Universidade Federal de Pernambuco

Centro de Ciências Biológicas

Programa de Pós-Graduação em Ciências Biológicas

Tese de Doutorado

Lectina de tilápia do Nilo (*Oreochromis niloticus*): identificação, purificação, caracterização gênica e avaliação de atividade imunomoduladora

Mércia Andréa da Silva Lino

Orientadora: Profª Drª Luana Cassandra Breitenbach Barroso Coelho (UFPE)

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2012

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À minha família, com todo o meu amor...

In the Name of Allah the Most Merciful, Beneficent and the Most Powerfull

SUMÁRIO

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RESUMO

Lectinas são proteínas ligantes de glicanos conhecidas para desempenhar papéis-chave na imunidade inata e resistência a doenças em peixes. O principal objetivo desse estudo foi identificar, purificar e caracterizar lectinas imuno-ativas presentes nos tecidos (baço e rim) e no soro da tilápia do Nilo, *Oreochromis niloticus*, um ciclídeo africano, considerado o carro-chefe da piscicultura brasileira. Inicialmente o DNA genômico obtido a partir dos tecidos da tilápia pelo protocolo CTAB 2% foi amplificado por PCR usando *primers* desenhados por homologia com diferentes organismos, purificado, sequênciado e analisado com ferramentas da bioinformática disponíveis em diferentes bancos de dados públicos. A partir dos tecidos renais foi possivel obter uma banda única, com concentração 40,5 ng/µL e alta qualidade (A260/A280 = 1,8), verificada em espectrofotômetro. A sequência (969pb) foi denominada receptor de lectina tipo C de *Oreochromis niloticus* (OniLCLR) e quando analisada pelo Blastn/NCBI, exibiu padrões não convencionais que reconhecem pequenos fragmentos de sequências de DNA, sem homologia tradicional (baixa identidade), no entanto, apresentou semelhanças com vários genes relacionados ao sistema imune e diferentes aspectos funcionais. OniLCLR contém ORF que codificam dois peptídios: um é homólogo ao domínio de lectina tipo C, membro da família A-10, denominado OniLCLEC10A (47% de identidade) e o outro homólogo ao Receptor-1 de lipoproteína oxidada de baixa densidade (25% de identidade), nomeado OniLORL1, ambos pertencentes à família dos receptores de lectina tipo C. As análises das relações entre as sequências, estruturas e funções desses peptídios revelaram que essas lectinas desempenham papéis essenciais como antígeno de superfície celular, inflamação e na arteriogênese, sugerindo seus envolvimentos no sistema imune da tilápia. Em outra abordagem, uma nova proteína denominada lectina de *Oreochromis niloticus* (OniL) foi purificada e parcialmente caracterizada a partir do soro do peixe. A purificação foi realizada por precipitação com sulfato de amônio (fração F20-40%) e pelo método cromatográfico em matriz de afinidade Con A Sepharose 4B, eluída com metil-α-D-manopiranosídeo (200 mM) em TBS. Foram avaliados parâmetros tais como especificidade a carboidratos, testes de temperatura e íons, eletroforese SDS-PAGE e PAGE. Ensaios imunológicos, *in vitro*, para avaliar a atividade imunomoduladora em culturas experimentais de esplenócitos de camundongos BALB/C também foram conduzidos. Índices de citotoxidade, níveis de produção de citocinas e o tipo de resposta imune celular foram avaliados. OniL apresentou massa molecular 17 kDa constituída por duas subunidades de 11 e 6,6 kDa, em condições redutoras, indicando a presença de pontes dissulfeto. OniL apresentou afinidade para os carboidratos metil-α-D-manopiranosídeo e D-manose. A atividade de Onil foi completamente abolida em temperaturas acima de 70 °C. A adição de EDTA diminuiu sua atividade e revelou que OniL é Ca^{2+} dependente. Os ensaios imunológicos demonstraram que OniL não apresenta citotoxicidade em esplenócitos de camundongos, induziu elevada produção de IFN-γ, baixa produção de IL-10 e não foi eficiente na liberação de óxido nítrico. A secreção de IFN-γ estimulada por OniL revelou diferenciação de células preferencialmente do tipo Th1, assim, sugere-se que esta proteína seja um potencial imunomodulador em mamíferos.

