundeuva*

NK – Células matadoras natural

NO – Óxido Nítrico

OfiL – Lectina de Cladônia de *Opuntia ficus* indica

OniL – Lectina *Oreochromis niloticus*

PAGE - Eletroforese em Gel de Poliacrilamida

PAMPs - Pradrões moleculares associados aos patógenos

pH – Potencial Hidrogeniônico

PHA - Lectina de *Phaseolus vulgaris*

PI – Iodeto de Propídeo

Ppel – Lectina da Semente de *Parkia pendula*

PpyLL – Lectina da Folha de Phthirusa pyrifolia

PRRs - Receptores de reconhecimento padrão

RBL – Lectina que se liga a L-rhamnose

RcaL – Lectina do soro de *Rachycentron canadum*

RPM – Rotações por Minuto

RT-PCR – Reação em cadeia da Polimerase – Trancriptase Reversa

ScLL – Lectina de *Synadenium carinatum*

SDS – Dodecil sulfato de sódio

SDS–PAGE - Eletroforese em Gel de Poliacrilamida com Dodecil Sulfato de Sódio

SejaBL – Lectina da Casca de *Sebastiania jacobinensis*

SHA –Atividade Hemaglutinante Específica

Th0 – Células T auxiliares

Th1 – Células T *helper* 1

Th2 - Células T *helper* 2

Th17 – Células T *helper* 17

TLR – Receptores *Toll-like*

TNF-α – Fator necrose tumoral-alfa

WGA - Lectina de *Wheatgerm agglutinin*

1 – INTRODUÇÃO

1.1 Generalidades do peixe Tilápia (*Oreochromis Niloticus*, Linaeus, 1766)

A tilápia (*O. niloticus*), nativo da África principalmente no Rio Nilo, é o peixe exótico mais produzido na aqüicultura brasileira com 140 mil toneladas ao ano (KUBITZA, 2011). Possui grande importância aquícola e econômica devido à elevada qualidade nutricional e rendimento do filé, rusticidade, resistência ao manejo, baixo nível trófico, apresentando crescimento rápido e uma excelente adaptação ao confinamento (BOYD et al., 2006). A tilápia nilótica é predominantemente herbíboro, de água quente, suportando muito bem as variações de salinidade e de temperatura, podendo ir de 12 a 30 °C (FUJIMURA & OKADA, 2007).

Estudos relacionados têm sido cada vez mais expressivos na área de Fisiologia/Morfologia observando o comportamento, crescimento e desempenho dos alevinos (ANDRADE et al., 2004) na endocrinologia, abordando a importância do hormônio do crescimento (MORI et al., 2007; MARTINEZ et al., 2012) na reprodução, descrevendo os estágios de desenvolvimento (FUJIMURA & OKADA, 2007), fertilização, maturação e desenvolvimento embrionário (DONG et al., 2004) e sistema imunológico (ACOSTA et al., 2008; ARGAYOSA e LEE et al., 2009).

Lectinas de peixes têm sido isoladas de soro, plasma, muco da pele e ovos para demonstrar a importância imunológica nesses organismos (DUTTA et al., 2005). O sistema imunológico de peixes envolve a resposta imune adquirida e a inata. Os peixes teleósteos apresentam características únicas, bem como dependência de temperatura para imunidade adquirida e algumas limitações em diversidade de anticorpo sintetizando apenas a IgM de todas as imunoglobulinas conhecidas (MAGNADÓTTIR et al., 2006; TSUTSUI et al., 2006). As funções imunes inatas podem ter um papel mais importante nesses animais que em outros vertebrados homeotérmicos, bem como aves e mamíferos (KUBITZA e KUBTIZA, 2004; TSOI et al., 2004).

1.2 Lectinas: considerações gerais

O termo Lectina criado por William Boyd, em 1954, vem da palavra latina" legere ", que significa "para selecionar". De uma forma mais abrangente, as lectinas são designadas como proteínas ou glicoproteínas que aglutinam células reconhecendo açúcares específicos com alto grau de especificidade através de pelo menos um domínio não catalítico (SHARON e LIS, 2007; SITOHY et al., 2007; LAM & NG, 2011). As lectinas têm habilidade de aglutinar células como eritrócitos (hemaglutinação), linfócitos e bactérias e ainda precipitam glicoconjugados (CORREIA e COELHO, 1995). As lectinas se ligam não somente a oligossacarídeos nas células (Figura 1), mas também a glicanos livres incluindo monossacarídeos (RABINOVICH et al., 2007).

Os carboidratos são as moléculas orgânicas mais abundantes encontradas na natureza e todos os organismos sintetizam e metabolizam (WADE, 1999). Isso vem ressaltar a importância dos carboidratos nos mais diversos sistemas biológicos e que tem despontado como um dos fatores principais na glicobiologia, juntamente com o estudo das lectinas para tentar compreender, por exemplo, aspectos glicobiológicos de doenças (GHAZARIAN et al., 2010).

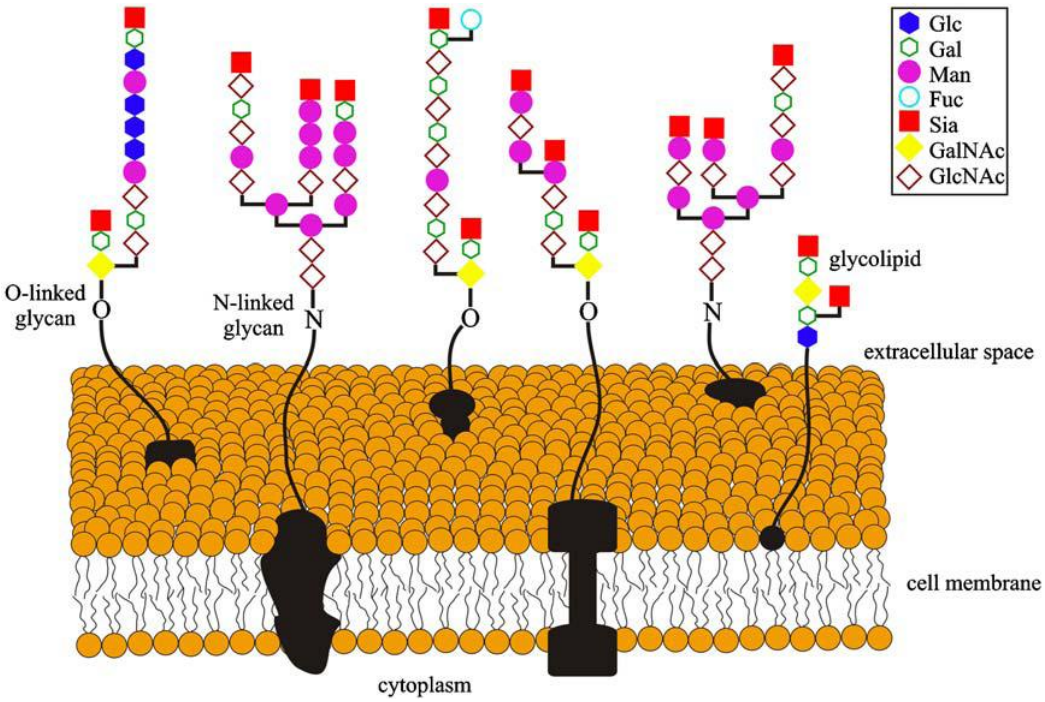

Figura 1: Esquema representativo demonstrando os diversos carboidratos expressos na membrana celular reconhecidos por lectinas. Fonte: Ghazarian et al., 2010.

As lectinas são amplamente distribuídas na natureza incluindo bactérias, fungos, plantas, vertebrados e invertebrados; podem ser solúveis ou ligadas a membranas (LIS & SHARON, 1986; JIMBO et al., 2007). Estas proteínas podem ser usualmente detectadas pela atividade hemaglutinante de uma variedade de eritrócitos. A capacidade hemaglutinante está limitada ao evento de ligação aos resíduos de aminoácidos que compõem as glicoproteínas e glicolipídeos da superfície da membrana celular chamados de Domínio de Reconhecimento a Carboidrato (*Carbohydrate Recognition Domain-CRD*) (SHARON e LIS, 2004; TSUITSUI et al., 2006a).

O CDR tem possui um padrão invariável e com resíduos de aminoácidos altamente conservados (RUSSEL e LUMSDEN, 2005). Essas características vêm sendo comprovadas em estudos estruturais de lectinas (SHARON e LIS, 2004), como exemplo, a estrutura tridimensional do complexo ECoRL-ligante da lectina da árvore Mulungu *(Erithrina cristagalli)* (SVENSSON et

al., 2002), específica para galactose e N-acetilgalactosamina apresenta uma constelação de três resíduos de aminoácidos, um ácido aspártico, uma asparagina e um aromático que foi essencial para ligação da lectina (ADAR & SHARON, 1996; MORENO et al., 1997; ADAR et al., 1998; LEMIEUX et al., 2000)

1.3 Purificação e caracterização de lectinas

1.3.1 Detecção e especificidade

O ensaio de atividade hemaglutinante é realizado para detectar a presença de lectinas numa determinada amostra. Neste ensaio, uma amostra contendo proteínas é incubada com eritrócitos "frescos" ou tratados enzimáticamente (tripsina, papaína ou neuroaminidase) ou quimicamente (glutaraldeído ou formaldeído) que aumenta a sensibilidade das células com a exposição dos carboidratos de superfície aonde a lectina se liga para formar uma "malha" de hemaglutinação, podendo ser visualisado a olho nu. A ausência de lectina na amostra resulta em precipitação dos eritrócitos. (CORREIA E COELHO, 1995; MO et al., 2000; COELHO et al., 2012).

Os eritrócitos expressam diferentes carboidratos na membrana celular e por isso muitas lectinas interagem ou não com determinados eritrócitos, o que caracteriza a especificidade (KENNEDY et al., 1995). Para investigar a especificidade destas lectinas é realizado um ensaio de inibição da atividade hemaglutinante com os diferentes carboidratos (mono/oligossarídeos) ou glicoproteínas; a ligação da lectina ao carboidrato específico não forma malha de hemaglutinação (COUTIÑO-RODRÍGUEZ et al., 2001; OKAMOTO et al., 2005; COELHO et al., 2012).

1.3.2 Processos de purificação

Inicialmente, o emprego de agentes químicos é necessário para o isolamento de lectinas, como solventes orgânicos (acetona) (MEDEIROS et al., 2010), ou *salting out* (sulfato de amônio) (SILVA et al., 2012; CARVALHO et al., 2012) podem ser utilizados para precipitação destas proteínas. Um grande número de lectinas vem sendo purificadas de diferentes organismos sob diferentes técnicas de purificação (LAM & NG, 2011).

Alguns critérios são levados em consideração para isolar quantidades em miligramas (mg) de lectinas como o teor no material e o uso de um método simples de purificação (CASTILLO-VILLANUEVA et al., 2007). Métodos cromatográficos utilizados na purificação tais como a cromatografia de troca iônica, gel filtração e afinidade (LAM & NG, 2011) são considerados os mais poderosos métodos para purificação e tem como base diferenças na carga, tamanho e afinidade de ligação; durante o processo, estas proteínas são ligadas em material sólido poroso com

propriedades químicas apropriadas (fase estacionária) mantido em uma coluna, pela qual é percolada uma solução tampão (fase móvel) (NELSON & COX, 2011).

A cromatografia de troca iônica é realizada de acordo com a carga da proteína em um determinado pH, esta é adsorvida na matriz, um polímero sintético, que contém grupos carregados ligados. O rompimento da interação da lectina com a matriz é dependente da mudança gradual dos valores do pH ou sal na fase móvel (Figura 2A) (NELSON & COX, 2011)

A cromatografia de gel filtração ou exclusão molecular separa biomoléculas de acordo com o tamanho molecular e se difundem através da uma matriz inerte porosa que possui grânulos de polímeros com cavidades de diferentes tamanhos. As moléculas maiores passam livremente através da coluna, pois não podem entrar nas cavidades aparecendo nas frações iniciais. As moléculas menores passam pelas cavidades tendo que percorrer um caminho maior através da coluna. Assim, as moléculas são separadas por ordem decrescente de peso molecular. (Figura 2B) (HAGEL, 1998; NELSON & COX, 2011).

O método de cromatografia de afinidade tem sido utilizado durante décadas como um meio seletivo para a purificação e análise de produtos químicos em sistemas biológicos (SCHIEL & HAGE, 200). A cromatografia de afinidade é um processo em que uma proteína ou outro agente bioativo é imobilizado sobre um suporte sólido onde as proteínas que têm afinidade a esse suporte ficam retidas (JONKER et al., 2011). Esta técnica é bastante utilizada para purificação de lectinas as quais se ligam a matriz acoplada a carboidratos ou glicoproteínas de acordo com a especificidade. Essas moléculas são eluídas com carboidratos ou glicoproteínas em solução, por exemplo a matriz Sephadex retém lectinas com especificidade a glicose, D-frutose, D-manose, sucrose e metil-β-D-glicopiranosídeo; a quitina é específica para N-acetil-D-glicosamina e oligassacarideos (Figura 2C) (KENNEDY et al., 1995).

Lectinas de plantas já foram imobilizadas em matrizes inertes e usadas em cromatografia de afinidade para purificação de glicoprotéinas, por exemplo a lectina Concanavalina A (Con A) (*Canavalia ensiformis*), uma metaloproteína que requer íons de metal incluindo manganês e cálcio para ligação (SUMMER & HOWELL, 1936). A Con A imobilizada em Sepharose tem especificidade a resíduos manosil e glicosil de polissacarídeos e glicoprotéinas, grupos hidroxila na posição do carbono 3, 4 e 6 de anéis D-glucopiranosil ou D-manopiranosil que também podem ser essenciais para ligação (YAHARA e EDELMAN, 1972). A tabela 1 mostra algumas lectinas imobilizadas e seus ligantes correspondentes utilizados em purificação de glicoproteínas.

Métodos eletroforéticos são utilizados para caracterizar estruturalmente as lectinas como também para revelar o grau de pureza das mesmas. A eletroforese em gel de poliacrilamida (PAGE) pode ser realizada usando um gel contendo Sulfato-sódico de Dodecila (SDS), β-mercaptoetanol ou ditiotreitol (DTT) (condições redutoras) que revela o grau de pureza, a composição, a massa

molecular de subunidades (REYNOSO-CAMACHO et al., 2003; PAIVA et al., 2006) e através de coloração específica, a natureza glicoprotéica (COELHO & SILVA, 2000; FENG et al., 2006).

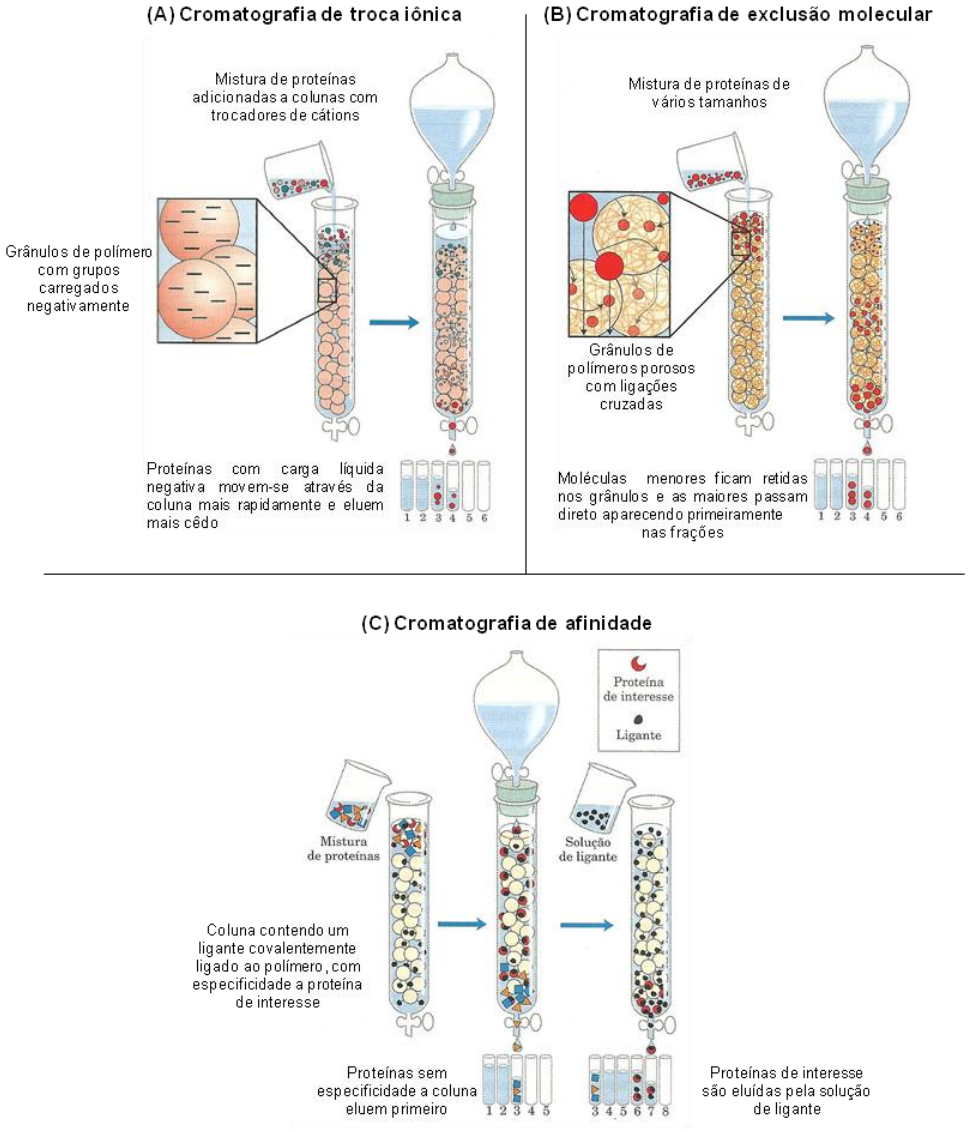

Figura 2: Esquema representativo dos tipos de cromatografia. (Nelson & Cox, 2011).

1.4 Importância e aplicações biológicas de lectinas

Estudo com lectinas se intensificaram quando se constatou que estas proteínas são extremamente valiosas para investigação de açúcares de superfíce celular avaliando o papel no crescimento, diferenciação e interação entre células bem como processos biológicos normais e

patológicos, assim, as lectinas repercutem como marcadores da identidade de células, um tema de grande importância para glicobiologia moderna (SHARON, 2007).

Tabela 1: Lectina imobilizadas para purificação de glicoproteínas e seus ligantes específicos.

Lectina	**Ligantes específicos**
Con A	*α-D-manosil e α-D-glicosil*
Lectina Lentil	*α-manopironaosídeo*
I-BSI *(Bandeiraea simplicifolia)*	*N-acetil-D-galactosaminil*
II-RCA *(Helix pomatia e Ricinuscommunis)*	*N-acetilgalactosamina*
WGA (wheatgerm agglutinin)	*N-acetil-glicosamina*
Jacalina	*α-galactopiranosídeos e metil-α-D-galactopiranosídeo*
I-RCA I *(Ricinus communis)*	*β-D-galactosil*
Cramoll 1,4 *(Cratillya mollis)*	*Glicose/manose*

Kennedy et al., 1995

Lectinas endógenas estão envolvidas numa variedade de processos biológicos (MODY et al., 1995; DRICKAMER, 1996). Estas são encontradas na forma solúvel ou ligadas às membranas (DRICKAMER, 1988). MBL (*Mannose Binding Lectin)* e fucolinas são lectinas que ativam a via das lectinas do sistema complemento ligando-se a superfície de patógenos opsonizando para facilitar a fagocitose; também regulam funções efetoras como aglutinação, imobilização (RUSSEL e LUMSDEN, 2005), migração e adesão, linfoproliferação, transdução de sinal mediada por células CD4+ e ativação de monócitos e macrófagos, essencial para resposta imune eficaz (PEUMANS & VAN DAMME, 1995; TAMMA et al., 2003; SAVAN & SAKAI, 2006; LEE et al., 2007).

Em nosso Laboratório de Glicoproteínas (Departamento de Bioquímica – UFPE) já foi purificada uma diversidade de lectinas de plantas e animais, e desde então a investigação em diversos sistemas biólógicos vem sendo cada vez mais promissora demonstrando o potencial aplicativo destas proteínas (Tabela 2).

Lectinas com diferentes especificidades foram utilizadas para investigar o padrão de carboidratos do epitélio intestinal de aves através da técnica histoquímica para explicar a ação de microorganismo no local, constatou-se que os glicoconjugados podem agir como sítios de ligação para os microorganismo (POHLMEYER et al 2005). Esta técnica também permite avaliar mudanças no perfil de carboidratos em células transformadas servindo como sondas histoquímicas no auxílio de diagnósticos laboratoriais em processos patológicos (BELTRÃO et al, 2003).