Palavras-chaves: Receptores de lectina tipo C, atividade imunomoduladora, sistema imune, tilápia do Nilo, *Oreochromis niloticus*, aquicultura.

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ABSTRACT

Lectins are glycan-binding proteins, known to play key roles in innate immunity and disease resistance in fish. The main objective of this study was to identify, purify and characterize immune-active lectins present in tissues (spleen and kidney) and serum of Nile tilapia, Oreochromis niloticus, an African cichlid, considered the flagship of the Brazilian fish farming. Initially the genomic DNA obtained from the tissues of tilapia by CTAB 2% protocol was amplified by PCR using primers designed by homology with different organisms, purified, sequenced and analyzed with bioinformatics tools available in different public databases. From the renal tissues was possible to obtain a unique band with concentration 40.5ng/μL and high quality (A260/A280 = 1.8), verified in spectrophotometer. The sequence (969pb) was named C-type lectin receptor of *Oreochromis niloticus* (OniLCLR) and when analyzed by BLASTN/NCBI, exhibited patterns unconventional recognizing small fragments of DNA sequences, without traditional homology (identity low), however, showed similarities with several genes related to the immune system and different functional aspects. OniLCLR encoding ORF contains two peptides: homologous to C-type lectin domain family member 10 A-like, *O. niloticus* called OniLCLEC10A (47% identity) and another is homologous to the low-density lipoprotein receptor-related protein 1, *Danio rerio* (25% identity), named OniLORL1, both belonging to the superfamily of receptors of type C lectin. The analyzes of relationships between sequences, structures and functions of peptides, revealed that these lectins play essential roles as cell surface antigen, inflammation and arteriogenesis, suggesting their involvement in the immune system of tilapia. In another approach, a new protein called *Oreochromis niloticus* lectin (OniL) was purified and partially characterized from tilapia serum. Purification was by ammonium sulfate precipitation (fractions F20-40%) and by chromatographic method in affinity matrix Con A Sepharose 4B, eluted with methyl-α-D-manopiranosídeo (200 mM) in TBS. We evaluated parameters such as specificity to carbohydrates, temperature and ions test, SDS-PAGE and PAGE. Immunological assays, in vitro, to evaluate the immunomodulatory activity in experimental cultures of splenocytes of mice BALB/C were also conducted. Cytotoxicity indices, cytokines production levels and cellular immune response type were evaluated. OniL presented molecular weight 17 kDa consisting of subunits of 11 and 6.6 kDa, under reducing conditions, indicating the presence of disulfide bonds. OniL exhibited carbohydrate affinity for methyl-α-D-mannose and D-manopiranoside. Activity was completely abolished above 70 °C temperature. EDTA addition decreased activity and revealed that OniL is Ca^{2+} dependent. The immunological assays revealed that OniL no cytotoxicity in mice splenocytes, induced IFN-γ high, low production of IL-10 and was not effective in the release of nitric oxide. IFN-γ secretion stimulated by OniL revealed differentiation cell Th1 preferentially, thus suggesting that this protein is an immunomodulatory potential in mammals.

Key words: C-type lectin receptor, immunomodulatory activity, immune system, *Oreochromis niloticus*, Aquaculture.

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LISTA DE ABREVIATURAS

AH – Atividade Hemaglutinante

ATP – Trifosfato de Adenosina

ATPase - Hidrólise do Trifosfato de Adenosina

BLAST - Ferramenta de Pesquisa Básica de Alinhamento Local

C – Controle

Ca^{2+} - Íon Cálcio

CC – Carboidrato Competidor

CCP - Proteína de Controle do Complemento

CD83 - Antígeno CD83

CLEC10A – Dominio da Lectina tipo C, Membro da Família 10 A

CLEC3B – Domínio da Lectina tipo C, Membro Família 3B

CLEC7A – Domínio da Lectina tipo C, Membro da Família 7A

CLR – Receptor de Lectina tipo C

ComaSeL – Lectina do Soro do *Colossoma macropomum*

Con A Sepharose 4B - Concanavalina A-Sepharose 4B

CRD - Domínio de Reconhecimento a Carboidrato

CRP – Proteína C Reativa

CTAB - Brometo de Cetil Trimetil Amônio

CTL - Lectina tipo C

CTLD - Domínio Semelhante a Lectina tipo C

DC - Células Dendríticas

DC-SIGN-like - Molécula de Adesão Intracelular Especifica de Célula Dendrítica

DNA - Ácido Desoxirribonucleico

DNOCS - Departamento Nacional de Obras Contra as Secas

dNTP - Trifosfatos Desoxirribonucleótido (dATP, dCTP, dGTP, dTTP)

E – Eritrócito

EDTA - Ácido Etilenodiamino Tetra-Acético

ELISA (do inglês: Enzyme Linked Immunosorbent Assay)

EMC – Carboidrato da Membrana do Eritrócito

EPD - Motivo de Ligação Glu-Pro-Asp

EPN - Motivo de Ligação Glu-Pro-Asn

EST - Etiqueta de Sequência Expressa

FAO - Organização das Nações Unidas para a Agricultura e Alimentação

FASTA – Precursor dos Programas de Alinhamento

GC - Guanina e Citosina

GC3 - Conteúdo de GC na Terceira Posição do Códon (Guanina Citosina)

GenBank – Banco de Dados Americano de Sequências de DNA e Proteínas

HA – Atividade Hemaglutinante

IAH – Inibição da Atividade Hemaglutinante

IFN-γ – Interferon Gama

IL1 – Interleucina 1

IL-10 – Interleucina 10

IL6 – Interleucina 6

kDa - Kilodalton

L – Lectina

LDL - Lipoproteína de Baixa Densidade

Lectina tipo F – Fucolectina

Lectina tipo I - Lectina da Superfamilia Imunoglobulina

MBP - Proteína de Ligação a Manose

MEGA - Análise Molecular Genética Evolutiva

$MgCl_2$ – Cloreto de Magnésio

NCBI – Centro Nacional de Informações sobre Biotecnologia

NK - Células Natural Killer

NKG2/CD94 - Receptores de CD94/NKG2

NO – Óxido Nítrico

OniL – Lectina de *Oreochromis niloticus*

OniLCLEC10A – Lectina tipo C de *Oreochromis niloticus* membro10A

OniLCLR - Receptor de lectina tipo C de *Oreochromis niloticus*

OniLORL1 - Receptor-1 de lipoproteína oxidada de baixa densidade de *Oreochromis niloticus*

ORF - Quadro de Leitura Aberta

ORL-1 - Receptor-1 de Lipoproteína Oxidada de Baixa Densidade

PAGE - Eletroforese em Gel de Poliacrilamida

PAMP - Padrões Moleculares Associados a Patógenos

PCR – Reação em Cadeia da Polimerase

pH – Potencial Hidrogeniônico

PRR - Receptores de Reconhecimento de Padrões

QPD - Motivo de Ligação Gln-Pro-Asp

QPS - Motivo de Ligação Gln-Pro-Ser

RBL – Lectina de Ligação L-Ramnose

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

Siglec-4 - Proteína 4 de Ligação ao Ácido Siálico

SP-A - Proteína Surfactante Pulmonar A

SP-D - Proteína Surfactante Pulmonar D

S-type – Lectina tipo S

TAE - Tris-Acetato-EDTA (Ácido Etilenodiamino Ttra-Acético)

TBS – Tampão Fosfato Salino

Th1 – Células T *helper* 1

Th2 - Células T *helper* 2

TNF – Fator de Necrose Tumoral

UV – Luz Ultravioleta

w/v – Relação Peso/Volume

YPG - Motivo de Ligação Tyr-Pro-Gly

YPT - Motivo de Ligação Tyr-Pro-Thr

Zn^{2+} - Íon Zinco

1 INTRODUÇÃO

Os peixes são vertebrados antigos e bastante diversificados que vivem em diferentes ecossistemas aquáticos. Eles convivem naturalmente com uma variedade de microorganismos e esse sucesso adaptativo é decorrente do seu sistema imunológico, cuja eficácia os protege contra o ataque de agentes patogênicos.

Embora os peixes apresentem um sistema de defesa composto por imunidade inata e adaptativa o estresse gerado pelas variações ambientais, aliado ao frequentemente manejo realizado nos sistemas de produção intensiva, pode diminuir a capacidade imunitária desses organismos e favorecer o surgimento de doenças.