Muitas lectinas utilizadas em estudo histoquímico são conjugadas à moléculas com propriedades enzimáticas, luminescentes ou quimioluminescentes como por exemplo, peroxidase,

isotiocianato de fluoresceína, ésteres de acridinas, criptato de európio (III), entre outros (CAMPOS et al., 2006; SOBRAL et al., 2010; LIMA et al., 2010; BANDEIRA et al., 2011).

Lectinas da aroeira (*Myracrodruon urundeuva*) são ativas e resistentes a enzimas digestivas e bactérias simbiontes presentes no intestino de cupins (*Nasutitermes corniger)* (NAPOLEÃO et al., 2011). Já as lectinas animais purificadas do veneno de serpente (*Bothrops leucurus)* tem atividade antibacteriana, para bactérias partogênicas e citotóxica, para células cancerígenas, respectivamente (NUNES et al., 2011). A lectina purificada do soro do peixe tambaqui (*Colossoma macropomun*) tem atividade antimicrobiana para bactérias *Aeromonas sobria, Aeromonas hidrofila,* e *Edwardsiella tarda* (CARVALHO et al., 2012).

Lectinas também são úteis para caracterizar diferentes estágios de desenvolvimento de Tripanossomatídeos (KENNEDY et al., 1995), atuar como composto citotóxico em *Trypassoma cruzi* (FERNANDES et al 2010), diferenciação de espécies de parasitas (WU et al., 2000), determinação de grupos saguíneos (MATSUI et al., 2001), produção de medicamento inteligentes, atuando em células específicas (CLARK et al, 2000; TORCHILIN et al., 2001) e promover mitogenicidade em linfócitos humanos (MACIEL et al., 2004).

Lectinas de plantas são amplamente estudadas para avaliar mecanismos imunomodulares. Em 1960 se deu a descoberta da primeira lectina do feijão (*Phaseolus vulgaris*), nomeada PHA, com atividade mitogênica (NOWEL, 1960). Diversas lectinas com características distintas e sítio específica já foram aplicadas para avaliar atividade imunomoduladora, linfoproliferação, transdução de sinal para diferenciação de células CD4+ bem como ativação monócitos e macrófagos (TAMMA et al., 2003; MACIEL et al., 2004; LEE et al., 2007; BAINS et al., 2005; MELO et al., 2011).

ConBr (*Canavalia braziliensis*) e KM+ (*Artocarpus integrifolia*), estimula a produção IFN-γ e IL-12p40, respectivamente, promovendo a produção de citocinas em camundongos BALB/c infectados conferindo ação protetora na infecção com *Leishmania* (*L. amazonensis* e *L. major*) (BARRAL-NETTO et al., 1996; PANUNTO-CASTELO et al., 2001); a lectina ScLL (*Synadenium carinatum*) induz produção de IFN-γ, IL-2 e TNF-α, citocinas importantes na diferenciação de células CD4+ em Th1 favorecendo a proteção em camundongos imunizados e desafiados com promastigotas de *L. amazonensis* (AFONSO-CARDOSO et al., 2007).

Cramoll 1,4, lectina do extrato de planta *Cratylia mollis* Mart, é considerada um potente agente imunomodulador, *in vitro,* em cultura de esplenócitos de camundongo induzindo produção de IFN-γ, atividade antinflamatória e supressão de óxido nítrico (MELO et al., 2010b); *in vivo,* induzindo ação proliferativa em cultura de esplenócitos de camundongos inoculados com esta lectina (MELO et al., 2011a).

Tabela 2: Lectinas animais e de plantas purificadas com suas atividades biológicas.

	Lectina	Fonte	Especificidade	Atividades Biológicas
Animais	**BlL** (Nunes et la., 2011)	Veneno de *Bothrops leucurus*	Galactose	Atividade antbacteriana (Nunes et al.,2011); Citotoxidade em células tumorais (Nunes et al., 2012)
	ComaSeL (Carvalho et al., 2012)	Soro de *Colossoma macropomum*	Glicose/ manose	Antimicrobiana (Carvalho et al., 2012)
	OniL (Silva et al., 2012)	Soro de *Oreochromis niloticus*	Glicose/ manose	Proliferativa / imunomoduladora (Silva et al., 2012).
	RcaL (Coriolano et al., 2012)	Soro de *Rachycentron canadum*	Glicose/ manose	Proliferativa / imunomoduladora (Coriolano et al., 2012).
Plantas	**BmoLL** (Coelho e Silva, 2000)	Folhas de *Bauhinia monandra*	Galactose	Inseticida contra *Anagasta kuehniella* (Macedo et al., 2007); Efeito citotóxico e genotóxico negativo (Sisenando et al., 2009). Diagnótico na infecção com Dengue (*Aedes aegypti)* (Andrade et al., 2011).
	BmoRoL (Souza et al., 2011)	Raízes secundárias de *Bauhinia monandra*		Antifúngica e termicida (Souza et al., 2011).
	ClaveLL (Silva et al., 2009)	Líquen de *Cladonia verticillaris*	N-acetil-glicosamina	Inseticida contra *Nasutitermes corniger* (Silva et a., 2009).
	CMoL (Santos et al., 2009)	Semente de *Moringa oleifera*	Galactose	Coagulante (Santos et al., 2009); Inseticida contra *Anagasta kuehniella* (Oliveira et al., 2011).
	Cramoll (Correia & Coelho, 1995)	Sementes de *Cratilya mollis*	Glicose/ manose	Mitogênica (Maciel et al., 2004); Antitumoral (Andrade et al., 2004); Citotóxidade em *Trymanossoma cruzi* (Fernandes et al., 2010); Estudo histoquímico em tecido protático humano (Lima et al., 2010); Imunomoduladora (Melo et al., 2010a); Proliferativa (Melo et al., 2011); Antihelmíntica contra *Schistosoma mansoni* (Melo et al., 2011a); Cicatrizante (Melo et al., 2011b); Antitrombótica e anticoagulante (Silva et al., 2011); Detecção de sorotipos do vírus da dengue (Oliveira et al.,2011); Tratamento de queimaduras de segundo grau (Pereira et al 2012).
	CrataBL (Araújo et a., 2012)	Casca de *Crataeva tapia*	Ovoalbumina	Inseticida contra *Nasutitermes corniger* (Araújo et a., 2012).
	MuHL (Sá et al., 2008)	Cerne de *Myracrodruon urundeuva*	N-acetil-glisocamina	Inseticida contra Nasutitermes corniger (Sá et al., 2008); Larvicida contra *Aedes aegypti* (Sá et al., 2009); Antimicrobiana (Sá et al., 2009b).
	MuLL (Napoleão et al., 2011)	Folhas de *Myracrodruon urundeuva*	N-acetil-glicosamina	Termiticida contra Nasutitermes corniger (Napoleão et al., 2011); Larvicida contra *Aedes aegypty* (Napoleão et al., 2012).
	MuBL (Sá et al., 2009)	Casca de *Myracrodruon urundeuva*	N-acetil-glicosamina	Termiticida contra *Nasutitermes corniger* (Napoleão et al., 2011); Antioxidante e inibição do crescimento de fusarium (Sá et al., 2009a); Larvicida contra *Aedes aegypti* (Sá et al., 2009).
	OfiL (Paiva et al., 2011**)**	Cladônias de *Opuntia ficus* indica	N-acetil-glicosamina	Inseticida contra *Nasutitermes corniger* (Paiva et al., 2011).
	Ppel (Beltrão, 2001)	Semente de *Parkia pendula*	Glisose/ manose	Inibição de cytomegalovirus (HCMV) (Favacho et al., 2007); Marcador histoquímico para tumor meningomesotelial (Beltrão et al., 2003).
	PpyLL (Costa et al., 2010)	Folha de Phthirusa pyrifolia	Caseina	Antibacteriana e antifúngica (Costa et al., 2010).
	SejaBL (Vaz et al., 2010)	Casca de *Sebastiania jacobinensis*		Tratamento da alergia (Vaz et al., 2010); Antifúngica (Vaz et al., 2011).

Em estudos com células cancerígenas as lectinas revelaram que Con A e ConBr têm efeito antiproliferativo em linhagens de células tumorais (leucemina) (FAHEINA-MARTINS et al., 2012). Lectina de esponja do mar (*Cinchyrella apion*) específica para lactose induz morte celular por apoptose em células HeLa (adenocarcinoma cervical humano) (MORAIS et al., 2012; RABELO et al., 2012).

1.5 Sistema imune: uma visão geral

Em humanos, as defesas contra patógenos encontram-se divida em três níveis: barreiras anátomo-fisiológicas, imunidade inata e imunidade adaptativa. As barreiras anatômicas constituem a primeira linha de defesa incluindo pele, mucosa, baixo pH do estômago, lisosimas bacteriolíticas em lágrimas, salivas e outras secreções. A imunidade inata aumenta a proteção oferecida pelas barreiras anátomo-fisiológicas (JANEWAY & MEDZHITOV, 2002; TURVEY & BROIDE, 2010).

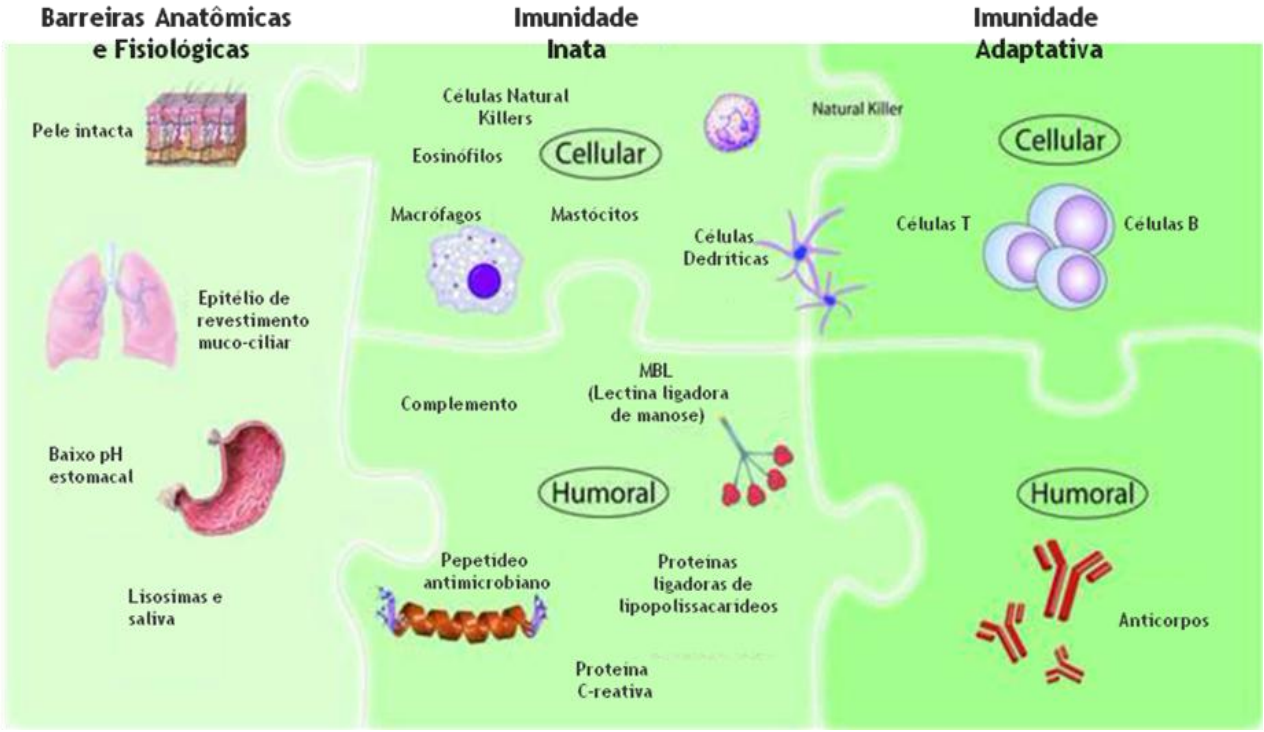

Figura 3: Níveis de defesas contra patógenos em humanos. Fonte: Turvey & Broide, 2010.

1.5.1 Imunidade Inata

O sistema imune inato tem ação imediata em resposta à exposição aos patógenos e depende de receptores invariantes limitados expressos na superfície de células imunológicas para detectar componentes microbianos comuns a esses agentes patogênicos gerando uma resposta inflamatória (TURVEY e BRODY, 2010).

As células que participam do sitema imune inato são neutrófilos, desepenham função fagocítica para destruição inicial dos micróbios; os macrófagos, também têm função fagocítica para destruição eficiente de micróbios e secreção de citocinas; e células natural *killer* (NK), capases de lisar a célula e ativar macrógafos pela liberação de citocina INF-γ (ABBAS et al., 2008).

Os fagócitos, também chamados de células apresentadoras de antígeno (APCs), possuem receptores de reconhecimento padrão (*pattern recognition receptor* - PRRs) ou TLR *(Toll-like receptors)* expressos na superfície e são capazes de reconhecer padrões moleculares (lipídeos, lipoproteínas, protéinas, glicanas e ácidos nucléicos) ditribuídos principalmente na superficie dos patógenos designados PAMPs (*Pathogen-associated Molecular Patterns*) (MEDZHITOV, 2007; KAWAI & AKIRA, 2010). Esses receptores também têm alta afinidade por moléculas de anticorpo, proteínas do sistema complemento e lectina que atuam como opsoninas revestindo os microorganismos contribuindo para fagocitose (ABBAS et al., 2008)

A produção de espécies reativas de oxigênio (EROs), originados do oxigênio molecular, induzida pela enzima fagócito oxidase e óxido nítrico (NO) localizados nos fagolisossomos também contribuem para eliminação de micróbios. EROs e NO são produzidos pela ativação dos receptores TRL *(Toll-like receptors)* por INF-γ que são altamente reativos na oxidação de macrógafos ativados. À mediada que os micróbios são fagocitados, são processados em peptídeos e apresentados às células T para iniciar a resposta imune adaptativa, desta forma, imunidade inata desempenha um papel central na ativação da resposta imunitária adaptativa subseqüente (ABBAS et al., 2008; TURVEY e BRODY, 2010).

1.5.2 Imunidade Adaptativa

O desencadear da resposta imune adaptativa se dá pela apresentação de antígenos processados por células fagocíticas (APCs), macrófagos e células dendríticas, pelo complexo de histocompatibilidade principal (MHC) classe II na superfície de fagócitos. Células T primárias (Th0), também chamadas de células T CD4+, se diferenciam em Th1, Th2 ou Th17 de acordo com o antígeno apresentado (KIDD et al., 2003; SAVAN & SAKAI, 2006).

A diferenciação celular irá desencadear uma cascata de sinalização com a liberação de diferentes tipos de citocinas (interleucinas, fator necrose tumoral, interferons e quimiocinas) originando clones de células efetoras com diferentes funções e ainda ativam macrófagos para eliminação de microorganismos; esse processo é o principal mecanismo da imunidade celular (KIDD, 2003; SAVAN & SAKAI, 2006; KAIKO et al., 2007, (ABBAS et al., 2008).

Citocinas incluem um grande número de fatores solúveis que são importantes reguladores do sistema imune, muitas delas tem a capacidade de se ligarem a carboidratos; são de baixo peso

molecular derivadas de glicoproteínas controlando a comunicação célula a célula durante a resposta imunológica (SAVAN & SAKAI, 2006).

A diferenciação de células Th1 se inicia quando células fagocíticas ativadas pela entrada de patógenos secretam IL-12, indutor essencial da imunidade mediada por célula, promovendo a diferenciação de células Th0 em Th1 e estas, subsequentemente, secretam IFN-γ, principal citocina ativadora de macrófagos o que permite proteção eficaz contra infecções intracelulares por bactérias e vírus. A secreção de IFN-γ inibe a diferenciação e proliferação de células Th2. Th1 ativadas também secretam IL-2 que age de forma autócrina para proliferação celular (KIDD, 2003; SIA, 2005; KAIKO et al., 2007; ABBAS et al., 2008; TAHVANAINEN, 2010; KOYASU & MORO, 2012).

Células Th0 se diferenciam em Th2 pela ação de IL-6, principal mediador secretada por células fagocíticas (APCs) ativadas. IL-4 que é secretada por Th2 agem em células fagocíticas para polarização (ativação) das mesmas para tornar eficiente a eliminação de patógenos. Th2 secretam IL-4 e IL-5 que vão ativar células B regulando a produção de anticorpos IgE específico para helmintos (SIA, 2005; KAIKO et al., 2007; DIENZ & RINCOM, 2009).

IL-10 é também secretada por macrófagos, células dendríticas, células B e vários outros conjuntos de células T CD4+ e CD8+. Possui sinalização autócrina em células dendríticas e pode atuar em células Th1 inibindo a proliferação e produção de citocinas IL-2, IFN-γ, IL-4, IL-5 e TNF-α por conseqüente inibição do MHC classe II (MOORE et al 2001). A figura 3 apresenta resumidamente o mecanismo de diferenciação das células Th0 bem com a secreção de citocinas.

De forma geral, células Th1 regulam a resposta immune celular, enquanto que células Th2, a resposta imune humoral. A cooperação da imunidade inata e adaptativa é essencial para proteção immune contra micróbios invasores (KAIKO et al., 2007).

As células efetoras Th17 foram descobertas nos últimos anos, estas secretam IL-17, IL-17F, IL-6 IL-22 e TNF-α e parecem desempenhar um papel essencial durante inflamação e ativação de neutrófilos no combate à infecções bactérias extracelulares. IL-6 particularmente, também está muito relacionada com a diferenciação das células Th17, estas células regem as respostas inflamatórias mediadas por neutrófilos (KOYASU & MORO, 2012).

Óxido nitric (NO) é um importante mediador da resposta immune (JAMES, 1995; SCHRODER et al., 2004). Este composto é produzido por macrófagos para controlar a proliferação de linfócitos agindo contra microorganismos, parasitas e células tumorais (MONCADA, et al., 2006). Muitos estudos têm demonstrado que o NO é preferencialmente produzido por células Th1 a partir do aumento da produção de INF-γ nestas células conferindo abilidade microbicida similar a de neutrófilos ativados por IFN-γ (SCHRODER et al., 2004; SILVA et al., 2012).

A apoptose é um processo no qual a célula dirige sua própria morte ativando vias intrínsecas (XAUS et al., 2001). Em macrófagos, a apoptose previne a colonização de patógenos intracelulares. Em contrapartida, muitos patógenos induzem a morte da célula hospedeira através da secreção de toxinas em macrófagos (TAMURA et al., 1997). Estudos revelaram que IFN-γ protege contra apoptose em células tratadas com esta citocina (BERNABEI et al., 2001)

Mecanismos estimulatórios induzidos por antígenos ou outros composto podem promover uma maior ativação de células T através do acúmulo de espécies reativas de oxigênio (EROs) e cálcio dependendo do tipo celular ou do estímulo, assim, a célula pode ser induzida a morte celular (SKULACHEV, 2006; MELO et al., 2010a)

Macrófagos eliminam bactérias, vírus, protozoários, helmintos e fungos primeiramente pela produção de EROs via indução da enzima NADPH oxidase (ROOS et al., 1992). EROs tem baixo peso molecular e facilmente penetra na parede celular microbiana causando destruição de patógenos. A produção de EROs acontece aparentemente em torno de 1 h após o estímulo (SCHODER et al., 2004).

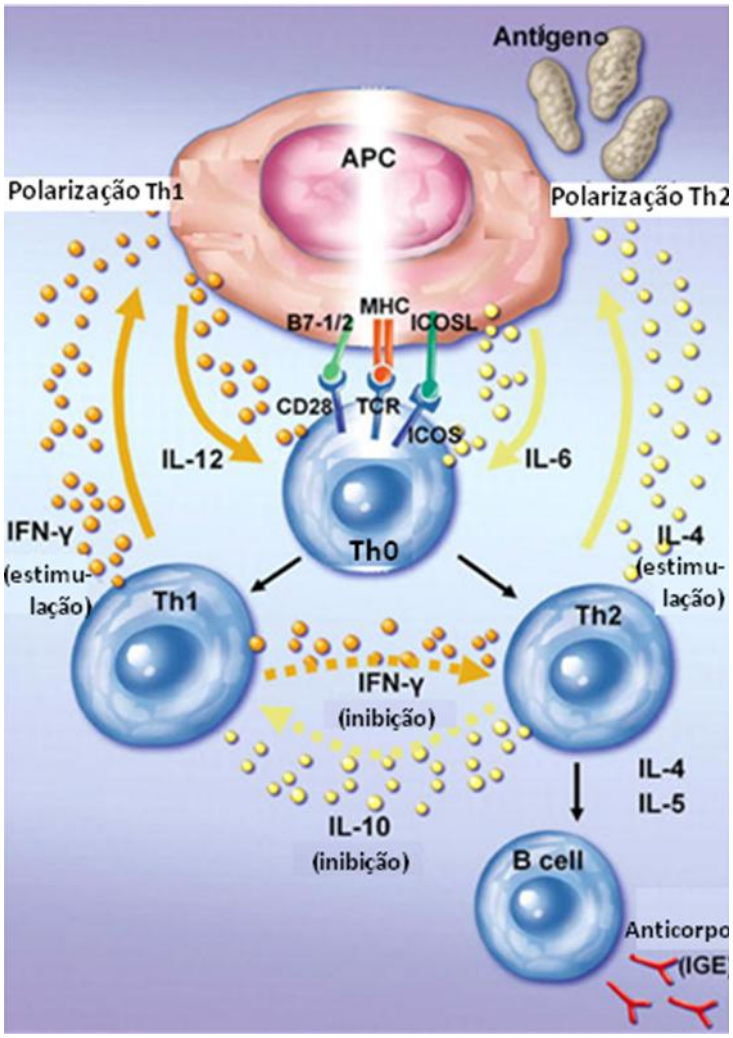

Figura 3: Diferenciação de células Th0 em Th1 / Th2 e mecanismo de ação das citocinas. Fonte: Sia, 2005.