Apesar de todos os esforços para produção de peixes saudáveis, as doenças infecciosas constituem-se uma ameaça em potencial e podem originar impactos ambientais e econômicos, portanto, muitos pesquisadores têm focado seus estudos no sistema imunológico dos peixes, tendo em vista uma melhor compreensão dos mecanismos de defesa desses vertebrados e assim contribuir com o desenvolvimento e a sustentabilidade do setor aquícola. A aquicultura é uma atividade que exerce um nobre papel, especialmente na produção de peixes que são fonte de proteínas de elevado valor para alimentação humana, entre outras finalidades.

As lectinas são proteínas consideradas moléculas-chave na defesa e resistência a doenças em peixes. Elas estão frequentemente relacionadas com a modulação das respostas imunes inatas consideradas as mais importantes para esses organiamos. Diversos estudos sobre as lectinas de peixes são conduzidos atualmente cujas estratégias utilizadas são fundamentadas, na maioria das vezes, em tecnologias de purificação de proteínas e/ou biomoleculares que investigam essas proeínas em diferentes tecidos e/ou fluidos corpóreos (soro, plasma, muco), contribuindo de forma simples para identificação, isolamento e caracterização desse diversificado grupo de moléculas.

Com o acelerado desenvolvimento da aquicultura brasileira os estudos das lectinas tornam-se fundamentais fornecendo importantes informações sobre o sistema imune das espécies nativas e exóticas, especialmente as de importância econômica para a piscicultura. Recentemente as lectinas do bijupirá *Rachycentron canadum* (RcaL), tambaqui *Colossoma macropomum* (ComaSeL) e pirarucu *Arapaima gigas* foram identificadas e isoladas no Laboratório de Glicoproteínas do Departamento de Bioquímica da Universidade Federal de Pernambuco, Brasil.

A tilápia do Nilo, *Oreochromis niloticus* é uma espécie exótica de origem africana cultivada em diversos países do mundo que vem se destacando de forma absoluta dentro do cenário aquícola brasileiro. Possui elevado valor econômico e é cultivada principalmente na região Nordeste do país, contribuindo deste modo para alimentação, geração de emprego e renda e com o desenvolvimento da piscicultura nacional. O estudo das lectinas da tilápia tem em vista fornecer informações sobre os

mecanismos inatos de defesa da espécie e contribuir melhoria dos cultivos. Segundo relatos o estudo dessas proteínas pode, por exemplo, servir como base para programas de melhoramento genético, para o desenvolvimento de peixes transgênicos que expressem níveis elevados de lectinas, especialmente em períodos frios, para prevenção de doenças em programas de saúde dos peixes e para geração de vacinas. Diversos trabalhos tem demonstrado que as lectinas protegem os peixes contra o ataque de diferentes patógenos, tais como fungos, bactérias e vírus. A identificação e caracterização de lectinas nos peixes podem também servir de base para produção de novas drogas e muitas outras aplicações biológicas.

Neste estudo, utilizando-se diferentes técnicas, foi possível identificar e caracterizar a partir do soro e tecidos da tilápia do Nilo, uma nova lectina denominada lectina de *O. niloticus* (OniL) e um gene que codifica pelo menos duas lectinas transmembranares que são membros da família dos receptores de lectina tipo C, o qual foi nomeado como receptor de lectina tipo C de *O. niloticus* (OnilCLR). Futuras aplicações biotecnológicas relacionadas às lectinas identicadas serão implementadas, dando continuidade aos estudos de caracterização e uma maior abrangência sobre as suas funções imunes na tilápia.

1.1 Sistema imune dos peixes: considerações básicas

Os peixes possuem elevada biodiversidade e ocorrem em diferentes ecossistemas aquáticos. Eles possuem plena capacidade de sobrevivência e adaptabilidade graças ao seu sistema imunológico, um componente ativo que assegura a proteção e defesa dos organismos contra diversos agressores e agentes patogênicos (TORT *et al.*, 2003).

São considerados os vertebrados mais primitivos que possuem sistema imune inato (ou natural) e adaptativo (ou adquirido), formado por uma cascata extremamente complexa de reações e vários constituintes, tais como: órgãos, células e moléculas, sinalizadores, etc (tabela 1), que em conjunto, contribuem para uma defesa notavelmente eficaz (MAGNADÓTTIR, 2006; MAGNADÓTTIR, 2010). Os peixes são excelentes modelos para estudo, uma vez que seu sistema imunológico apresenta notáveis semelhanças com os mamíferos (TORT *et al.*, 2003; TRAVER *et al.*, 2003).