O sistema complemento é essencial para defesa inicial contra patógenos e quando ativado conduz uma cascata proteolítica eficiente; a via das lectinas do sistema complemento se dá pela interação de lectinas ligadoras de manose (MBL - *Mannose Binding Lectin*) e fucolinas localizadas no plasma e superfície celular, reconhece carboidratos conservados nas superfíce dos patógenos, os PAMPs (WALPORT, 2001a; WALPORT, 2001b), pela ativação por serino-proteases (MASP) para eliminação de patógenos (TURNER, 1996; GORDON, 2002; RICKLIN et al., 2010). A ligação da lectina na superfície desses agentes patogênicos induz opsonização facilitando a fagocitose e lise do patógeno bem como a geração de uma resposta inflamatória clássica pela produção de moléculas pró-inflamatórias (WALPORT, 2001; DUNKELBERGER & SONG, 2010).

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3 - OBJETIVOS

3.1 Geral

Investigar a variedade de lectinas de peixes, purificar e caracterizar uma lectina presente no soro de tilápia (*Oreochromis niloticus*) e avaliar, *in vitro*, a atividade imunomodulatória em esplenócitos de camundongos.

3.2 Específico

- Compilar informações relacionadas às funções imunológicas, aplicações biológicas, classificação de lectinas de peixes;
- Obter e pré-purificar o soro da tilápia (extrato bruto) por fracionamento com sulfato de amônio;
- Detectar uma lectina através da atividade hemaglutinante (AH) e inibição da atividade hemaglutinante (IAH) das diferentes frações obtidas;
- Purificar uma lectina por processos cromatográficos à partir da fração de maior rendimento;
- Caracterizar uma fração eluída através da inibição da AH com carboidratos e glicoproteínas, estabilidade térmica, influência de íons e eletroforese em gel de poliacrilamida;
- Avaliar os índices de citotoxidade e proliferação de diferentes concentrações de lectina em cultura de esplenócitos de camundongos;
- Investigar atividade imunomoduladora induzida pela lectina observando os níveis de produção de citocinas (IL-2, IL-6, IL-10, IFN-γ) e óxido nítrico;
- Identificar o tipo de resposta imune celular (Th1 – Th2).

4 - CAPÍTULO I

4.1 Artigo Científico 1

FISH LECTINS: A BRIEF REVIEW

Capítulo de livro aceito para publicação em: ***Advances in Zoology Research***

---------- Forwarded message ----------
From: **Aimee Himelfarb** <Aimee@novapublishers.com>
Date: 2012/6/25
Subject: Re: Review Chapter Abstract (Environment Research Updates)
To: Luana Cassandra Breitenbach Barroso Coelho <lcbbcoelho@gmail.com>

Dear Dr. Coelho:

With regard to your second chapter entitled "**Fish Lectins: A Brief Review**", please note, we could include this manuscript in the next volume of our continuing series "Advances in Zoology Research. Volume 5". If you are agreeable to the placement of your chapter therein, please send the fully completed submission in either MSW word or Latex format directly to: aimee@novapublishers.com at your earliest convenience. This title will start compilation in late August, 2012...

Kind regards,

Aimee Himelfarb
aimee@novapublishers.com

FISH LECTINS: A BRIEF REVIEW

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ABSTRACT

Studies on fish lectins in recent decades are motivated to unravel the role of these proteins in the innate immune system of these vertebrates. Lectins are carbohydrate-binding proteins found in viruses, prokaryotes and eukaryotes. These proteins can agglutinate cells, and precipitate polysaccharides, glycoprotein or glycolipids mediating different biological processes such as cell-cell interactions, glycoprotein traffics and clearance, induction of apoptosis, antibacterial and antiviral activity, mitogenic activity and antitumor activity. Lectins are believed to mediate pathogen recognition in fish immune system with important roles in innate immune response. In addition to the function of defense against microorganisms, there is evidence that these fish lectins have also an important role in fertilization, embryogenesis and morphogenesis. Mitogenic and antiproliferative activities have also been identified in various fish species. The classification of animal lectins is diversified and is based on structural, functional and evolutionary studies. Currently there are a very large number of animal lectin families that have already been identified; most of them occur in fish such as galectins, C-type, Pentraxins, Calnexins, I-type, F-type and L-rhamnose. The purification, structural and functional characterization of fish lectins has been approached; most of these studies reinforce the role of lectins in innate immune system in these animals. This review deals with different families of animal lectins found in body fluids, cells and tissues of fish. Some properties, functions, and biological events of such proteins will also be presented. The analysis of lectin roles are mainly related to the innate immune response in fish of economic importance and certainly will contribute to increasing knowledge on the subject and generate appropriate technologies to improve the development of aquaculture.

Key words: Fish lectin; Classification; Lectin purification; Lectin function.

1.INTRODUCTION

Lectins are groups of proteins characterized by their ability to bind carbohydrates with considerable specificity (Nilsson, 2007). These proteins are present in virus, bacteria, cyanobacteria and yeast (Loris, 2002; Loris, 2009; Veelders et al., 2010; Huskens et al., 2010; Xu et al., 2012), plants and animals (Ferreira et al., 2011; Nunes et al., 2012). Lectins can agglutinate cells and precipitate polysaccharides, glycoprotein or glycolipids (Lis and Sharon, 1998; Zhang et al., 2009). These properties enable lectins to mediate different biological processes such as cell-cell interactions (Gabor et al., 2004), induction of apoptosis (Perillo et al., 1995; Vervecken et al., 2000), cytotoxic activity (Kawsar et al., 2010; Silva et al., 2012), antibacterial and antiviral activity (Araújo et al., 2012; Napoleão et al., 2012), antiproliferative activity for cancer cells (Bah et al., 2011), mitogenic activity (Maciel et al., 2004; Bah et al., 2011) and antitumor activity (Andrade et al., 2004). Lectins have been investigated in marine bioresources by their various pharmacological applications to develop new drugs (Ogawa et al., 2011).

The lectin term, from Latin *lectus*, which means chosen, was introduced by William Boyd and Elizabeth Shapleigh in 1954 reflecting etymologically their property to agglutinate selectively blood cell groups (Boyd and Shapleigh, 1954). Initially the study of lectins was focused only on plants, since it was believed that these proteins occurred only in the plant kingdom. The first agglutinin was identified in the crude extract from *Ricinus communis* by Stillmark, in 1888, when searching effects of plant toxicity (Sharon and Lis, 1989, 2004). The first plant lectins have been isolated and characterized from *Phaseolus vulgaris* (Takahashi et al., 1967), *Phaseolus lunatus* (Galbraith and Goldstein, 1970; Gould and Scheinberg, 1970) and *Canavalia ensiformis* (Lloyd, 1970). In the late twentieth century the field of glycobiology had a major breakthrough due to the recognition of the lectin presence in animal tissues by the scientific community (Sharon, 2008). Probably the first mammalian lectin was identified by Stockert et al. (1974) in rabbit liver and, also, the first serum lectin was identified by Ashwell and Morell (1974) as the hepatic asialoglycoprotein receptor.

Animal lectins provided great advances in the field of Glycobiology, decoding the glycocode and contributing to the development of various areas of basic and applied bioscience (Sharon, 2008; Varki et al., 2009; Kumar and Mittal, 2011).

The association of lectins with carbohydrates is a primary event in some biological processes such as infection and metastasis (Bouwman et al., 2006; Rambaruth and Dwek, 2011). In addition to this important role in cellular recognition, the interaction of lectins with carbohydrates has been explored in various fields of research where saccharide specificity is essential. These proteins are invaluable tools for the study of simple or complex carbohydrates, in solution or on cell

surface, as well as for cell characterization (Sharon, 2008) due to the ability to discriminate among the myriad of complex carbohydrate structures (Drickamer and Taylor, 1993).

Lectins have been classified based on specific carbohydrates which they recognized, but with the growing number of lectins being discovered, classification is also based on structural information. Ultimately, with the increasing interest, a burst of information about structures, properties and biological functions of lectins are available and can be accessed in different databases (Krengel and Imberty, 2007; Frank and Schloissnig, 2010; Kumar and Mittal, 2011).

This article reviews the different families of animal lectins found in body fluids, cells and fish tissues. Some properties, functions, and biological events of such proteins will be presented. This study intended to gather general information about lectins occurring in several fish's species, their purification, characteristics, immunological roles, among other biological functions, and potential biotechnological applications for aquaculture.

2. FISH LECTINS AND FUNCTION

Lectins are proteins that recognize cells through carbohydrate binding sites. Each year the understanding of lectins has grown and various fish lectins have been characterized (Vasta et al., 2011; Ogawa et al., 2011). This diversity and characterization led to an expansion of its definition to any protein that has a non-catalytic carbohydrate-recognition domain (CDR). There are effective lectins that do not agglutinate cells, such as membrane-bound proteins, and have only a carbohydrate recognition domain (Ewart et al., 2001). The lectin binding to carbohydrate in the CRD occurs through weak interactions such as hydrogen bonding, ionic attractions, hydrophobic and van der Waals forces; these interactions can make the specific and transient nature of protein-ligand bonds (Nelson and Cox, 2011) (Figure 1).

The lectins of animal origin have been classified according to CRD comparison. The CRD found in each particular lectin type share a pattern of amino acid residues highly conserved and invariable (Ewart et al., 2001; Loris, 2002; Suzuki et al., 2003). Furthermore, different properties such as a requirement of divalent cations or reducing environment for ligand binding led to its classification in several major families (Vasta et al., 2011).

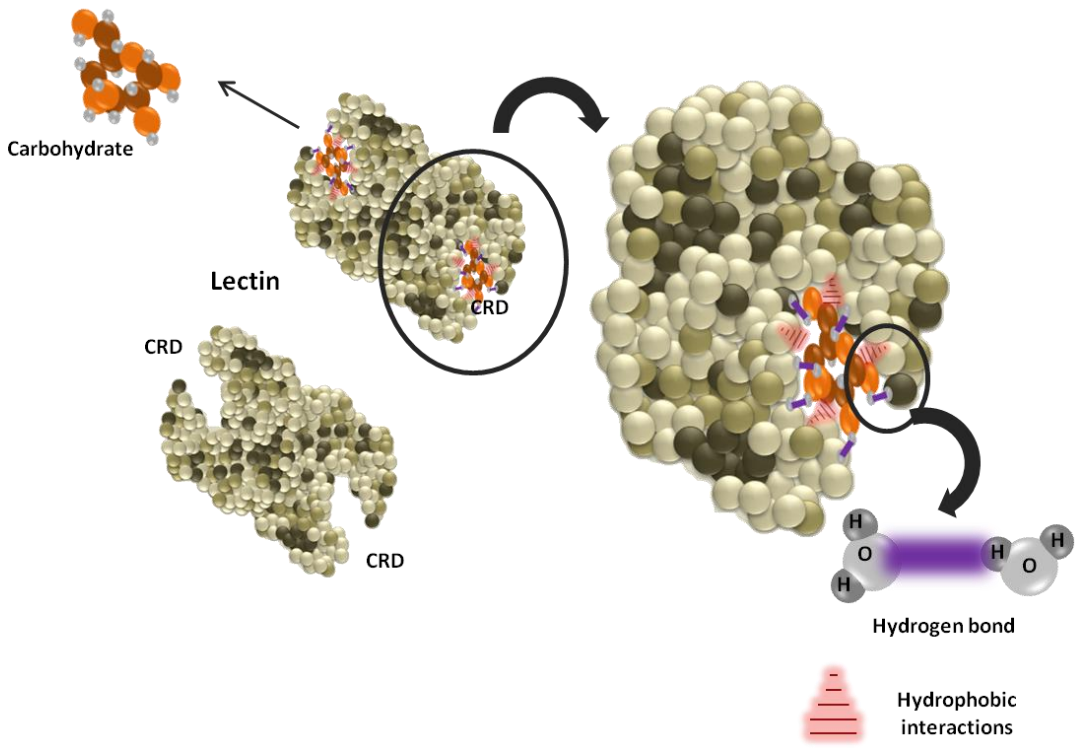

Figure 1. Scheme illustrating the binding of lectin to the carbohydrate through the Carbohydrate Recognition Domain. The carbohydrate-lectin interaction involves, among other non-covalent forces, the formation of hydrogen bonds and hydrophobic interactions.

The number of studies investigating the role of fish lectins using both immunological and molecular biology techniques has been growing (Shiina et al., 2002; Magnadottir et al., 2010; Bah et al., 2011). Humoral and membrane-associated lectins from host are critical recognition molecules that may facilitate the establishment of favorable mutualistic interactions with colonizing microbes, or initiate innate and adaptive responses against potentially pathogenic microorganisms (Vasta et al., 2011). In addition, fish lectins mediate other functions, such as agglutination, fertilization, immobilization with complement-mediated opsonization and deaths of pathogens (Ewart et al., 2001, Dong et al., 2004; Russell and Lumsden, 2005).

Some fish lectins may be present in the intracellular compartments mediating processes, such as splicing of RNA to protein folding and trafficking proteins (Vasta et al., 2011). Fish lectins can still be released to extracellular compartment having two destinations: cell surface or soluble components in biological fluids (Hébert, 2000; Vasta et al., 2011).

The immune system of vertebrates involves the innate and adaptive immune response. The innate immune system possesses several proteins acting, such as lysozyme and lectins. The lysozyme acts by attacking and disrupting the cell wall polysaccharides of different bacterial species killing microorganisms. Major events in innate immune defense include the recognition of

microbial targets for lectins, such as collectins. These proteins recognize foreign cells as "non-self" through the carbohydrates expressed on the surface acting as opsonins and encouraging their destruction by complement and/or phagocytic cells (Fock et al., 2001; Dutta et al., 2005; Battison and Summerfield, 2009; Imamichi and Yokoyama, 2010).

The importance and roles of innate immune components such as circulating lectins is well recognized in other vertebrates such as mammals (Fock et al., 2001; Russell and Lumsdem, 2005). Fishes differ from mammals in relation to the acquired immune response; then lectins and other innate immune effectors may have much more important roles.

There are several studies on the role of lectins in the immune system of fish. Several fish lectins are believed to mediate pathogen recognition in the immune system. There is a mannose binding lectin isolated from the serum of Atlantic salmon that has antibacterial activity against *Aeromonas salmonicida* and according to Ottinger et al. (1999), this lectin has similarity in structure and activity with the mannose-binding lectin of mammal which in turn plays a key role in innate immunity. A lectin isolated from the ovaries of cobia (*Rachycentron canadum*) showed antibacterial activity against *Escherichia coli* and no antifungal activity for *Coprinus comatus*, *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Rhizoctonia solani* (Ngai and Ng, 2007). Studies with mucus isolated from lectin catfish (*Silurus asotus*) showed that the gene of this lectin, in RT-PCR assay was not induced by bacterial stimulation in vivo; agglutination activity against the pathogenic bacteria *A. salmonicida* suggested that this lectin plays an important role in self-defense against bacteria on the skin surface of the catfish (Tsutsui et al., 2011). A lectin isolated from egg chum salmon (*Oncorhynchus keta*) showed different patterns of hemagglutinating activity inhibition to rabbit erythrocytes when Gram-negative bacteria lipopolysaccharides were used, such as *A. salmonicida*; *E. coli* and *Bacillus subtilis* bacteria were agglutinated (Shiina et al., 2002). Another lectin from Chinook salmon roe (*Onchorhynchus tshawytscha*) showed no antifungal activity or agglutination towards *Valsa mali*, *Helminthosporium. maydis*, *Mycosphaerella arachidicola*, *Setospaeria turcica* and *Bipolaris maydis* (Bah et al., 2011). In addition to the role of defense against microorganisms, there is evidence that these lectins have an important role in fertilization, embryogenesis (Dong et al., 2004; Vasta et al., 2011) and morphogenesis (Ahmed et al., 2004; Dutta et al., 2005).

Opsonization activity has also been reported in fish. The salmon serum lectin was found to be an opsonin for *A. salmonicida*. It enhanced the phagocytosis of heat-killed *A. salmonicida* by macrophages in a dose-dependent manner (Ottinger et al., 1999). Other lectin isolated from serum of sea bass (*Dicentrarchus labrax*), whose localization and expression occurs in hepatocytes and intestinal cells, also revealed that the exposure of *E. coli* formalin-killed to this lectin enhanced their phagocytosis by *D. labrax* peritoneal macrophages relative to unexposed controls (Salerno et al.,

2009). The opsonization assay with another lectin isolated from serum of the gilt head bream (*Sparus aurata*) showed that this lectin binds to formalin-killed *E. coli* and enhances their phagocytosis by peritoneal macrophages (Cammarata et al., 2012). A C-type lectin isolated from conger eel (*Conger myriaster*) showed that when it was bound to microspheres significantly enhanced their phagocytosis in conger eel macrophages (Tsutsui et al., 2007). These findings suggest that these lectins act as opsonins and may play an important role in innate immunity.

There are lectins that have other bioactivities such as mitogenic and antiproliferative activity. Some lectins induced lymphocyte proliferation or modulated several immune functions; these mitogenic lectins are useful as reagents to study lectin interactions with lymphocyte cells in vitro (Maciel et al., 2004). The grass carp (*Ctenopharyngodon idellus*) roe lectin exhibited mitogenic activity toward murine splenocytes with a potency lower than that of the plant lectin Con-A (Ng et al., 2003). The lectins isolated from the ovary of cobia (*Rachycentron canadum*), also showed mitogenic activity toward mouse splenocytes (Ngai and Ng., 2007). The lectin of salmon (*Oncorhynchus tshawytscha*) showed no mitogenic activity towards murine splenocytes, but it showed antiproliferative activity, which reduced the proliferation of human breast tumor (Bah et al., 2011).

Acquired immunity is reduced at low temperatures even in eurythermal fish; studies suggest that components of the innate immune system of fish can be less affected by temperature (Ewart et al., 2001; Magnadottir et al., 1999). If these findings could be extended to the components of the innate immune system increasing of innate immunity would be the route of choice for the generation of higher disease resistance in fish. In addition, fish lectins play important roles in many biological systems. The knowledge gained from the study of these lectins as a bioactive compound with activity to human tumor cells points towards its potential use in biotechnological applications (Lam and Ng, 2011).

3. CLASSIFICATION AND DIVERSITY OF FISH LECTINS

Lectins are structurally diverse molecules (Shirai et al., 2009); this structural complexity, inherent to these proteins, reflects in a large number of families. The structures of lectins are important for describing the characteristics of glycan classes found in several species and currently, animal lectins are incorporated into different categories, grouped by shared evolutionary origin and/or similarity of structural folds (Russell and Lumsden, 2005; Lin et al., 2009). In this section we try to summarize the main animal lectins that also occur in fish, highlighting their main characteristics and general functions.

The first classification of animal lectins divided these proteins into two categories S-type and C-type based on structural information of the protein portion responsible for interaction with carbohydrate, CRD (Drickamer, 1988). Since then, new lectin groups have emerged based mainly on structural information of the CRD. The variety of functions of animal lectins could be considered in general terms to be recognition molecules within the immune system. More specifically, lectins have been implicated in a direct first-line defense against pathogens, cell trafficking, immune regulation and prevention of autoimmunity (Kilpatrick, 2002). Table 1 shows the main categories of animal lectins with their relevant features and examples of function and/or biological activities.

The S-type lectins are designated as thiol-dependent proteins of intra-and extracellular localization recognizing mainly β-galactosides (Drickamer, 1988). However the need for thiol reducing agents is not very clear for some lectins of this group; a consensus of the term galectin to this group is due to common characteristics, such as ability to binding beta-galactosides and Ca^{2+}-independent activity (Arason, 1996; Kilpatrick, 2002). The galectins are abundant in cytosol, can be divided into three types, the proto-type (galectin 1, 2, 5, 7, 10, 11, 13 and 14), chimaera-type (galectin 3) and tandem-repeat type (galectin 4, 6, 8, 9 and 12). Galectin-1 contains two CRD as a homodimer is bound by a high-affinity receptor and acts as an autocrine inhibitor of cell growth. Galectin-4 has two CRD connected by a link peptide, whereas galectin-3 has one CRD connected to two domains which causes the molecule to form multimers (Dumic et al., 2006). All three major galectin types, proto, chimera, and tandem-repeat, are present in teleost fish. Galectin-3 sequences have been determined in pufferfish genome *Tetraoodon nigroviridis* (AL301540) and zebrafish EST *Danio rerio* (BM034940) been used in comparative studies with human galectin-3 (HSPC159) (Cooper, 2002). In addition, homology screening of fish databases reveals many other galectin-like sequences in catfish (*Ictalurus punctatus*), flounder (*Paralichthys olivaceus*), trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), zebrafish (*D. rerio*), and two species of pufferfish (*Takifugu rubripes* and *T. nigroviridis*) (Cooper, 2002).