Apesar da complexidade, o sistema imune dos peixes pode ser afetado por fatores internos (variações genéticas, idade, sexo, etc) e externos, tais como, mudanças ambientais (temperatura, pH, O_2, nível de CO_2, nitrito, carga orgânica, poluentes), fatores nutricionais. Quando cultivados, os peixes são submetidos a elevadas taxas de estocagem à medida que os sistemas de produção se intensificam. Excessivas práticas de manejo e variações ambientais abruptas podem aumentar o estresse que por sua vez, pode alterar o padrão fisiológico normal e adaptativo do organismo e,

consequentemente, suas respostas imunes (inatas e adaptativas) são profundamente afetadas (MAGNADÓTTIR, 2006; MAGNADÓTTIR, 2010).

O sistema imune inato é o mais primitivo sendo considerado o mais importante, pois é usado por todos os organismos na defesa contra invasão microbiana (TORT *et al.*, 2003). A imunidade inata se constitui a vanguarda da defesa imunológica portanto é cruciaial na resistência às doenças nos peixes, considerando que esses vertebrados possuem apenas as IgM de todas as imunoglobulinas (EWART *et al*., 2001; KILPATRICK, 2002; MAGNADÓTTIR 2006; MAGNADÓTTIR, 2010).

Tabela 1. Sistema imune inato dos peixes

COMPONENTES		**ELEMENTOS BÁSICOS CONSTITUINTES**
I	**Barreiras Físicas/Natuarais**	Escamas, superfície epitelial e muco (branquias, pele, intestino)
II	**Componentes Humorais**	Complemento (vias clássica e alternativa), lisozima, lectina, peptideos antimicrobianos, transferrina, interferon, proteína C reativa, fatores de ativação de macrófagos
III	**Componentes Celulares**	Células fagocíticas semelhantes a monócitos/macrófagos, granulócitos (neutrófilos, eosinófilos e basófilos), células Natural Killer, linfócitos B e T, complexo principal de histocompatibilidade

Fonte: Adaptado de MAGNADÓTTIR (2006); MAGNADÓTTIR (2010).

As lectinas são definidas como proteínas ou glicoproteínas de ligação a glicanos e ocorrem em todos os organismos vivos. Elas interagem com seus ligandos sem modificá-los (figura 1) e medeiam diferentes processos fisiológicos e patológicos (RUDIGER & GABIUS, 2001; SHARON & LIS, 2001; SÁ *et al*., 2009; SANTOS *et al*., 2011).

As lectinas são consideradas ferramentas essenciais para explicar diferentes mecanismos imunológicos inatos dos peixes e, recentemente, tem sido identificadas como reguladoras críticas das respostas adaptativas em mamíferos (COOK *et al*., 2005; NGAI & NG, 2007). Os estudos sobre as propriedades características das lectinas, suas funções, evolução gênica e potenciais aplicações biológicas se intensificaram consideravelmente nos últimos anos (OGAWA *et al.*, 2011; VASTA *et al*., 2011).