The C-type superfamily includes the C-type lectins (CTL) and proteins containing C-type lectin-like domain (CTLD). CTL require calcium ions in binding to carbohydrate. The calcium domain is highly conserved in all members of the family, however, usually differ in the types of recognized carbohydrates and within the CRD. Key conserved residues that bind sugars, include the Glu-Pro-Asn (EPN) or Gln-Pro-Asp (QPD) motifs, in vertebrates (Zelensky and Gready, 2005; Cummings and McEver 2009).

CTLD refer to protein domains that are homologous to CRD of the C-type lectins, or which have structure resembling the structure of the prototypic C-type lectin CRD, regardless of their ability to bind sugars. Many CTLD have evolved to specifically recognize a variety of ligands, including carbohydrates, inorganic ligands (Ca2CO3), lipid, specific snake venoms, fish antifreeze

and bird egg-shell proteins (Zelensky and Gready, 2005). C-type domains are normally found in animal lectins from serum, extracellular matrix, and membranes (Vijayan and Chandra, 1999). C-type lectins have been identified in various fish species such as sea lamprey *Petromyzon marinus* (Ourth et al., 2008), japanese flounder *Paralichthys olivaceus* (Kondo et al. 2007), venomous fish *Thalassophryne nattereri* (Lopes-Ferreira et al., 2011) and grass carp *Ctenopharyngodon idellus* (Liu et al., 2011), *inter alia*.

C-type lectins are functionally diverse and have been implicated in various processes including cell adhesion, tissue integration and remodeling, platelet activation, complement activation, pathogen recognition, endocytosis, and phagocytosis, cytotoxic effect, mitogenic and antibacterial activities, besides of specific antibody production (Ngai and Ng, 2007; Kerrigan and Brown, 2009; Komegae et al., 2011; Saraiva et al., 2011).

Selectins and collectins are members of the C-type lectins superfamily. MBL can also be classified within the superfamily of C-type lectin according to characterization with CTL or CTLD. Mannose-binding lectin (MBL) is an important component of innate immunity in mammals, extensively studied (Turner, 2003; Gadjeva et al., 2004). This lectin has affinity for mannose, fucose and *N*-acetyl glucosamine (GlcNAc); MBL is calcium-dependent to sugars that have hydroxyl groups on carbon-3 and carbon-4 orientated in the equatorial plane of the pyranose ring (Turner, 1996). MBL is an acute-phase protein produced by hepatocytes and increases in response to an infection or inflammatory response. The protein acts directly as an opsonin promoting phagocytosis of foreign material to which it has bound triggering the lectin pathway of complement activation via MBL associated serine proteases (Arnold et al., 2006; Ourth et al., 2008). MBL, identified in several fish species, may play important immune functions. Channel catfish *Ictalurus punctatus* showed up-expression with Gram-negative bacterium infection (Zhang et al., 2012), African catfish *Clarias gariepinus* with antimicrobial activity (Argayosa et al., 2011) and tilapia fish *Oreochromis niloticus* inducing cytokine production (Silva et al., 2012).

Pentraxins are composed of multiple subunits with size varying between 20–25 kDa and one CRD per subunit. These lectins show Ca^{2+}-dependent binding to saccharides on bacterial cell surfaces and exist in serum as acute phase proteins, indicating their role in defense system (Magnadóttir et al., 2010). They may also be membrane associated, CRP, with preference for phosphorylcholine; or exist as a component of the extracellular matrix, SAP, with preference for phosphoethanolamine (Kilpatrick, 2002). Pentraxins showed opsonin activity in snapper *Pagrus auratus* displaying a functional role in the host defense fish (Cook et al., 2005); they were also detected in serum of pangasius *Pangasianodon hypophthalmus* (Huong-Giang et al., 2010) and Atlantic cod, *Gadus morhua* (Gisladottir et al., 2009).

Table 1. Main categories of animal lectins, features, functions and biological activities

Categories	Main features	Function/Biological activity
S-type lectins (Galectins)	Binding β-Galactosides; Ca^{2+} independent activity	Inflammatory responses; development, differentiation, morphogenesis, tumor metastasis, apoptosis; cell growth control and apoptosis (Fukumori et al., 2007)
C-type lectin	Ca^{2+} dependent activity, conserved Ca^{2+} biding site	Innate immunity (collectins); promote phagocytosis, complement activation (MBL); Cell adesion (selectins): Lymphocyte homing (L-selectin); Leukocyte trafficking to sites of inflammation (E- and P-selectins); Cell growth control and apoptosis (Kerrigan and Brown, 2009; Arnold et al., 2006; Ourth et al., 2008)
Pentraxins	Ca^{2+} dependent, exist in serum as acute phase protein	Recogniton of foreign or aberrant cell glycosylation (Endocytosis or initiation of opsonization or complement activation) (Kilpatrick, 2002; Magnadottir et al., 2010)
Calnexin	Intracelular lectin	Folding mechanism and misfolded protein retention in endoplasmic reticulum (Williams, 2006); stress-induced apoptosis (Takizawa et al., 2004)
I-type lectins	Structural similarity to the immunoglobulin superfamily, affinity for sialic acid	Immune and neural system; Cell-cell interactions; Cell routing (Varki and Angata, 2006)
F-type lectins or Fucolectin	Affinity for L-fucose, Ca^{2+} independent, non glycosylated	Molecular recognition in innate immunity (Salerno et al., 2009)
L-rhamnose binding lectins	Binding L-rhamnose, two or three homologous CRD in tandem of about 95 at 100 amino acids residues	Carbohydrate metabolism regulation, fertilization, cell proliferation, cytotoxicity, and opsonisation, respiratory burst stimulation, microbicidal activity (Terada et al., 2007; Watanabe et al., 2009; Franchi et al., 2011)

Calnexin and calreticulin are related proteins that represent a group of intracellular lectins, proteins of the endoplasmic reticulum that interacts transiently with glycoproteins and might participate in the folding mechanism but more probably acts to retain misfolded proteins in the endoplasmic reticulum. Possess a lectin site that recognizes an early oligosaccharide processing intermediate on the folding glycoprotein, Glc1Man9GlcNAc2 (Williams, 2006). Calnexin and calreticulin have a high-affinity Ca^{2+}-binding site and bind Zn^{2+} at sites within the globular domain; both bind ATP, although no ATPase activity has been detected (Leach et al., 2002). They have been identified in mammals, plants, fish salmonids as rainbow trout, *O. mykiss*, cyprinids (Kales et al., 2004; Kales et al., 2007; Bielek 2008). In mammals, calreticulin has numerous physiological and

immunological functions in the eukaryotic cell such as regulation of intracellular calcium homoeostasis, lectin binding and oxidative stress responses (Michalak et al., 1999; Kales et al., 2004). The calreticulin gene is little studied in teleost fishes but has been characterized in channel catfish *I. punctatus*; *e*ven with 72% identity with mammalian calnexins some characteristics indicate that assembly of class II molecules MHC in the catfish probably proceeds via different steps than occur in mammals (Fuller et al., 2004).

Lectins with structural similarity to the immunoglobulin superfamily were called I-type lectin; they constitute a category of lectins that mediates cell-cell interactions through the recognition of specific sialylated glycoconjugates (Powel and Varki, 1995). The sialic acid-binding Immunoglobulin superfamily lectins (Siglecs) are a structurally distinct subclass of I-type lectins. They are integral membrane proteins, preferentially expressed on the plasma membrane (Angata and Brinkman-Van der Linden, 2002). Structurally different than the Siglecs, but also apparently recognizing sialic acids are CD83 (Scholler et al., 2001) and cell adhesion molecule L1 (Kleene et al., 2001). A genomic sequence of a Siglec-4 was identified in two fishes Fugu, *T. rubripes* and zebrafish, *D. rerio* (Lehmann et al., 2004). Several lines of evidence suggest important roles of Siglec-4 in the maintenance of myelin integrity and the regulation of neuronal growth (Spencer et al., 2003).

F-type is a lectin group specific for α L-fucose, Ca^{2+}-independent and non-glycosylated. This protein category was named fucolectin by Honda et al., (2000). The ell fucolectin show a structure unique among the known lectins. The northern blot analysis revealed the presence of seven types of clones, three of which from the liver, and coding for similar but distinct proteins with 180 amino acid residues (Honda et al., 2000). Fucolectins have been identified as immunorecognition molecules in invertebrates and vertebrates such as pearl oyster, *Pinctada martensii* (Chen et al., 2011), rock bream, *Oplegnathus fasciatus* (Park et al., 2012) and gilt head bream *S. aurata* (Cammarata et al., 2012). Tandem-repeated types of F-type lectins are found in modern teleosts such as Nile tilapia (*O. niloticus)*, Japanese sea perch (*Lateolabrax japonicus*) and striped bass (*Morone saxatilis*) (Argayosa and Lee, 2009; Bianchet et al., 2010; Qiu et al., 2011). F-type lectin CRD motifs are absent in genomes of higher vertebrates such as reptiles, birds, and mammals (Ogawa et al., 2011).

The group of lectin binding to L-rhamnose (RBL) was proposed based on their binding specificity characteristic of the carbohydrates and molecular structure, which consists of two or three homologous CRD in tandem of about 95 at 100 amino acid residues with characteristic topology and a series of conserved motifs (Tateno et al. 2002; Terada et al., 2007). The RBL are classified into five subgroups based on the domain architecture, hemagglutinating activity for human erythrocytes and carbohydrate specificity (Nitta et al., 2007). These proteins have been

identified in various types of fish fat-east dace, *Tribolodon brandti* (Jimbo et al. 2007), spanish mackerel, *Scomberomorus niphonius* (Terada et al. 2007) and sweet fish (ayu), *Plecoglossus altivelis* (Watanabe et al. 2008) interacting with various types of bacteria, such as *Staphylococcus epidermidis, E. coli* and *Pseudomonas aeruginosa* (Franchi et al., 2011). It is also suggested to be involved in inflammatory reactions by functioning as agents of recognition and trafficking of cells to sites of inflammation as well as activate the inflammatory cascade by regulating the expression of cytokines (Watanabe et al., 2009).

4. PURIFICATION AND CHARACTERIZATION OF FISH LECTINS

Isolation and characterization of lectins are of paramount importance for elucidation of the basic properties and biological functions of these proteins. A considerable number of lectins from fish has been isolated from serum (Carvalho et al., 2012; Silva et. al., 2012), plasma (Mitra and Das, 2002), mucus (Tsutsui et al., 2011), gill (Pan et al., 2010) and eggs (Shiina et al., 2002; Ngai and Ng, 2007).

Serum samples containing lectins can be subjected to partial purification by methods such as fractionation dependent on pH (Suzuki et al., 1979) or salt, especially ammonium sulfate, followed by exhaustive dialysis (Sage and Green, 1972). Currently, almost all lectins are purified by affinity chromatography (Correia and Coelho, 1995; Coelho and Silva., 2000; Santos et al., 2009; Leite et al., 2012; Nunes et al., 2011; Souza et al., 2011), a technique that relies on the ability of lectins to bind carbohydrates specifically and reversibly (Lis and Sharon, 1981). Often other chromatographic methods are needed to obtain a lectin with high purity such as ion exchange chromatography (Bah et al., 2011; Dutta et al., 2005) and molecular exclusion (Silva et al., 2009).

Lectins have the ability to agglutinate cells such as erythrocytes (hemagglutination), lymphocytes and bacteria, being also able to precipitate glycoconjugates (Correia and Coelho, 1995; Silva et al., 2011). Thus a useful assay for identifying a lectin in a given sample such as serum or tissue homogenate is performed using an hemaglutination assay with human or other animal erythrocytes (Figure 2a and 2b). To be considered a lectin, the hemagglutination activity should be inhibited (Figure 2c) when added to the assay environment a mono or oligosaccharide (Coelho et al., 2012).

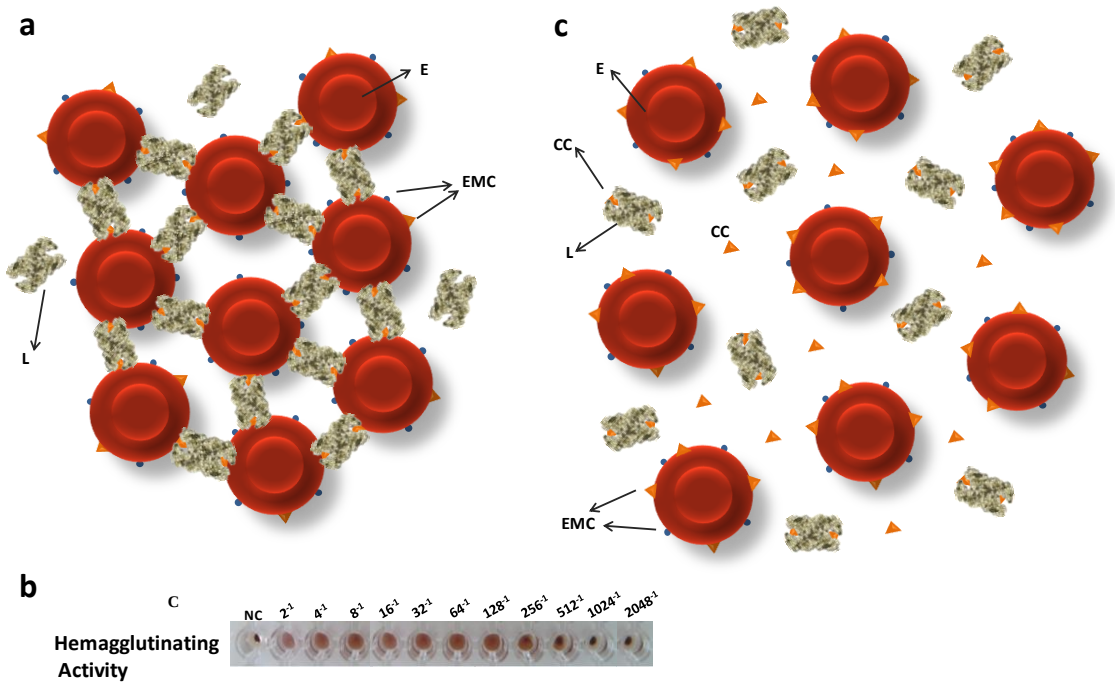

Figure 2. Hemagglutinating and inhibition of hemagglutinating activity assay (HA). The presence of lectin is revealed by the formation of a hemagglutination net due to lectin binding to erythrocyte surface carbohydrate (**a**). HA is performed in 96-wells microtiter plates. Lectin preparations (50 µL) are serially two-fold diluted and an equal volume of erythrocyte (2.5%) suspension is added to each well. Plates are incubated at room temperature for 40 min. Activity corresponds to the last dilution in which hemagglutination is visualized; in this case 512^{-1} (**b**). HA inhibition is revealed when lectin sample is incubated with carbohydrate prior to erythrocytes. Specific carbohydrate binding to lectin abolishes net formation (**c**). E – Erythrocyte, EMC - erythrocyte membrane carbohydrate, L – lectin, CC – competitor carbohydrate, C – control.

The gene evolution or expression of fish lectins has been investigated to elucidate their origin and their occurrence in different tissues (Shirai et al., 2009; Mistry et al., 2001). In certain experiments, the fish are defied against pathogens and observed in vivo, and subsequently, leads to gene expression analysis comparing healthy and infected animals under experimental conditions (Jorgensen et al., 2008).

Our group has been working with lectins besides of protease inhibitors of exotic and Brazilian autochthonous fish. The first protease inhibitor of tilapia (*O. niloticus*) was identified and showed antibacterial activity (Leite et al., 2012).

A mannose recognizing lectin from *O. niloticus* serum was purified and partially characterized. This lectin (OniL) did not show cytotoxicity against splenocytes and induced higher IFN- γ production. OniL is a potencial immunomodulator which has preferentially Th1-type immune response (Silva et al., 2012). Another lectin was identified from serum of cobia (*Rachycentron canadum*) with specificity for methyl-α-D-mannopyranoside (Coriolano and Coelho,

2012). Lectin was identified in the serum of the Amazonian fish tambaqui (*Colossoma macropomum*). This lectin (ComaSeL) showed antimicrobial activity against pathogenic bacteria to freshwater fish. ComaSeL is seasonal and its serum concentration in cold periods is extremely low; high mortality of these fish for fungal and bacterial infections occurs in cold seasons (Carvalho et al., 2012). The temperature is the main environmental factor that stimulates changes in the immune response of many species of fish, affecting both innate and acquired immunity. The effects of seasonality on the immunological and hematological parameters of the Amazonian fish pirarucu (*Arapaima gigas*) are under investigation.

5. PERSPECTIVES

The fish innate immune system includes lectin(s) which could increase innate immunity efficiency. Pathogens are present in aquatic environments; the generation of fish with enhanced disease resistance would be of great value to the industry to increase productivity and therefore economic gains.

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5 - CAPÍTULO II

5.1 Artigo Científico 2

Purification and Characterization of a Mannose Recognition Lectin from *Oreochromis niloticus* (Tilapia Fish): Cytokine Production in Mice Splenocytes

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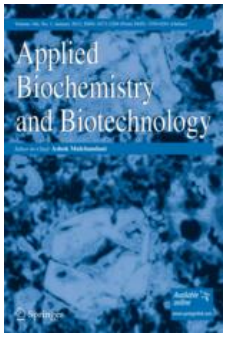

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Purification and Characterization of a Mannose Recognition Lectin from *Oreochromis niloticus* (Tilapia Fish): Cytokine Production in Mice Splenocytes

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Abstract The aim of this work was to purify and partially characterize a mannose recognition lectin from Nile tilapia (*Oreochromis niloticus*) serum, named OmiL. OmiL was isolated through precipitation with ammonium sulfate and affinity chromatography (Concanavalin A–Sepharose 4B). In addition, we evaluated carbohydrate specificity, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) profiles, and in vitro immunomodulatory activity on mice splenocyte experimental cultures through cytotoxic assays and cytokine production. The ammonium sulfate fraction F2 showed the highest specific hemagglutinating activity (331) and was applied to affinity matrix. Adsorbed proteins (OmiL) were eluted with methyl-α-D-mannopyranoside. OmiL, a 17.4-kDa protein by SDS–PAGE constituted by subunits of 11 and 6.6 kDa, showed highest affinity for methyl-α-D-mannopyranoside and D-mannose. Immunological assays, in vitro, showed that OmiL did not show cytotoxicity against splenocytes, induced higher IFN-γ production

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and lower IL-10 as well as nitrite release. In conclusion, OniL lectin was successfully purified and showed a preferential Th1 response in mice splenocytes.

Keywords *Oreochromis niloticus* - Tilapia - Lectin purification - Immunomodulatory activity

Introduction

Lectins are a group of sugar-binding proteins that specifically recognize carbohydrate structures and agglutinate various cells through binding to cell surface glycoconjugates and, in contrast to antibodies, do not produce an immune response [1, 2]. The carbohydrate-binding property used to determine lectin class is promoted by the carbohydrate recognition domain (CRD), with invariant and highly conserved amino acid residues at a characteristic pattern [3]. CDR binds specifically to carbohydrate molecules expressed on pathogens helping in their rapid clearance by enhancing opsonization, phagocytosis and increasing oxidative burst activities [4, 5].

Mammalian liver lectin was discovered in 1974, and since then, a large number of researchers have intensively investigated animal lectins, especially of mammals. Animal lectins are classified into several families based on the structure of the CRD [2]. Lectins have been studied in various teleost fishes. The detail of correlation to the fish immunity, however, is unclear [6]; it is considered to be the first line of defense against infection and hence the universal form of host defense [4]. Fish serum lectins can be effectively purified by affinity chromatography using matrices that are conjugated to specific carbohydrates [7, 8] or to neoglycoproteins [9].

Some fish lectins with biological relevance are those isolated from hus almon serum, important for binding to the surface of *Vibrio anguillarum* and *Aeromonas salmonicida* [8] and a fish mucosal lectin able to bind parasitic metazoans [10]. It seems that fish lectins have an important role in fertilization, morphogenesis, and embryogenesis, in addition to their role on the defense against microorganisms [11, 12]. Argayosa and Lee [13] described the isolation and partial characterization of fucose-binding proteins from the Nile tilapia serum showing the property to agglutinate pathogens as *Aeromonas hidrophyla* and *Enterococcus faecalis*.

Mannose-binding C-type lectins (MBLs) are important components of the innate immunity in mammals [14, 15]. Some collectins opsonize microorganisms and also activates the lectin complement pathway [16]. The levels of mannose-binding lectin increase in response to an infection or inflammatory response. The innate immune system has become important for studying fish immunity [17, 18].

Teleost fish MBLs are able to recognize mannose, *N*-acetyl-d-glucosamine, and glucose [19]; these particular lectins have been reported in trout, salmon, carp, rohu fish, channel catfish, blue catfish [8, 20–22], and now here in the serum of the Nile tilapia (*Oreochromis niloticus*).

Immunological assays are important to evaluate biological responses that new compounds can induce in organisms; however, few studies had investigated the immunomodulatory profile induced by fish lectins. This paper describes the isolation by affinity chromatography of a mannose recognition lectin from *O. niloticus* serum lectin (OniL), its partial characterization, and immunomodulatory activity. Hemagglutinating activity inhibition assays with simple sugars and glycoproteins, cytotoxicity assay, as well as in vitro measurement of cytokine production and nitrite analysis in mice splenocytes were also performed.

Materials and Methods

Blood Collection and Serum Separation

The tilapia (*O. niloticus*) blood collection was performed in the *Laboratório de Fisiologia e Ecologia de Peixes, Estação de Aqüicultura Continental Prof. Johei Koiki, Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco*. The blood from a juvenile tilapia was collected using a 3-mL syringe through its caudal vein. Immediately after collection, the blood was kept in glass tubes and maintained for 16 h (overnight) at 4 °C. Subsequently, after blood coagulation, the serum was removed with the aid of a Pasteur pipette and centrifuged at 1,300×*g* for 5 min at 4 °C. The serum was again centrifuged under the same conditions. Subsequently, the serum was kept at −20 °C until being used, when it was removed from cold storage and centrifuged.

Saline Fractionation

The serum was precipitated with ammonium sulfate (0–20%, fraction 1; 20–40%, fraction 2 (F2); 40–60%, fraction 3; and 60–80%, fraction 4) under saturation according to Green and Hughes [23]. The fractions were dialyzed against Tris-buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, 20 mM $CaCl_2$, at pH 8.0). After dialysis, the hemagglutinating activity was determined for each fraction.

Hemagglutinating Assay

The evaluation of hemagglutinating activity (HA) was performed in microtiter plates according to Correia and Coelho [24]. Lectin preparations (50 μL) were serially twofold diluted in 0.15 M NaCl before addition of 50 μL 2.5% (*v*/*v*) suspension of rabbit glutaraldehyde-treated erythrocytes or human erythrocytes (types A, B, AB, and O). The titer was expressed as the highest dilution exhibiting hemagglutination. The HA was defined as the inverse of the last dilution at which the sample still showed hemagglutination. Specific HA (SHA) was defined as the ratio between the titer and protein concentration (milligrams per milliliter).

Purification of *O. niloticus* Serum Lectin

The F2 (20–40%) was chromatographed (0.5 mg of protein) on a Concanavalin A–Sepharose 4B (Sigma-Aldrich, USA) column previously equilibrated with 10 mM $CaCl_2$ and 10 mM $MnCl_2$ in TBS at pH 8.0. The fraction was applied to a 3-mL column which was then washed with TBS to 280 nm. The lectin elution was performed with methyl-α-D-mannopyranoside (200 mM) in TBS. Two-milliliter fractions were collected at a flow rate of 20 mL/h. Adsorbed fractions with the highest absorbance were pooled (OniL) and submitted to dialysis against TBS at 4 °C. Protein was determined according to Bradford [25]; OniL activity was assayed using a 2.5% (*v*/*v*) suspension of glutaraldehyde-treated rabbit erythrocytes.

Carbohydrate-Binding Specificity

The evaluation of hemagglutinating activity inhibition used OniL and carbohydrates (D(+) glucose, D(+) mannose, galactose, methylglucopyranoside, methylmannopyranoside, methyl-α-D-galactopyranoside, *N*-acetylglucosamine) or glycoprotein (fetuin, asialofetuin, ovalbu-

min, casein, and azocasein) solutions. The assay was performed according to Correia and Coelho [24] in 96-well microtiter plates. The inhibitory assays were similar to the hemagglutinating assay with the addition of the incubation step (15 min, at 28 °C) to provide a lectin–inhibitor interaction before erythrocyte addition. Minimal inhibitory concentrations were determined and corresponded to the lowest carbohydrate or glycoprotein concentration able to neutralize hemagglutinating activity of OniL. The highest carbohydrates and glycoprotein concentrations used were 200 mM and 500 μg/mL, respectively.

Effect of Different pH, Temperatures, and Ca^{+} on Lectin HA

OniL pH stability was evaluated through incubation in the following buffers for 12 h at 4 °C: 100 mM glycine–HCl (pH 2.0–3.0), 100 mM acetate (pH 4.0–5.0), 100 mM sodium phosphate (pH 6.0–7.0), 100 mM Tris–HCl (pH 8.0), and 100 mM glycine–NaOH (pH 9.0–10). Thermal stability was evaluated with previously heated OniL for 10 min at various temperatures (25 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90, and 100 °C). To determine Ca^{2+} dependence, OniL was incubated overnight in TBS (pH 8.0) containing 25 mM ethylenediaminetetraacetic acid (EDTA). Aliquots of the lectin solution were incubated with different concentrations of Ca^{2+} (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mM) in TBS, placed in 96-well microtiter plates, and incubated overnight at room temperature. HA was measured for all experiments using 50 μL of a 2.5% (*v*/*v*) suspension of glutaraldehyde-treated rabbit erythrocytes.

Lectin Characterization

Polyacrylamide gel electrophoresis (PAGE) was performed for native and acidic proteins according to Davis [26]. OniL molecular weight was estimated using 10% (*w*/*v*) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli [27] under nonreducing conditions as well as in the presence of β-mercaptoethanol. Molecular mass markers (myosin, 212.0 kDa; β-galactosidase, 116.0 kDa; phosphorylase, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 48.0 kDa; carbonic anydrase, 31.0 kDa; soybean trypsin inhibitor, 21.4 kDa; lysozyme, 14.4 kDa; aprotinin, 6.5 kDa) were used. The gels were stained with Coomassie Brilliant Blue.

Animals

Male BALB/c mice (6–8 weeks old) were raised at the animal facilities of the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil) and maintained at the animal facilities of the Centro de Pesquisa Aggeu Magalhães da Fundação Oswaldo Cruz in Recife, Brazil. All mice were treated and sacrificed in accordance with the Comissão de Experimentos com Animais de Laboratório da Fundação Oswaldo Cruz (Ministério da Saúde, Brazil, 0266/05).

Preparation of Splenocytes

Splenocytes were obtained according to Pereira and collaborators [28]. After killing the animal with CO_2 gas, the spleen of each mouse was removed aseptically and placed in a Falcon tube containing RPMI 1640 with fetal calf serum (complete medium). In a vertical flow, each spleen was transferred to a Petri dish where they were soaked. The cell suspensions obtained were transferred to Falcon tubes containing approximately 10 mL of incomplete medium per spleen. Homogenates were overlaid onto a Ficoll-PaqueTM PLUS layer, with density adjusted to 1.076 g/mL, and centrifuged at 1,000 ×*g* for 25 min at room

temperature. The interface cell layer containing immune cells was recovered by Pasteur pipette, washed twice in PBS, and centrifuged two times at 500×g for 10 min. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was greater than 98%.

In Vitro Cytotoxicity Assays

The cytotoxicity of the lectins (OmiL and Concanavalin A) was determined using BALB/c mice splenocytes (6×10^5 cells/well) cultured in 96-well plates in RPMI 1640 media (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FCS; Cultilab, Campinas, SP, Brazil) and 50 μg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil). The *Canavalia ensiformis* (Concanavalin A) was purchased from Sigma-Aldrich (St Louis, MO, USA). Each lectin was evaluated at six concentrations (100, 50, 25, 10, 5, and 1 μg/mL), in triplicate in two independent assays. Cultures were incubated in the presence of [3H]-thymidine (Amersham Biosciences; 1 μCi/well) for 24 h at 37 °C and 5% CO_2. After this period, the content of the plate was harvested to determine the [3H]-thymidine ($[^3H]TdR$) incorporation using a beta-radiation counter (β-matrix 9600, Packard). The toxicity of the compounds was determined by comparing the percentage of [3H]-thymidine incorporation (as an indicator of cell viability) of lectin-treated wells in relation to untreated wells. Saponin (0.05%), known for higher cytotoxicity, was used as a positive control. Non-cytotoxic concentrations were defined as those causing a reduction of [3H]-thymidine incorporation below 30% in relation to untreated controls.

Measurement of Cytokine Levels in Splenocyte Supernatants

Splenocytes were cultured in 24-well plates (TPP) at a density of 10^6 cells/well. Cytokines were quantified in 24-, 48-, 72-h and 6-day supernatants from cultures stimulated with OmiL at 10 μg/mL and Concanavalin A (Con A) at 2.5 μg/mL or maintained only in culture medium (control). The levels of interleukin-10 (IL-10) and interferon-gamma (IFN-γ) were measured by sandwich ELISA, according to the manufacturer's suggested protocols. The monoclonal antibodies used were from Kit OptEIA (BD Biosciences), being previously titered. Plates with 96 wells (Nalge Nunc International Corporation) were sensitized with specific anti-cytokine antibodies (according to the manufacturer's instructions) and incubated overnight at 4 °C. Cytokine standards were added after serial dilution from their initial concentration (8,000 pg/mL, for both IL-10 and IFN-γ). After washes, 50 μl of all samples and standards was added in duplicate and the plate incubated for 2 h at room temperature. Subsequently, the specific antibodies were combined with biotin (according to the manufacturer's instructions) and incubated for 1 h 30 min at room temperature. Revealer solution was added containing 2.2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt. The reaction was blocked with 1 M sulfuric acid, and the reading was carried out in a spectrophotometer (Bio-Rad 3550, Hercules, CA) at 415 nm. Sample concentrations were calculated in the linear region of the titration curve of cytokine standards, and final concentrations were expressed in picograms per milliliter, using the Microplate Manager Version 4.0 software (Bio-Rad Laboratories).

In Vitro Nitrite Analysis

Mice spleen cells were used to evaluate the concentration of nitrite, while treated with Con A (2.5 μg/mL) and OmiL (10 μg/mL) after 24, 48, 72 h and 6 days of incubation. Culture

media were carefully collected for subsequent measurement by the colorimetric Griess method. NO concentration was estimated by the standard curve (3.12–100 μmol mL^{-1}).

Statistical Analysis

Data were analyzed using nonparametric tests. To detect differences between groups, the Mann–Whitney *U* test was used. All results were expressed by mean values of groups ± standard deviation and were analyzed considering the value of $p<0.05$ as statistically significant.

Results

Purification of OniL

The *O. niloticus* serum lectin, OniL, was purified, and immunological assays were performed to analyze the biological response of this new lectin against mice splenocytes. Nile tilapia serum was subjected to ammonium sulfate precipitation and only F2 (SHA, 330.3) showed hemagglutinating activity (Table 1). OniL (SHA, 94.7) was obtained from F2 in a single-step purification on a Con A–Sepharose 4B affinity column (Fig. 1). OniL was eluted with methyl-α-D-mannopyranoside (200 mM) in TBS; fractions of OniL retained 31.3% of chromatographed lectin activity, and the obtained purification factor was 0.29 (Table 1).

OniL agglutinated rabbit (titer, 64^{-1}) as well as A, B, AB, and O human erythrocytes (titer, 8^{-1}, 64^{-1}, 4^{-1}, 32^{-1}, respectively). OniL HA was detected between a pH range of 7.0 and 11.0 and was fully preserved after heating for 10 min at 25 °C, 30 °C, 40 °C, 50 °C, and 60 °C. However, it was completely neutralized after heating at 70 °C. Addition of EDTA diminished activity revealing that OniL is a calcium-dependent lectin. The *O. niloticus* lectin appeared to need a Ca^{2+} optimal concentration of 15 mM, and further increase in Ca^{2+} concentration had no effect on lectin activity.

HA inhibition assays examined the binding specificity of OniL using carbohydrates and glycoproteins (Table 2). The methyl-α-D-mannoppyranoside and D-mannose were the best inhibitors.

Table 1 Summary of OniL purification

Sample	Serum	F2	OniL
Volume (mL)	15	3.2	8
Total protein (mg)	16.05	4.96	5.41
Hemagglutinating activity	512^{-1}	512^{-1}	64^{-1}
Total hemagglutinating activity	7,680	512	512
Specific hemagglutinating activity	478.5	330.3	94.11
Yield (%)[a]	–	100	31.1
Purification (folds)	–	1	0.28

Hemagglutinating activity assays and specific hemagglutinating activity were performed with rabbit erythrocytes. The concentration of more than 5 mg of OniL was obtained from 15 mL of serum when it was submitted to 20–40% ammonium sulfate fractionation followed by Concanavalin A–Sepharose 4B affinity chromatography

[a] Percentage of total activity recovered

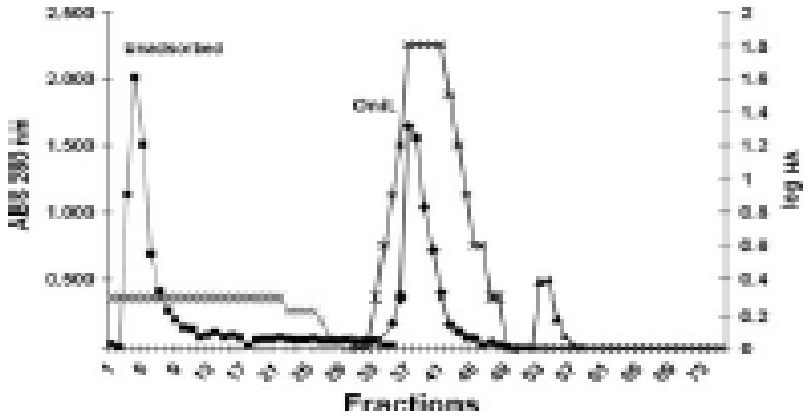

Fig. 1 Affinity chromatography of the tilapia serum on Con A–Sepharose 4B. The F2 was applied to the column (3 mL) and pre-equilibrated with Tris-buffered saline (TBS, 20 mM Tris–HCl containing 150 mM and NaCl 20 mM CaCl2, pH 8.0) at a constant flow rate of 20 mL/h. Bound protein was eluted with buffer containing 200 mM alpha-D-mannopyranoside; Fractions (2.0 mL) were collected and evaluated for hemagglutinating activity (*HA*). Absorbance (*ABS*) at 280 nm (*black squares*) and log HA (*multiplication signs*) are represented

The molecular weight of OmiL, purified after Con A–Sepharose 4B chromatography, was 17 kDa by SDS–PAGE under nonreducing conditions (Fig. 2b). In the presence of a reducing agent, OmiL was revealed as a dimeric protein constituted by subunits of 11 and 6.6 kDa linked by disulfide bonds (Fig. 2c). Native acidic protein showed a unique polypeptide band (Fig. 2d).

OmiL Was Not Cytotoxic Against Mice Splenocytes

The cytotoxicity threshold was expressed as the highest concentration tested that was not cytotoxic for the splenocytes. Saponin and Concanavalin A were used as positive controls. Saponin (0.05%), known for its cytotoxicity, demonstrated a higher inhibition. Con A, used

Table 2 Minimum inhibitory concentrations of carbohydrates and glycoproteins

Inhibitor	Minimal inhibitory concentration*
Galactose	100
N-acetylglucosamine	25
Mannose	6.25
Methyl-α-D-mannopyranoside	6.25
Methyl glycopyranoside	25
Asialofetuin	15.63
Azocasein	31.25
Casein	1.25
Fetuin	62.5
Ovalbumin	31.25

The hemagglutinating activity inhibition assays were performed with rabbit erythrocytes and in triplicate

*Minimal inhibitory concentration corresponds to the lowest carbohydrate or glycoprotein concentration able to neutralize hemagglutinating activity of OmiL. The values are expressed in millimolar for carbohydrates and micrograms per milliliter for glycoproteins. The highest carbohydrate and glycoprotein concentrations used were 200 mM and 500 μg/mL, respectively

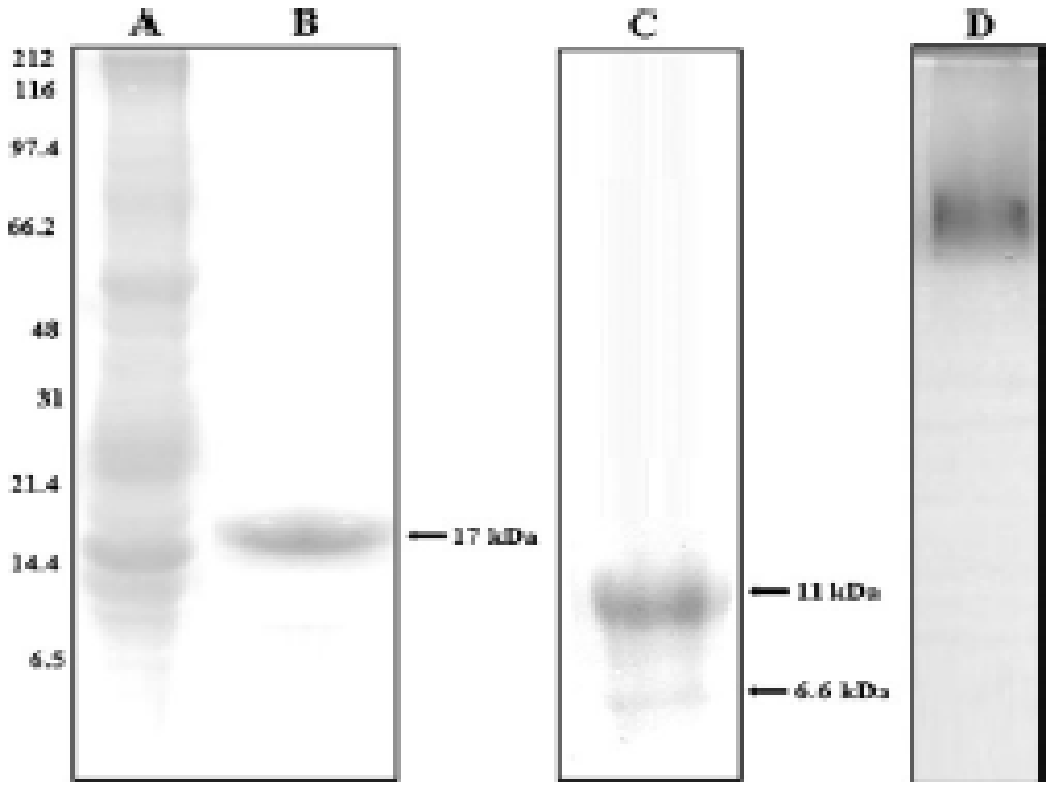

Fig. 2 Polyacrylamide gel electrophoresis (10%, w/v) in the presence of sodium dodecyl sulfate (SDS) of 20–40% ammonium sulfate fraction, F2 (a), and OmiL (40 μg) from Con A–Sepharose 4B affinity chromatography in the absence (b) and presence of reducing agent β-mercaptoethanol (c). d PAGE for native acidic proteins of native OmiL. Molecular weight markers (a): myosin (212.0 kDa), β-galactosidase (116.0 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (48.0 kDa), carbonic anydrase (31.0 kDa), soybean trypsin inhibitor (21.4 kDa), and lysozyme (14.4 kDa); the gels were stained with Coomassie Brilliant Blue

as a reference for immunological assays, did not show toxic activity below 50 μg/mL concentrations, and OmiL was not cytotoxic at either experimental concentration (Table 3).

OmiL Stimulated Higher IFN-γ in Mice Splenocytes

OmiL induced higher IFN-γ in mice splenocyte cultures. Figure 3a–d shows that OmiL, similar to Con A, induced stronger IFN-γ production at all experimental times in relation to the control. In addition, at 24 h of assay, OmiL produced greater stimulus than Con A (Fig. 3a). IL-10 was also stimulated by the lectin, but at lower values. In fact, only at 72 h of assay did OmiL induce statistically different values for IL-10 (50.8±18 and 16.7±5.5 for OmiL and control, respectively, $p<0.05$). Con A also induced IL-10 production and was superior to the control cultures, but similar to OmiL, only at 72 h of assay were these values statistically different (86±6.6 and 16.7±5.5 for Con A and control, respectively, $p<0.05$).

Nitric oxide was the other chemical mediator analyzed in this study. However, OmiL was not able to induce NO release in statistically significant values in mice splenocytes. On the other hand, Con A stimulus induced higher NO production at 24 h and 6 days (45±5 and 86±6.6, respectively), but only at 6 days did Con A show statistically different values in relation to the control (48± 15).