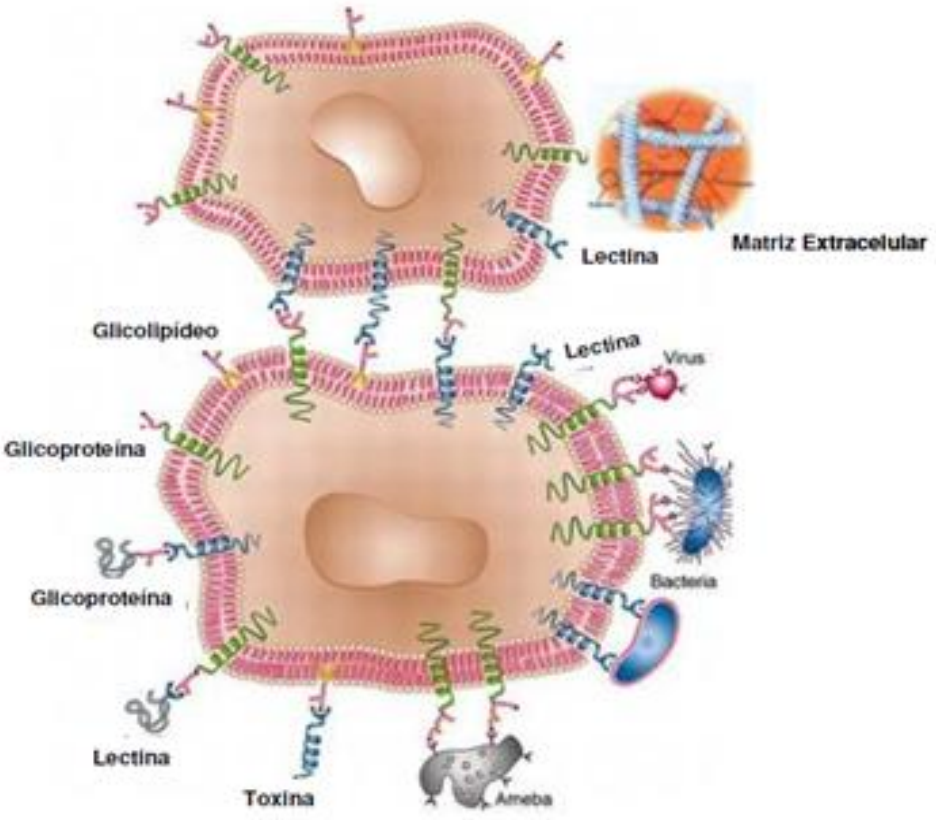

Figura 1. Interações das lectinas na superfície celular. As lectinas interagem com os seus ligandos por meio de diferentes formas, por exemplo, proteína-proteína, proteína-lipídios, proteína-ácidos nucléicos, toxinas, fixação de microrganismos. *Fonte: SHARON & LIS (2004) - Baseada em um esquema original de BioCarbAB (Lund, Sweden).*

1.2 Lectinas como mediadoras da resposta imune inata

As respostas imunes inatas (inespecíficas) são imediatas e obtidas através de um grupo de receptores de reconhecimento de padrões (PRR), tais como receptores de lectina tipo C (GEIJTENBEEK & GRINGHUIS, 2009), Receptores Toll-like (IWASAKI & MEDZHITOV, 2004) entre outros, que são proteínas ou glicoproteínas presentes em membranas celulares ou citosol que se ligam especificamente a moléculas sinalizadoras, desencadeando reações em cascata dentro das células (MEDZHITOV & JANEWAY, 2000; FUJITA, 2002; MOGENSEN, 2009).

Os receptores reconhecem padrões moleculares associados aos patógenos PAMP (lipopolissacárideos, glicoproteínas, DNA e RNA viral, peptídeos bacterianos e glucanos fúngicos), presentes na superfície celular dos microorganismos, mas não no hospedeiro, identificando, sequestrando e destruindo tais organismos, via complemento ou ataques citotóxicos (GEIJTENBEEK & GRINGHUIS, 2009; MOGENSEN, 2009).

Os receptores de lectina tipo C (CLR) compreendem uma grande família de receptores capazes de reconhecer glicanos presentes nos agentes patogênicos, através do domínio de reconhecimento a carboidratos (CRD) (WEIS & DRICKAMER, 1998; ZELENSKY & GREADY, 2004; ZELENSKY & GREADY, 2005).

O CRD compõe a estrutura típica para a maioria dos membros das lectinas tipo C. Sua estrutura é altamente conservada e mantém características específicas da sequência primária de aminoácidos ou da estrutura terciária. As interações do tipo lectina-carboidrato, exibem elevada especificidade e baixa afinidade e podem adquirir uma maior avidez quando se oligomerizam em múltiplos sítios de ligação (VASTA & AHMED, 2008; VARKI *et al*., 2009; VASTA *et al*., 2011).

De acordo com CUMMINGS & McEVER (2009), o CRD consiste de 110 a 180 resíduos de aminoácidos com vários motivos conservados; frequentemente contém duas regiões α-hélices, duas folhas-β e alguns *loops* moleculares. O CRD é fortemente associado a sítios de ligação de cátions bivalentes, e na sua estrutura, existem aproximadamente quatro sítios de ligação ao $Ca^{(2+)}$ entre os quais, dois são essenciais para a ligação a carboidratos e usados para previsão da especificidade de ligação das lectinas tipo C. O motivo de ligação ao açucar encontra-se em um dos *loops*.