Table 3 Percentile of cytotoxicity induced by OniL

Compounds	Concentrations (μg/mL)					
	100	50	25	10	5	1
	Inhibition (%)[a]					
OniL	–	–	–	–	–	–
Con A	58	–	–	–	–	–
Saporin[a]	94	91	90	89	88	88

Assay using splenocytes of BALB/c mice cultured, in vitro, with OniL, Con A, saporin, and unstimulated cells labeled with [3H]-thymidine

[a] Non-cytotoxic concentrations (−) were defined as those causing a reduction of [3H]-thymidine incorporation below 30% in relation to untreated controls

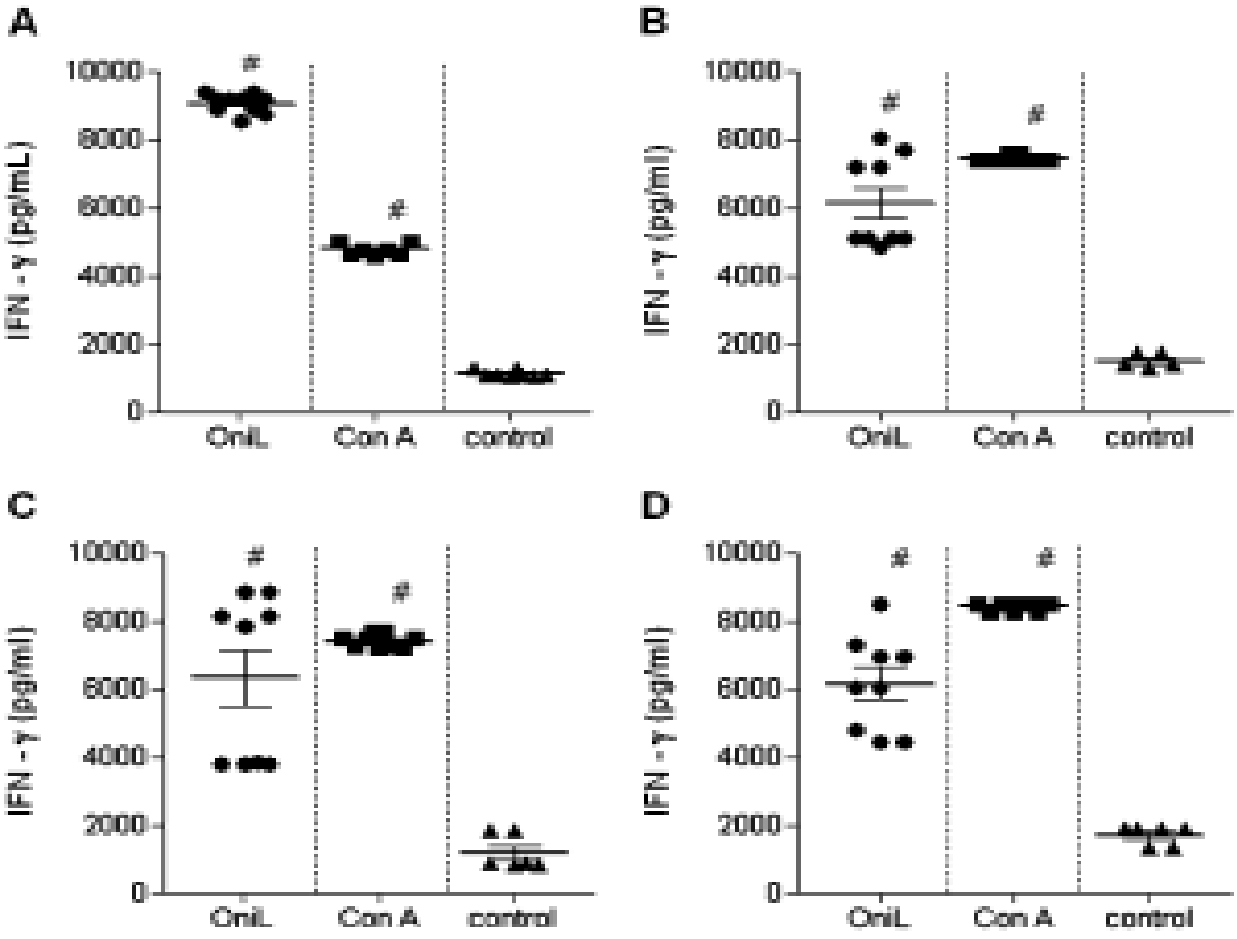

Fig. 3 IFN-γ production induced by OniL and Con A lectins on mice splenocyte cultures after 24 (a), 48 (b), 72 h (c) and 6 days (d). OniL and Con A induced higher and statistically different values of IFN-γ production at all experimental times in relation to control. At 24 h OniL stimulus was also superior to that of Con A (a). *Horizontal bars* represent the average of four independent experiments per group. *$p < 0.05$

Discussion

Mannose-binding lectin acts as a first line of defense against many microbial pathogens and has been reported in trout, salmon, carp, rohu, channel catfish, and blue catfish [22]. Here, a mannose recognition lectin, OmiL, has been purified and characterized from Nile tilapia using a simple protocol.

Argayosa and Lee reported the isolation and partial characterization of fucose-binding proteins from Nile tilapia serum. In the present study, OmiL showed highest affinity for mannose derivatives. The inhibition by other carbohydrates and glycoproteins indicates that OmiL carbohydrate-binding sites are also able to recognize these other molecules, although with low affinity than for methyl-α-D-mannopyranoside and D-mannose. The data agree with the report that fish serum lectin might have α-methyl glucose or α-methyl mannose motifs to be recognized by Con A [11]. Absar and collaborators reported the isolation and purification of a mannose recognition protein from the crude extract of the ventral portion of potka fish which was also specifically inhibited in the presence of mannose and its derivatives, a mannose-specific protein [29]. The specific hemagglutinating activity of F2 was higher than that of OmiL probably because in the fraction there are other lectins. It is probable that the chromatography on Con A–Sepharose selected OmiL and the other lectins did not adsorb on matrix. In the chromatogram (Fig. 1), it could be seen that proteins with hemagglutinating activity were also present in unadsorbed material.

The buffer used to purify OmiL contained calcium necessary to bind lectin to the chromatography column. A C-type mannose-binding lectin from sea lamprey plasma was also shown to be calcium-dependent after being eluted in a mannan–agarose affinity column with EDTA [30] Another study showed that the activity of this serum lectin from Indian catfish was also found to be Ca^{2+} dependent as evidenced by a complete loss of activity in the presence of EDTA; the loss of Ca^{2+} by calcium chelators could cause irreversible damage to the active sites of lectin [11]. Mannose is one of the dominant carbohydrates found on the bacterial cell surface; in fact, collectin recognizes the mannose patterns in bacterial oligosaccharides [31].

The thermal instability of *O. niloticus* lectin was in line with the results obtained for STL1 and STL2 lectins isolated from steelhead trout (*Oncorhynchus mykiss*). STL1 completely lost its hemagglutinating activity after heating for 90 min at 50 °C, whereas half of STL2 activity was retained under the same conditions; the activity was completely abrogated following treatment for 10 min at 70 °C [32]. Moreover, grass carpa (*Ctenopharyngodon idellus*) lectin lost half of its activity after incubation for 10 min at 40 °C, and the activity was completely abolished after incubation for 5 min at 80 °C [11].

OmiL showed two polypeptide bands of 11 and 6.6 kDa, apparent molecular masses in SDS–PAGE under reducing conditions, indicating the presence of disulfide bonds. Mannose-binding C-type lectins were previously isolated from channel catfish and blue catfish sera with a molecular weight range of 62–66 kDa [30]. Pufflectin-4 was also purified from skin mucus extract of fugu by mannose-affinity chromatography. SDS–PAGE showed an apparent molecular weight of 13 kDa interacting specifically with D-mannose [33].

In fish species, a possible involvement of the mannose receptor in phagocytosis by leucocytes has also been suggested for the gilthead seabream, *Sparus aurata* [34]. Mannose-binding lectin plays a crucial role in the first line of host defense [35]; thus, the fish lectins may also act as important modulators of acquired immune responses [36] binding to foreign mannose at the initiation of the complement [37, 38].

OmiL enhanced immunological response through cytokine production in mice splenocytes; a preferential Th1 response was observed through higher INF-γ production.

These results are similar to other fish lectins that investigated L-rhamnose-binding lectins isolated from chum salmon (*Oncorhynchus keta*) and from grass carp. In these studies, both lectins induced pro-inflammatory cytokine production and exerted a mitogenic effect on mice splenocytes [11, 39]. However, few studies have shown immunological properties induced by fish lectins. Salerno and collaborators investigating F-type lectin from sea bass (*Dicentrarchus labrax*) showed its function as an opsonin in plasma and intestinal mucus [40]; Lam and Ng observed that the purified grass carp lectin was mitogenic toward murine splenocytes and peritoneal exudate cells [37].

In summary, OmiL was purified and used in immunological assays; the lectin showed a preferential Th1 profile. OmiL is a potential immunomodulatory compound since mannose recognition lectin from fish serum can be active in mammals.

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6 - CAPÍTULO III

5.1 ARTIGO CIENTÍFICO 3

LECTIN FROM TILAPIA FISH (*Oreochromis niloticus)* SERUM INDUCES STIMULATORY ACTIVITY ON MICE SPLENOCYTES

Artigo a ser submetido ao periódico ***Scandinavian Journal of Immunology***

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Lectin from tilapia fish (*Oreochromis niloticus)* serum induces proinflamtory cytokines production and proriferation on mice splenocytes

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ABSTRACT

Lectins, proteins with distinct carbohydrate specificities and structural characteristics, have been used for immunomodulatory and proliferative activities, but few studies show immunological profile induced by fish lectins. A lectin from serum of tilapia fish *Oreochromis niloticus* (OniL) was purified by affinity chromatography on Concanavalin A-Sepharose 4B. This *in vitro* study analyzed cellular proliferation and cytokine production in mice splenocytes cultures treated with OniL (2.5, 5 and 10 µg/mL) and *Canavalia ensiformis* lectin, Concanavalin A, Con A (2.5 µg/mL). The unstimulated culture plate was used as a negative control. Cellular proliferation assay was performed by [^{3}H]-thymidine incorporation. Cytokine production was measured by enzyme-linked immunoadsorbent assay (ELISA) and IL-2 and IL-6 cytokines were quantified. Cell viability assay was performed by Annexin V-FITC/PI. Annexin-FITC$^-$/PI$^+$ cells were considered necrotic cells and Annexin-FITC$^+$/PI$^-$ represented splenocytes in the early stage of apoptosis. All nonparametric assays were used in statistical analysis. Higher and statistical indexes of proliferation were induced by OniL in relation to control cells. OniL showed higher mitogenic activity in relation to control and Con A for all analyzed concentrations. Higher IL-2 and IL-6 production was promoted by OniL; sometimes this production was also superior to Con A. Results of viability assay showed that OniL did not promote cell damage. Beyond, Con A induced higher apoptosis and necrosis in both 24 and 48 h in relation to control and fish lectin cultures. OniL induced higher proliferative response; this lectin can be used as a mitogenic agent in immunostimulatory assays.

Keyword: *Oreochromis niloticus* lectin, tilapia fish, cytokines, splenocytes, proliferative activity.

1 INTRODUCTION

An increasing body of evidence points to a central regulatory role for glucose in mediating cellular processes [1]. The structural variability and complexity of cell surface glycans allows them to function as signaling, recognition and adhesion molecules, normal embryonic development,

differentiation, growth, contact inhibition, disease development, metastasis, intracellular traffick in gand localization, rate of degradation, membrane rigidity, cell–cell recognition, cell signaling, host–pathogen interaction during infection and host immune response [2].

The immune response is started through molecular events which may occur through lectin and carbohydrate interactions. Lectins with distinct characteristics and specificity have been used to evaluate their immunomodulatory activity, lymphoproliferation, CD4-mediated signal transduction and functional activation of monocytes and macrophage-like cells [3, 4].

The first lectin to be described to immunological evaluations was the bean lectin from *Phaseolus vulgaris* (PHA) [5]. Proliferative and immune responses are mediated by specific cytokines and cells that determine effective functions of immune system compounds [6]. These molecules regulate local and systemic immune inflammatory and regulatory events. This include interleukins, tumor necrosis factors, interferons, colony stimulating factors and chemokines essential to differentiation of Th cell to Th1, Th2 and Th17 to develop an effective immune response [7, 8].

Differentiation of naive CD4 T cells from a common precursor cell into distinctive Th1 and Th2 cell subsets is mediated by a complex interplay between the cytokine environment and receptor-ligand interactions between a naive T cell and an antigen presenting cell (APC) [9]. Most lectins may display Carbohydrate Recognition Domain (CRD) in combination with other domains. These proteins not only recognize carbohydrates on the surface of potential pathogen, but also mediate several effector functions including agglutination, immobilization, and opsonization of microbial pathogens, and complement pathway/phagocyte activation [10].

L-2 is a pleiotropic cytokine with a very broad array of actions, including its abilities to drive T-cell proliferation, augment cytolytic activity, promote Treg cell development, and mediate activation-induced cell death. This cytokine also regulates other key cytokine receptors to allow responsiveness to IL-12 and promoting Th1 differentiation, while repressing thus IL-6 responsiveness, thereby inhibiting Th17 differentiation [11].

Interleukin-6 is directly related to T cell naive differentiation B cell and antibody production. Over the years it has become increasingly clear that IL-6 has also a profound effect on CD4 T cells. Resting, naïve CD4 T cells undergo apoptosis *in vitro* cell culture soon after isolation from lymphoid mouse tissues suggesting the presence of survival factors in vivo. IL-6 has such anti-apoptotic properties as it prolongs CD4 T cell survival *in vitro* most likely by retaining Bcl-2 expression in the isolated T cells [12].

Lectins have been investigated as mitogenic components and constitute invaluable tools to study the biochemical changes associated with lymphocyte activation and proliferation of various immune cells [13], beyond mitogenic activities in human lymphocytes [14] and immunomodulatory in mice splenocytes [15].

Our group has been working with fish lectins showing the biological importance, such as antimicrobial activity of mannose-specific lectin purified from serum of the Amazonian tambaqui fish (*Colossoma macropomum*), ComaseL [16]. A mannose recognition lectin was purifyed from Tilapia fish serum (*Oreochromis niloticus*), named OniL; the fish lectin revealed some immunological properties. In fact, Onil was not cytotoxic against mice splenocytes and was capable to induce preferential Th1 response in cell cultures [17]. In this current study it was analyzed, *in vitro*, the mitogenic action, proliferative cytokine production and cell viability in mice splenocytes treated with OniL lectin.

2 MATERIALS AND METHODS

2.1 Animals

Mice were experimental animals used for immunological assays (BALB/c, male, 30 days old, 5/group). Mice were raised at the animal facilities of the Fundação Oswaldo Cruz (Rio de Janeiro, Brasil) and maintained at the animal facilities of the Centro de Pequisa Aggeu Magalhães/Fundação Oswaldo Cruz (Recife, Brasil). The guidelines of the Comitê de Ética para Uso de Animais Experimentais da Fundação Oswaldo Cruz/FIOCRUZ (Ministério da Sáude, Brasil) were followed.

All mice suffered euthanization and were treated in accordance with the guidelines of the Comissão de Experimentos com Animais de Laboratório da Fundação Oswaldo Cruz (Ministério da Saúde, Brasil, 0266/05).

2.2 Lectins

The serum of tilapia fish was precipitated using ammonium sulphate (20-40% w/v); this fraction with highest hemagglutinating activity was dialyzed against Tris buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, 20 mM $CaCl_2$, at pH 8.0). The fraction containing 0.5 mg of protein was submitted to affinity chromatography on Concanavalin A-Sepharose 4B (Sigma) gel column previously equilibrated with 10 mM $CaCl_2$ and 10 mM $MnCl_2$ in Tris Buffered Saline (TBS), at pH 8.0. OniL elution was performed with methyl-α-D-mannopyranoside (200 mM) then dialysed against TBS [17].

2.3 Preparation of splenocytes

The splenocyte isolation assay was performed in accordance to Pereira end collaborators [18]. After euthanizing the animals with CO_2 gas, the spleen of each mouse was aseptically removed and placed in a Falcon tube containing RPMI 1640 with fetal calf serum (complete medium). In a vertical flow, each spleen was transferred to a Petri dish where they were soaked. The cell suspensions obtained from each spleen were transferred to Falcon tubes containing approximately 10 ml of incomplete medium. Spleen homogenates were overlaid onto a Ficoll-PaqueTM PLUS layer, with the density adjusted to 1.076 g/mL, and centrifuged at 1000 x g at room temperature for 25 min. The interface cell layer containing immune cells was recovered by Pasteur pipette, washed twice in phosphate-buffered saline (PBS) and centrifuged twice at 500 g for 10 min. Cells were counted in a Neubauer chamber; cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was >98%.

2.4 Cell proliferation assay

The cell proliferation assay was performed in accordance to Melo end collaborators [19]. Mice splenocytes isolated ($4x10^5$ cells/well) were cultured (at 37 ºC and 5% CO_2) in triplicate in 96-well culture plates (TPP, St. Louis, Switzerland). Cells of each group were treated, *in vitro*, for 72 h with Con A (2.5 µg/mL) or OniL (2.5, 5 and 10 µg/mL) to evaluate the proliferative activity of Onil in comparison to Con A. The unstimulated culture plate was used as a negative control. Into each culture well, 0.5 µCi of thymidine - [3H]-TdR (Amersham Biosciences, Boston, MA, USA) was added and proliferation was assessed by [3H]-TdR incorporation. At the end of this period, the material was collected via automatic cell collector (Skatron Instruments, Sterling, VA, USA) and deposited on glassfibre paper (Whatman International Ltd., Maidstone, UK). The incorporation of [3H]-thymidine was determined through emitted β radiation, expressed in rate per minute (RPM). Results were expressed by the stimulation indexes (SI), defined as the RPM arithmetic average of stimulated cultures, divided by the arithmetic average of unstimulated cultures, ±standard deviation. The cut-off was determined by the control group medium ±two standard deviations. SI values ≥ 3 were considered representative of positive proliferation.

2.5 Cytokine evaluation in culture supernatants

Splenocytes isolated were cultured in 24-well plates (TPP) at a density of 10^6 cells/well for 24, 48, 72 h and 6 days. Each well received Con A (2.5 µg/mL) or OniL (10 µg/mL) lectins, and supernatants from cultures stimulated *in vitro* with lectins were obtained at end of 24, 48, 72 h and 6 days. Cells maintained only in culture medium (unstimulated culture) were also obtained as a negative control. IL-2 and IL-6 cytokines were quantified by ELISA, according to the manufacturer's suggested protocols. The monoclonal antibodies used were from kit OptEIA (BDBiosciences, Mountain View, CA, USA), being previously tittered. Plates with 96 wells (Nalge Nunc International Corporation, Roskilde, Denmark) were sensitized with specific anticytokine antibodies (according to the manufacturer's instructions) and incubated 'overnight' at 4 ºC.

Cytokine standards were added after serial dilution from their initial concentrations (according to the manufacturer's instructions). After washes, 50 µL of all samples and standards were added in duplicate and the plate was incubated for 2 h at room temperature. Subsequently, the specific antibodies were combined with biotin (according to the manufacturer's instructions) and incubated for 1 h 30 min at room temperature. Revealer solution was added containing 2.2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS). The reaction was blocked with 1 M sulphuric acid, and the reading was carried out with a spectrophotometer (Bio-Rad 3550, Hercules, CA, USA) at 415 nm. Sample concentrations were calculated in the linear region of the titration curve of cytokine standards, and final concentrations were expressed in pg/ml, using the Microplate Manager Version 4.0 software (Bio-Rad laboratories).

2.6 Analysis of cell viability by annexin V-FITC and propidium iodide staining

Splenocytes (10^6 cells) were stimulated with lectins Con A (2,5 µg/mL) e OniL (10µg/mL) for 24 and 48 h. After these experimental times, cells were centrifuged at 4 ºC, 450 g for 10 min. After discarding the supernatant, 1 ml of PBS 1X was added to the precipitate and this was then centrifuged at 4 ºC, 450 g for 10 min. After discarding the supernatant, the pellet was resuspended in a binding buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and annexin V conjugated with fluorescein isothiocyanate (FITC) (1:500) and propidium iodide (PI, 20 µg/ml; 10^6 cells) were added to each labelled cytometer tube. Flow cytometry was performed in a FACSCalibur (Becton Dickinson Biosciences) and analyzed using Cell Quest Pro software (Becton Dickinson). Result analysis was performed in graphs by dot plot. Annexin-$FITC^-$/PI^+ cells were considered necrotic cells and Annexin-FITC+/PI^-) represented splenocytes in the early stage of apoptosis. Double negatives were considered viable cells.

2.7 Statistical analysis

Data were analyzed using nonparametric tests. To detect the differences between groups, the Mann–Whitney *U*-test was used. The student t-test was used to analyze the cell viability assay. All results were expressed as mean values of groups ±standard deviation and were analyzed considering the value of $P < 0.05$ as statistically significant.

3 RESULTS

3.1 OniL shows higher values in lymphoproliferative assay through [3H]-TdR incorporation

OniL promoted mice splenic cell proliferation treated with different concentrations of this fish lectin. Con A, a plant lectin and a compound considered as standard mitogen in immunological assays, was used as positive control. Higher and statistical indexes of proliferation were induced by OniL, in all concentrations, in relation to control cells (figure 1).