Nos vertebrados, as diferentes especificidades a carboidratos são ocasionadas por pequenas variações estruturais no motivo de ligação, por exemplo, o motivo EPN (Glu-Pro-Asn) indica afinidade ao açúcar manose e o motivo QPD (Gln-Pro-Asp) para galactose. Sete tipos de motivos incluindo EPN e QPD já foram identificados em moluscos: EPD (Glu-Pro-Asp), QPG (Gln-Pro-Gly), QPS (Gln-Pro-Ser), YPG (Tyr-Pro-Gly) e YPT (Tyr-Pro-Thr) (WANG *et al*., 2011).

Apesar de possuir homologia estrutural, a dobra tipo C é bastante variada e não está limitada necessariamente ao reconhecimento de carboidratos. Os membros com domínios semelhantes às lectinas tipo C (CTLD), por exemplo, evoluíram para reconhecer uma variedade de ligandos incluindo proteínas, lipídios, carbonato de cálcio e venenos específicos de ofídios. Os CTLD são proteínas extracelulares muito comuns em animais (ZELENSKY & GREADY, 2005).

São conhecidas cerca de 17 subfamílias da superfamilia tipo C (figura 2): I-*Lecticans*; II-*Asialoglycoproteins and DC Receptors*; III-*Collectins*; IV-*Selectins*; V-*NK Cell Receptors*; VI-*Mult* CTLD *Endocytic Receptors*; VII-REG *proteins*; VIII-*Chondrolectin, Laylin*; IX-*Tetranectin*; X-*Polycystin 1*; XI-*Attractin*; XII-*Eosinophil Major Basic protein* (EMBP); XIII-DGCR2 (*Di-George Syndrome*); XIV-*Thrombomodulin*; XV-*Bimlec*; XVI-SEEC e XVII-CBCP/Frem1/QBRICK (ZELENSKY & GREADY, 2005; CUMMINGS & McEVER, 2009).