3.2 Production of proliferative cytokines was induced by OniL on mice splenocytes cultures

OniL and Con A induced significant production of IL-2 cytokine in mice splenocytes cultures in all experimental times (figure 2A, B, C and D). It was also observed that OniL was superior to control after 48 and 72 h of assay. IL-6 production was similar in relation to IL-2 production. For all experimental times, OniL induced higher IL-6 production in relation to control (figure 3A, B, C and D).

3.3 OniL did not promoted significant cell damage

Results of viability assay showed that mice splenocytes cultures treated with OniL (10 µg/mL) did not promoted significant cell damage in all the experimental times. However, the cells treated with Con A (2.5 µg/mL) induced higher cell damage, especially at 24 h of assay. In fact, Con A induced higher apoptosis, late apoptosis and necrosis at 24 h and higher apoptosis at 48 h of assay (figure 4A and B).

4 DISCUSSION

Lectins are involved in the modification of various cellular interactions owing to the differential glycosylated state of particular counter-receptors. Lectin-glycan interactions in the control of immune cell homeostasis. Certain glycoreceptors expressed on lymphocytes surface can be recognized by C-type lectins, galectins and siglecs, depending on their glycosylation status. Synapse formation between APCs and T cells can be regulated by galectin-3 and by DC-SIGN and ICAM-3 [20].

Emerging evidences establishes that the immune system employs carbohydrates as recognition determinants for various cellular interactions. The best understood case is provided by the selectin family L-, E- and P-selectin which are found on leukocytes. These receptors mediate adhesion by recognising specific carbohydrates-based ligands on partner cells by virtue of C type lectin domains [21].

Several reports have shown that lectins play important roles in the immune response of invertebrates and vertebrates either by recognizing exposed glycans of potential pathogens or by their immunoregulatory roles. The binding to carbohydrates on the surfaces of immunocompetent cells [22] is a pivotal step for initiating cellular signalling pathways [23].

Cytokines are low molecular weight cell derived glycoproteins controlling the cell to cell communication on a variety of target cells responsible for an immune response regulating host defence network. These molecules are also responsible for Th cell differentiation; balance between Th1, Th2 and Th17 is important to establish an effective immune response and proliferative action [6, 7, 8].

Mitogenic lectins are invaluable tools to study the biochemical changes associated with activation and proliferation of various immune cells [13, 14, 19]. Here we report the mitogenic response in mice splenocytes cultures induced by OniL. The lymphoproliferative assay by timidine [^{3}H]-TdR incorporation mice splenocyte allowed to observe that OniL was a potent mitogenic inductor towards mice splenocyte culture treated with different concentrations of fish lectin as well

as Con A, a lectin well known as immunomodulating compound (figure 1). Watanabe and collaborators as well as Ng and collaborators observed that lectins induced pro-inflammatory cytokine production and exert a mitogenic effect on mice splenocytes [24, 25] and this immunomodulatory mechanism can be initiated by lectin-carbohydrate interactions [26].

In study present, using proliferative parameters of IL-2 and IL-6 mitogenic cytokines by quantitative ELISA the levels of these cytokines induced by OniL were evaluated in mice splenocyte culture. The data showed that OniL induced high production of cytokine IL-6 in mice splenocyte cultures when compared to control (figure 3C). More recently, we demonstrated that the immunological profile induced by OniL can promote a proinflammatory reaction in mice splenocytes *in vitro* showing a preferential Th1 profile [17]. IL-6 is a pleiotropic cytokine showing essential roles in immunity, hematopoiesis and inflammation [27] and it is considered an important modulator of CD4 T cell [12]. Dienz and Rincon were the first to show that indeed IL-6 can modulate the Th1/Th2 balance towards Th2. IL-6 present during antigen stimulation of CD4 T cells promotes autocrine IL-4 production which further enhances Th2 differentiation through an autofeedback loop [12].

Our results demonstrated that the secretion of IL-2 by splenocytes was confirmed by the proliferation of mice splenocytes (figure 1). This study was relevant, since the secretion of IL-2 is considered as a mediator of cellular signaling in the immune system [28]; it is a cytokine with important immunomodulatory role.

The T cell proliferation when IL-2 initially binds a high mannose-type glycan and a specific peptide sequence of the IL-2 receptor γ-subunit sequentially forming a high affinity complex of IL-2/IL-2 receptor α-, β-, and γ-subunits [29]. Based on these reports we suggest that splenocyte proliferation induced by OniL can be stimulated by the carbohydrate recognition in mice splenocyte surfaces. A recent approach glycans study expressed on immune cells surface, they undergo changes when the functions and expression of endogenous proteins or lectins (C-type lectins,

galectins and siglecs) are then regulated at the site of inflammation affecting directly signaling during T cells and dendritic cells activation [20].

Gamma interferon (IFN-γ), a cytokine with diverse roles in the innate and adaptive immune responses has a myriad of effects in both host defense and immune regulation, including antiviral activity, antimicrobial activity, and antitumor activity as well as Th1 immune responses; it also regulates T cell differentiation, activation, expansion, homeostasis, and killing survival [17, 30].

OniL induced *in vitro* high levels of IFN-γ, low IL-10 release and Th1 preferential response in mice splenocytes [17]. Figures 2 and 3 showed that OniL induced high levels of IL-2 and IL-6 revealing proliferative activity in mice splenocytes. In similar studies, the grass carp (*Ctenopharyngodon idellus*) ovaries and lectin induced higher mitogenic response in murine splenocytes and peritoneal exudates by IL-2 and INF-γ release [31]. The roe lectin of the same species of fish, exhibited mitogenic activity toward murine splenocytes with a potency lower than that of the plant lectin Con A [32].

Previous study by our group has demonstrated by immunological assays that OniL did not show cytotoxicity against splenocytes [17]. This report in viability assay showed that mice splenocyte cultures treated with OniL did not cause cellular damage when compared to control (figure 4). However, cells treated with Con A induced cellular damage. Con A induced higher apoptosis and cell death in relation to control. Studies have shown inhibitory control mechanisms on effector T cells, such as induction of apoptosis by galectin binding to glycosylated CD45, CD43 or TIM-3 or action through C-type lectin-glycan interactions between tolerogenic APCs and effector T cells [20].

A schematic representation of OniL immunomodulatory action mechanism in mice splenocytes is shown in figure 5, inducing proliferation. In contrast, mistletoe lectin is extensively used in traditional medicine as an immunomodulating agent and biological response modifier to stimulate an increase in apoptosis in neutrophils, inducing cell death [33, 34]. Another lectin with immunostimulatory effect, Cramoll 1,4, also induced a proliferative response through cellular and

humoral parameters in mice previously inoculated, showed higher necrosis compared with Con A which showed more apoptotic cells [19]. The present study demonstrated that OniL modulates Th1 CD4+ preferential differentiation by upregulation of IL-2 and IL-6 in mice splenocyte cultures and can be considered as a new modulator in various physiologic processes as well as a mitogenic agent end does not promote significant cell death.

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6 FIGURE LEGENDS

Figure 1: Mitogenic response towards Mice splenocytes treated with different OniL concentrations. A positive control is also shown using Con A. OniL revealed higher mitogenic activity in relation to control and Con A for all analyzed concentrations. OniL at 5 µg/mL was also superior to Con A (2.5 µg/mL). The mice splenocyte proliferation was measured by [3H]-thymidine incorporation as described in Materials and Methods (item 2.4). Results were expressed by the proliferation indexes (PI), and PI ‡ 3 were considered positive for proliferation. Points represent the average of 6 independent experiments per group. * $p < 0.05$.

Figure 2: IL-2 production induced by OniL in Mice splenocytes cultures. A, B, C and D were 24, 48, 72 h and 6 days of assay, respectively. OniL and Con A induced IL-2 production in relation to control in all experimental times, however with 48 and 72 h OniL stimulation was higher, whereas Con A was superior under 24 h and 6 days. Points represent the average of 6 independent experiments per group. ***** $p < 0.05$.

Figure 3: IL-6 production induced by OniL in Mice splenocytes cultures. A, B, C and D were 24, 48, 72 h and 6 days of assay, respectively. OniL induced higher IL-6 production control under 24, 72 h and 6 days. Only at 48 h Con A also stimulated mice splenocytes hight IL-6 production in comparison to OniL. Points represent the average of 6 independent experiments per group. ***** $p < 0.05$.

Figure 4: Viability assay with mouse splenocyte cultures treated with OniL 24 h (a) and 48 h (b). OniL (10 µg/ mL) did not promote cellular damage, but Con A (2.5 µg/mL) did. The latter lectin induced higher apoptosis (24 and 48 h) in relation to control and OniL. Points represent the average of five independent experiments per group. ***** $p < 0.05$.

Figure 5: **Schematic representation of immunomodulatory mechanism from OniL splenocyte proliferation.** OniL recognized glycans expressed on the splenocyte surface by Carbohydrates Recognition Domain (CRD) specific site and proliferative activity mediated by high levels of IL-2, IL-6, IFN-γ and low IL-10 production that are essential cytokines for differentiation of Th cell to Th1 induces effective immunomodulatory response. The mitogenic cytokines released by interaction between OniL and splenocytes did not promote cell damage. The figure was based on previous knowledge [8, 9, 12, 17, 26] and present results.

7 FIGURES

Figure 1

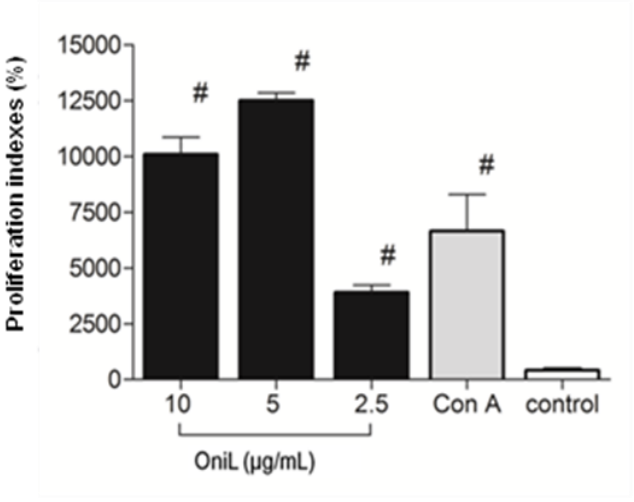


Figure 2

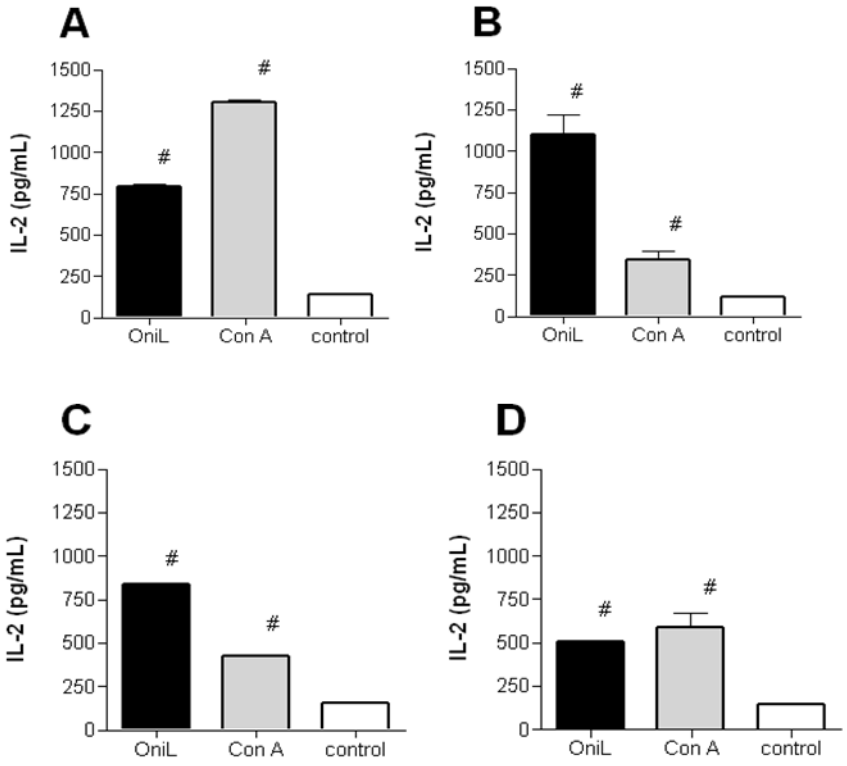


Figure 3

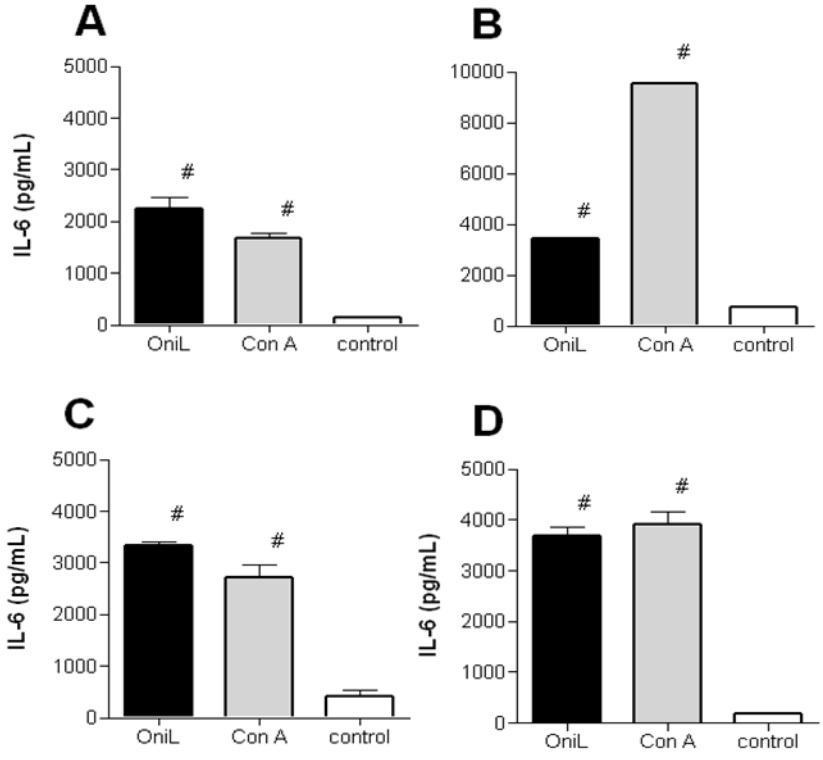


Figure 4

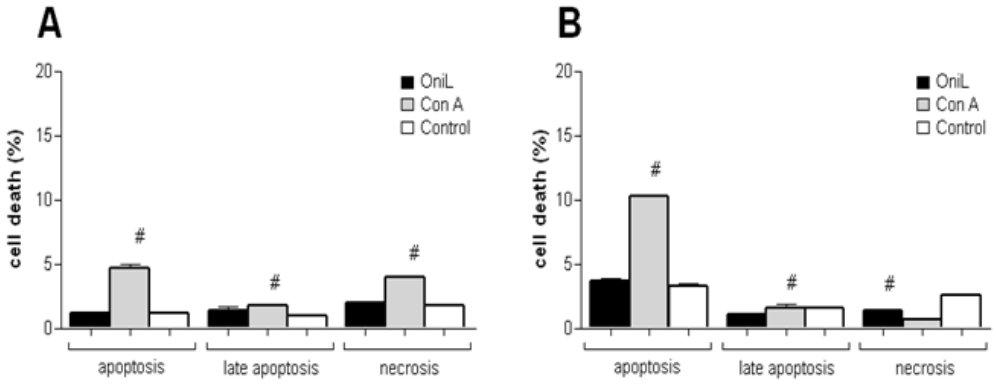
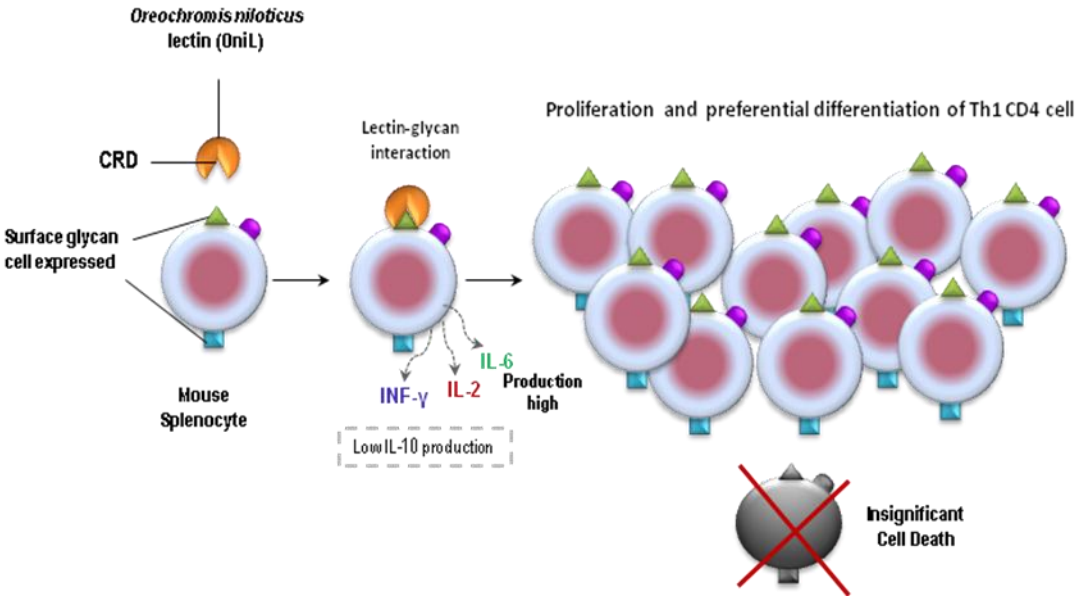

A
B
cell death (%)
OniL
Con A
Control
apoptosis
late apoptosis
necrosis
#

Figure 5


Oreochromis niloticus
lectin (OniL)
CRD
Surface glycan
cell expressed
Mouse
Splenocyte
Lectin-glycan
interaction
INF-γ
IL-2
IL-6
Production
high
Low IL-10 production
Proliferation and preferential differentiation of Th1 CD4 cell
Insignificant
Cell Death

7 – CONLUSÕES

- O diversificado repertório de lectinas (galectinas, pentraxinas, calnexinas, tipo C, tipo I, tipo F, L-rhamnose, dentre outras) encontrados em fluidos corporais, células e tecidos aumenta a eficiência da imunidade inata conferindo aos peixes uma maior resistência a doenças;

- A lectina OniL, presente no soro de tilápia (*O.niloticus*) foi purificada e caracterizada parcialmente pelo método cromatográfico em matriz de afinidade Con A Sepharose 4B e mostrou elevada afinidade para os carboidratos metil-α-D-manopiranosídeo e D-manose;

- As características bioquímicas de OniL revelaram que essa proteína aglutinou eritrócitos humanos e de coelho; a atividade hemaglutinante foi detectada entre o pH 7,0 e 11 sendo potencializada na presença de íons cálcio e abolida apenas em temperaturas superiores a 70 °C;

- OniL apresentou massa molecular aparente de 17 kDa; na presença de um agente redutor apresentou duas subunidades de 11 e 6,6 kDa indicando a presença de pontes dissulfeto;

- OniL não conferiu citotoxidade em esplenócitos de camundongos, induziu elevada produção de citocinas IFN-γ, IL-2 e IL-6, baixa produção de IL-10 e supressão de óxido nítrico (NO);

- A habilidade de OniL reconhecer resíduos de manose na superfície de esplenócitos permitiu a secreção de citocinas pro-inflamatórias por essas células, principalmente IFN-γ e IL-2, exercendo um efeito mitogênico conduzindo a uma resposta immune celular preferencialmente do tipo Th1; OniL não induziu morte celular significativa.

8 ANEXOS

8.1 Comprovação de aceite do artigo 1

Fwd: Decision on your manuscript #ABAB-3527R1

Luana Cassandra Breitenbach Barroso Coelho lcbbcoelho@gmail.com 23/10/11

---------- Forwarded message ----------
From: **Applied Biochemistry and Biotechnology** <abab@engr.ucr.edu>
Date: 2011/10/22
Subject: Decision on your manuscript #ABAB-3527R1
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Dear Dr. Luana Cassandra Coelho:

I am pleased to inform you that your manuscript, "Purification and characterization of a mannose-recognition lectin from Oreochromis niloticus (Tilapia fish): cytokine production in mice splenocytes" has been accepted for publication in Applied Biochemistry and Biotechnology.

For queries regarding your accepted paper, please click the following link http://www.springer.com/12010; then click on "Contacts", and then "Production Editor", complete the query form and click "Submit".

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Congratulations and best regards,

Ashok Mulchandani
Applied Biochemistry and Biotechnology

8.2 Normas do Periódico – Artigo 3

Scandinavian Journal of Immunology

Edited by:
R. Jonsson and H.-G. Ljunggren

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XXXVIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular – SBBq 16 a 19 de Maio de 2009 - Hotel Monte Real Resort, Águas de Lindóia, São Paulo

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Partial Purification and Characterization of a Serum Lectin from Tilapia Fish (*Oreochromis niloticus*)

Silva, C.D.C.[1]; Lino, M.A.S.[1]; Bezerra, R.F.[1]; Santos, A.J.G. [2]; Maciel de Carvalho, E.V.M.[1]; Coelho, L.C.B.B.[1]

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Lectins are proteins or glycoproteins that recognize carbohydrates with a high degree of specificity. They agglutinate cells, precipitate polysaccharides, glycoproteins and glycolipids. Several studies concerning lectins isolated from serum, plasma, mucus and skin of fish have shown the biological importance of these proteins in fish immunology. The main aim of this work was the partial purification and characterization of tilapia fish (*Oreochromis niloticus*) serum lectin(s) by fractionation with ammonium sulfate (0-20%, F1; 20-40%, F2; 40-60%, F3; 60-80%, F4), carbohydrate specificity, affinity chromatography (Concanavalin A-Sepharose 4B) and polyacrylamide gel electrophoresis containing sodium dodecyl sulphate (10% SDS-PAGE). The serum lectin was inhibited by galactose and lactose; it was partially purified through ammonium sulfate precipitation. The electrophoresis of the fractions was distinct, under reducing and non-reducing conditions. F2, with higher hemagglutinating activity (HA, 512^{-1}), was applied to the affinity chromatography. The adsorbed proteins were eluted with 200 mM N-methyl-glucosamine. Active peak fractions were pooled, dialyzed and electrophoresed. Hence, it can be concluded that tilapia serum lectin(s) was partially purified by two steps and included within the family of the galectins.

Keywords: Lectin; Purification, Affinity chromatography; Tilapia fish.
Support: CNPq.

XXXVIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular – SBBq 16 a 19 de Maio de 2009 - Hotel Monte Real Resort, Águas de Lindóia, São Paulo

PURIFICATION AND CHARACTERIZATION OF LECTIN(S) FROM TAMBAQUI AMAZON FISH

Maciel de Carvalho, E.V.M.[1*]; Bezerra, R. F.[1]; Silva, C.D.C.[1]; Lino, M.A.S[1].; Bezerra, R. S.[1], Santos, A. J. G.[2], Correia, M. T. S.[1], Coelho, L. C. B. B[1].

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Lectins constitute a group of proteins that recognize and bind specifically to carbohydrates. These proteins have a significant role in the immune responses of hosts. They bind specifically to carbohydrate molecules expressed on pathogens and help their opsonization and phagocytosis. The tambaqui (*Colossoma macropomum*) is a native fish from the Amazon Region, and it is one of the most important species in Brazilian pisciculture. In the presente work, the tambaqui serum lectin was partially purified through the saline precipitation of ammonium sulfate. The obtained fractions were analyzed and the one with the biggest hemagglutinating activity (AH), fraction 0-50% (AH: 2048^{-1}), was chosen in order to perform the following steps. The fraction 0-50% (F1) was partially inhibited by fucose, galactose and methyl-α-D-galactose carbohydrates. F1, with 34,03 mg/mL (Bradford method) was subjected to chromatography affinity in Con A Sepharose 4B; the adsorbed and eluated with 200 mM N-methyl-glucosamine showed one peak. Active fractions were pooled, dialysed and subjected to a DEAE-Sepharose ion exchange column chromatography, a weak anion exchanger. Proteins were eluted with a saline gradient from 0 to 150 mM NaCl. This elution presented one peak with AH. Through these steps of purification, it was possible to eliminate several bands resulting in a profile with 5 bands only. Therefore, it can be concluded that the lectins present in the serum of tambaqui are acid proteins able to recognize galactose and fucose. In the near future, we intend to subject this material to a molecular exclusion chromatography and to a mass spectrometry.

Keywords: *Colossoma macropomum*; Lectin; Tambaqui, Purification.

Financial support: CNPq.

XXXIX Annual Meeting of Brazilian Biochemistry and Molecular Biology Society – SBBq

Foz do Iguaçu, PR, Brazil, May 18 to 21, 2010

Identification and Parcial Caracterization of Mannose-Binding Lectin (MBL) of Serum from Nile tilapia (*Oreochromis niloticus)*

Silva, C.D.C.[1], Lino, M.A.S.[1], Coriolano, M. C. [1], Santos, A.J.G. [2], Maciel de Carvalho, E.V.M.[1], Coelho, L.C.B.B.[1]

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Lectins are proteins or glycoproteins that recognize carbohydrate with a high degree of specificity by, at least, two binding sites. They agglutinate cells, precipitate polysaccharides, glycoproteins and glycolipids. Soluble plasma lectins are a first-line of host defense that can initially recognize pathogens, therefore, have shown the biological importance of these proteins in fish immunology. MBL is an important constituent of the innate immune system and 1 of the 30 or so proteins of the complement system and have been reported in trout, salmon, carp, rohu fish, channel catfish, blue catfish. MBL was the identification and parcial characterization of mannose bind lectin of serum lectins nile tilapia (*O. niloticus*), with precipitation with ammonium sulfate (0-20%, F1; 20-40%, F2; 40-60%, F3; 60-80%, F4), carbohydrate specificity, affinity and polyacrylamide gel (7,5% SDS-PAGE). F2, the higher hemagglutinating activity (HA, 512-1), was applied to the affinity chromatography and the adsorbed proteins were eluted with 200mM Metil-α-D-mannopyranoside. The electrophoresis of the fractions was distinct, under reducing and non-reducing conditions indicating the presence of disulfide bonds. The only peak of adsorbed material with activity was revealed in electrophoresis native proteins. This study describes the presence of MBL in serum from Nile tilapia.

Keywords: MBL, Identification, Affinity Chromatography, Tilapia.
Support: CNPq.

XXXIX Annual Meeting of Brazilian Biochemistry and Molecular Biology Society – SBBq

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Purification and Partial Characterization of a Galactose Lectin from *Beijupirá* Fish (*Rachycentron canadum*) Serum

Coriolano, M. C. [1], Silva, C.D.C.[1], Lino, M.A.S.[1], Santos, A.J.G. [2], Maciel de Carvalho, E.V.M.[1], Coelho, L.C.B.B.[1]

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Lectins constitute a group of proteins that have a significant role in host immune response of most animals. They bind specifically to cell surface carbohydrates, acting as opsonins, stimulating pathogen destruction by macrophages. The bacteria recognition is a property reported by many fish lectins. A galectin recognizing bacteria was identified in salmon eggs. The main aim of this work was the purification and partial characterization of a galactose lectin from *Beijupirá* fish (*Rachycentron canadum*) serum, through fractionation with ammonium sulfate (0-20%, F1; 20-40%, F2; 40-60%, F3; 60-80%, F4), hemagglutinating activity (HA), carbohydrate specificity, affinity chromatography, SDS-PAGE (7.5%, w/v) and native protein electrophoresis. F3 showed the highest HA (256^{-1}) and was totally inhibited by galactose (200 mM); after affinity chromatography in guar gel column a peak with activity was obtained. The electrophoresis of the fractions was distinct, under reducing and non-reducing conditions. One active peak of adsorbed material was revealed in electrophoresis to native proteins. Therefore, it can be concluded that a lectin present in the *Beijupirá* serum is an acidic protein that recognizes galactose being a member of the galectin group.

Keywords: fish lectins, immune response, galectin, purification, characterization.
Support: CNPq, FACEPE, PRONEX/FACEPE.

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Foz do Iguaçu, PR, Brazil, May 18 to 21, 2010

Knowledge Transposition from Tropical Fish Serum Proteins to Fundamental Education Students Through Biochemical Models

Maciel de Carvalho, EVM[1]; Bezerra, RF[1]; Leite, KM[1]; Fuchs, J[1]; Cahú, GGOM[1]; Silva, CDC[1]; Lino, MAS[1]; Coriolano, MC[1]; Correia, MTS[1]; Paiva, PMG[1]; Souza, SR[2]; Coelho, LCBB[1]

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The subject was represented and discussed at The National Week of Science and Technology, UFPE, an initiative from The Ministry of Science and Technology to encourage children and people in science and technology activities. The work aimed to renew the importance to transmit knowledge from simple, imaginative, biochemical models and interactive teaching. The stand tool contained an aquarium with fishes, five scale models showing peptide bond, carbohydrate inhibited lectin molecule, hemagglutination reaction, lectin-bacterium surface interaction and enzyme-substract-inhibitor. Posters described tropical fish importance and methods applied to obtain fish serum and organs to purify lectins and protein inhibitors as well as to extract tissue DNA; notions were transmitted on fish immunology and diseases. The students were attracted and impressed with the exotic fishes most cultivated in Brazil; they asked if it is necessary to kill the fish to extract lectin and about lectin importance. Students were also interested to know if all fish enzyme/inhibitors are favorable to the own fish organism. The work succeeded to inform and stimulate future scientists in the field and to awake their scientific curiosity.

Keywords: Biochemical Models, Fish Proteins, Interactive Teaching
Supported: CNPq, CAPES, FACEPE, PRONEX/FACEPE.

Sociedade Brasileira de Bioquímica e Biologia Molecular – SBBq

X Reunião Regional Nordeste SBBq – Pesquisa Científica no Semiárido e Desenvolvimento Sustentável
Salvador- BA 30/10 a 01/11 de 2010

Isolation and Partial Characterization of a Potential Mannose-Binding Lectin from Nile Tilapia (*Oreochromis niloticus*) plasma

Silva, C.D.C.[1], Lino, M.A.S.[1], Coriolano, M. C.[1], Bezerra, R. S.[1], Santos, A.J.G.[2], Maciel de Carvalho, E.V.M.[1], Coelho, L.C.B.B.[1]

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Lectins are a group of sugar-binding proteins that recognize specific carbohydrate structures and agglutinate various cells by binding to cell-surface glycoconjugates. Mannose-Binding Lectin (MBL) is an important constituent of innate immune system and is able to recognize mannose present on pathogen surface. In teleost fish, it is suggested that MBL is the first-line of host defense that can activate the complement system and therefore has biological importance in fish immunology. The main aim of this work was the partial purification and characterization of tilapia fish (*Oreochromis niloticus*) plasma lectin(s) by fractionation with ammonium sulfate (0-20%, F1; 20-40%, F2; 40-60%, F3; 60-80%, F4), carbohydrate specificity, affinity chromatography (Concanavalin A Sepharose 4B) and polyacrylamide gel electrophoresis containing sodium dodecyl sulphate (7.5% SDS-PAGE). F2, totally inhibited by methyl-α-D-mannopyranoside (200 mM) and D-mannose (200 mM), had the highest specific hemagglutinating activity (SHA, 331) and was applied to the affinity chromatography. Adsorbed proteins were eluted with methyl-α-D-mannopyranoside. The electrophoresis of fractions was distinct, under reducing and non-reducing conditions indicating the presence of disulfide bonds. The unique active peak of adsorbed material was revealed in electrophoresis for native proteins. In conclusion, this study describes the partial purification and characterization of potential MBL from Nile tilapia plasma.

Keywords: MBL, Affinity Chromatography, *Oreochromis niloticus*.
Support: CNPq, PRONEX/FACEPE.

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A Galactose Lectin from *Beijupirá* Fish (*Rachycentron canadum*) Plasma: Purification and Partial Characterization

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Lectins represent a diverse group of multivalent sugar binding proteins of non-immune origin. They are ubiquitous in nature, have a unique property to bind to carbohydrate moieties on cell surfaces, and play an important role in cellular events like agglutination, proliferation, opsonization, signal transduction, metastasis and apoptosis. The discovery of fish lectins has added a new dimension in lectin biology and fish immunology. Lectins have so far been isolated from serum, plasma, skin mucus, egg surfaces and egg components of fish. Lectins with affinity for galactose appear to have important roles in modulating immune responses in fish. A galectin recognizing bacteria was identified in salmon eggs. The main aim of this work was the purification and partial characterization of a galactose lectin from *Beijupirá* fish (*Rachycentron canadum*) plasma, through fractionation with ammonium sulfate (0-20%, F1; 20-40%, F2; 40-60%, F3; 60-80%, F4), hemagglutinating activity (HA), carbohydrate specificity, affinity chromatography, SDS-PAGE (7.5%, w/v) and native protein electrophoresis. F3 showed the highest HA (256^{-1}) and was totally inhibited by galactose (200 mM); after affinity chromatography in guar gel column a peak with activity was obtained. The electrophoresis of the fractions was distinct, under reducing and non-reducing conditions. One active peak of adsorbed material was revealed in electrophoresis to native proteins. Therefore, it can be concluded that a lectin present in the *Beijupirá* plasma is an acidic protein that recognizes galactose being a member of the galectin group.

Keywords: fish lectins, immune response, galectin, purification, characterization.
Support: CNPq, FACEPE, PRONEX/FACEPE.

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IDENTIFICATION OF MANNOSE BINDING LECTIN (MBL) IN TISSUES OF NILE TILAPIA (O*reochromis niloticus*)

Lino, M.A.S.[1]; Silva, C.D.C.[1]; Vila Nova, M.X.[1]; Fuchs, J.[1]; Coriolano, M.C.[1]; Freitas, A.C.[2]; Bezerra, R.S.[1]; Santos, A.J.G.[3]; Maciel de Carvalho, E.V.M.[1]; Coelho, L.C.B.B.[1]

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Mannose binding lectin (MBL) is a C-type lectin that plays an important role in the first line of host defense. The lectin is well studied in various teleost fishes, although the detail of correlation to the fish immunity is unclear. Teleost fish MBL is able to recognize mannose, N-acetyl-D-glucosamine (GlcNAc) and glucose. The main objective of this work is to detect the presence of the MBL gene in tissues of Nile tilapia (*Oreochromis niloticus*) a species with high economic importance to aquaculture in Brazil. Experimental assays were performed to optimize the amplification of the gene expression of MBL by PCR (Polymerase Chain Reaction). Samples of total genomic DNA isolated from kidney tissue of Nile tilapia were subjected to PCR with two pairs of primers MBL(1) and MBL(2). These primers were designed from the database homology existing in the sequences of MBL fishes selected from the National Center for Biotechnology Information (NCBI). The use of MBL(2) primer resulted in a single band amplified with high sharpness, approximately 550 bp, suggesting analogy with MBL identified in *Danio rerio*. The lectins are important for the innate immune system of fish; they can recognize units of sugars on the surface of many microorganisms allowing its opsonization and phagocytosis. The detailed analysis of innate immune molecules related to fish, including their function and structure, will generate new technologies that can be applied to improve aquaculture.

Financial Support: FACEPE, CNPq, CAPES.

Key words: Innate immunity; C-type lectin; mannose binding lectin; *Oreochromis niloticus*; aquaculture.

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Purification and Characterization of a Mannose-recognition Lectin from *Oreochromis niloticus* (Tilapia fish): Cytokine Production Induced in Mice Splenocytes

Silva, CDC [1]; Coriolano, MC [1]; Lino, MAS [1];Moutinho, CLM [3]; Souza, SR [1]; Maciel de Carvalho, EVM [1]; Santos, AJG [2]; Pereira, VRA [3]; Coelho, LCBB[1]

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Lectins are carbohydrate binding proteins which can mediate immunological functions. Immunological assays are important to evaluate biological responses of fish lectins but few studies had investigated immunomodulatory profile induced by these molecules. The aim of this work was to purify and partially characterize a mannose recognition lectin from serum of Nile tilapia fish, *Oreochromis niloticus* lectin, OniL, through precipitation with ammonium sulfate, affinity chromatography, carbohydrate specificity, and SDS-PAGE. *In vitro* lectin immunomodulatory activity on mice splenocyte experimental cultures was investigated through cytotoxic assays and cytokine production. F2, totally inhibited by methyl-α-D-mannopyranoside, had the highest specific hemagglutinating activity (SHA, 331) and was applied to the affinity chromatography. Adsorbed proteins were eluted with methyl-α-D-mannopyranoside. OniL SDS-PAGE (7.5 % gel) under reducing conditions showed an apparent molecular weight of 17.6 kDa with a single band. Immunological assays, *in vitro*, showed that OniL did not reveal cytotoxicity against splenocytes; the lectin induced nitrite release, higher IFN-γ production and lower IL-10 level. OniL was purified and characterized showing preferential Th1 response in mice splenocytes.

Keywords: *Oreochromis niloticus*, lectin purification, immunomodulatory activity.
Supported by: CNPq, FACEPE.

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Immunomodulatory Response on Mice Splenocytes Induced by a Lectin Isolated from Bijupirá fish (Rachycentron canadum) Serum, RcaL

Coriolano, M. C.[1]; Silva, C. D. C.[1]; Melo, C. M. L.[2]; Bezerra, R. S.[1]; Santos, A. J. G.[3]; Pereira, V. R. A. [2]; Coelho, L. C. B. B.[1]

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Lectins constitute a group of proteins that have a variety of functions in host immune response of most animals. The aim of this work was the purification and characterization of a serum lectin from *Bijupirá* fish (*Rachycentron canadum*), RcaL. Furthermore, we evaluated its cytotoxicity against mice splenocytes and immunomodulatory activity over cytokine and nitric oxide production. Fractionation with ammonium sulfate (F3), hemagglutinating activity (HA), carbohydrate specificity, affinity chromatography, SDS-PAGE (7.5%, w/v) and native protein electrophoresis (PAGE) were performed; also cytotoxicity against mice splenocytes and immunomodulatory assays were evaluated. F3 showed the highest HA (128^{-1}) and was totally inhibited by methyl-α-D-mannopyranoside (200 mM); after affinity chromatography, a peak with activity was obtained. Under reduced conditions a main polypeptide band of 19.2 kDa was revealed in SDS-PAGE; PAGE confirmed RcaL as an acidic protein revealed in a single peak. Cytotoxic and immunomodulatory assays with RcaL in mice splenocyte cultures showed that the lectin was not cytotoxic and induced higher IFN-γ and nitric oxide production in splenocyte cultures. Purified RcaL induced preferential Th1 response suggesting an action as immunomodulatory compound.

Word Keys: *Rachycentron canadum*, fish lectin, immune response.
Supported by: FACEPE, CNPq.

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Protective Effect of *Oreochoromis niloticus* Lectin (OniL) Against Oxidative Stress Caused by Tert-Butyl Hidroperoxide (t-BOOH) in B16-F10 murine melanoma cells

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Oxidative stress occurs when antioxidant mechanisms are overwhelmed by ROS; the resulting damage can lead to cell death, necrotic or apoptotic. t-BOOH is an organic hydroperoxide, broadly used to induce oxidative stress in a variety of cells. Numerous antioxidant methods have been developed to evaluate antioxidant activity and to explain protective function and biochemical mechanism of t-BOOH to ROS levels and antioxidants. This study aimed to evaluate the protective action of *Oreochoromis niloticus* lectin (OniL) against oxidative stress induced by t-BOOH as well as to measure viability, ROS and death of B16-F10 murine melanoma cells. Melanoma (10^6 cells) was treated with OniL; after 24 h cell oxidative stress was induced with 400 µM t-BOOH for 2 h. The cell viability was determined by trypan blue exclusion assay; cells were counted in a Neubauer chamber. Cell viability was evaluated by annexin V conjugated with fluorescein isothiocyanate and propidium iodide. Annexin-FITC- / PI+ cells were considered necrotic and annexin-FITC+/PI- represented cells in apoptosis. ROS mitochondrial production was marked with MitoSOX Red probe (5 µM) and monitored by flow cytometer. Nonparametric assays were used in statistical analysis. OniL had protective action in melanoma against toxicity induced by t-BOOH and decreased necrotic cells, but did not influence ROS production. We conclude that OniL protects against cell death caused by t-BOOH.

Word Keys: Lectin, Oxidative Stress, Protective Effect.
Supported by: CAPES, CNPq, FACEPE and FACEPE/PRONEX

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Cidade Universitária Zeferino Vaz, 19 de junho de 2012.

DECLARAÇÃO DE ESTÁGIO

Declaro para os devidos fins que a aluna Cynarha Daysy Cardoso da Silva, Doutoranda pelo Programa de Pós-Graduação em Ciências Biológicas/UFPE, estagiou no Laboratório de Bioenergética da Faculdade de Ciências Médicas da UNICAMP nos períodos de 5 de julho a 4 de setembro 2011, 01 de novembro a 16 de dezembro 2011 e de 09 a 30 de abril de 2012. Nestes períodos Cynarha realizou estudos de atividade biológica da lectina OniL, presente no soro do peixe Tilápia (*Oreochromis niloticus*), sobre respiração, fosforilação oxidativa e transporte de íons de cálcio em mitocôndrias isoladas de fígado de rato e de viabilidade e morte celular em cultura de células tumorais. Estes estudos envolveram as técnicas de espectrofluorimetria, respirometria e citometria de fluxo.

Atenciosamente,

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