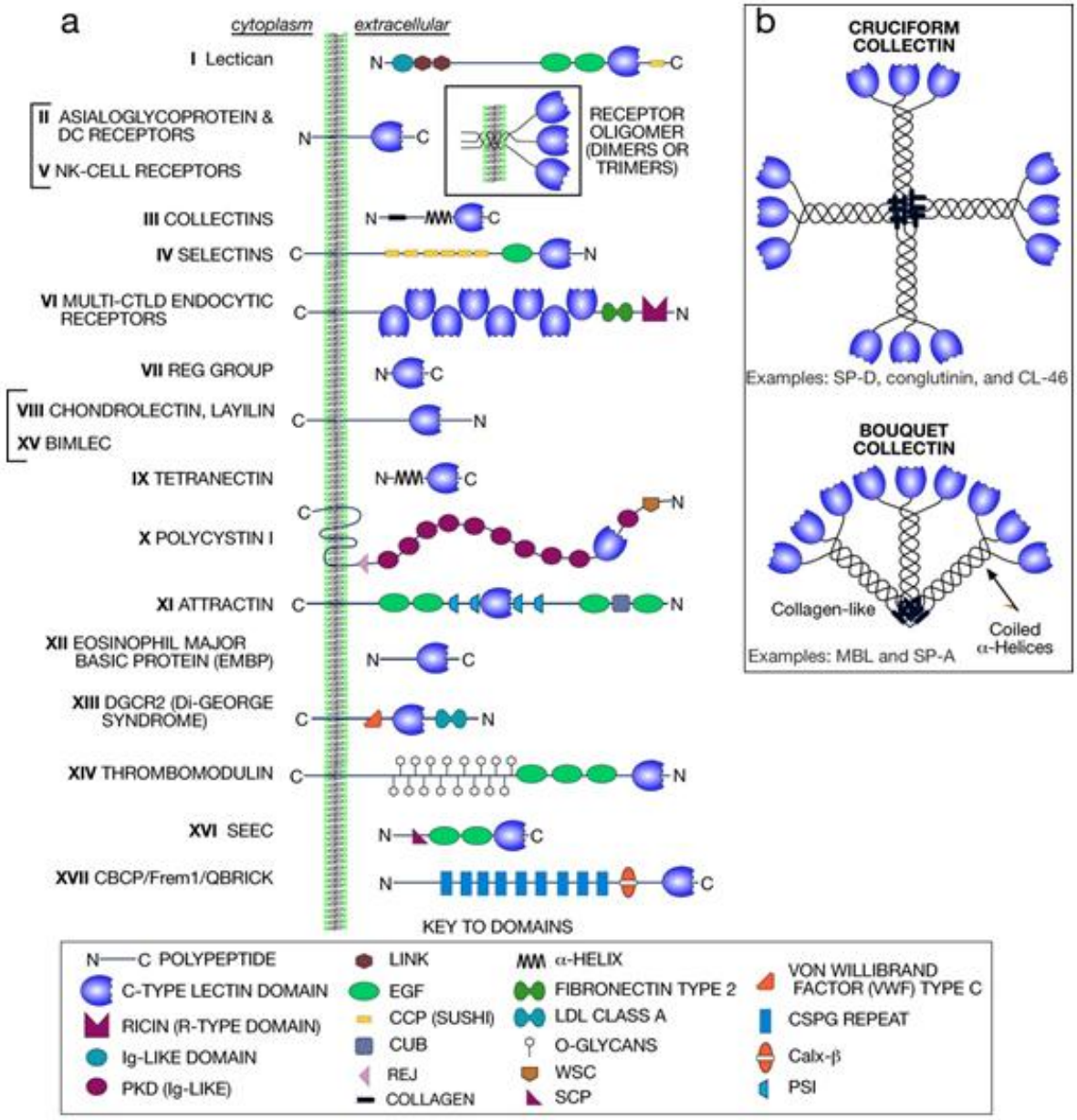

Figura 2. Diferentes familias de lectinas tipo C e domínios estruturais. Dezessete subfamilias são definidas por relações filogenéticas e estruturas dos domínios. Algumas famílias são proteínas solúveis e outras são proteínas transmembranares. As colectinas (III) formam estruturas oligoméricas em forma de cruz e *bouquet*. Cada domínio é denominado como indicado na chave: células dendríticas (DC); células natural killer (NK); domínio semelhante às lectinas tipo C (CTLD); proteína de ligação a manose (MBP); surfactantes (SP-A e SP-D); proteína de controle do complemento (CCP). *Fonte: CUMMINGS & McEVER, 2009.*

A dobra tipo C tem sido encontrada em mais de 1000 proteínas e com base na sua estrutura molecular, os CLR podem ser classificados em dois grupos de proteínas transmembranares e um

grupo de proteínas solúveis (secretadas). Diversos membros da superfamília tipo C são estudados em peixes (tabela 2).

Tabela 2. Membros da superfamília de lectinas tipo C estudados em peixes

MEMBROS	ORGANISMOS	REFERÊNCIAS
Lecticans	*Danio rerio*	(KANG *et al.*, 2004)
Asialoglicoproteinas e Receptores de células dendríticas DC	*Danio rerio* *Osmerus lanceolatus* *Oncorhynchus mykiss*	(LIN *et al.*, 2009) (HOSONO *et al.*, 2005) (ZHANG *et al.*, 2000)
Colectinas	*Gadus morhua* *Ictalurus punctatus* *Oncorhynchus mykiss* *Scophthalmus maximus* *Epinephelus coioides* *Petromyzon marinus* *Ictalurus punctatus* *Conger myriaster*	(RAJAN *et al.*, 2011) (ZHANG *et al.*, 2012) (KANIA *et al.*, 2010) (ZHANG *et. al.*, 2010) (WEI *et al.*, 2010) (OURTH *et al.*, 2008) (OURTH *et al.*, 2007) (TSUTSUI *et al*., 2007)
Selectinas	*Sparus aurata*	(CHAVES-POZO *et al.*, 2008)
Receptores de células Natural Kiler (NK)	*Oreochromis niloticus*	(KIKUNO *et al.*, 2004)
Tetranectina	*Carcharhinus springeri*	(NEAME *et al.*, 1992)
Polycystina	*Danio rerio*	(OBARA *et al*., 2006)

As proteínas transmembranares tipo I, contêm vários CRD ou domínios CRD semelhantes; as proteínas transmembranares tipo II tipicamente, contêm um único domínio CRD (CUMMINGS & McEVER 2009). Os CLR são expressos pela maioria dos tipos celulares incluindo, macrófagos, plaquetas e células dendríticas imaturas e estão envolvidos no reconhecimento de vírus, fungos, bactérias e helmintos (SARAIVA *et al.*, 2011; SILVA *et al.*, 2012).

1.3 Diversidade das lectinas animais

As lectinas animais são proteínas extremamente diversas. Até o momento, não existe nenhum consenso inteiramente aceito entre os autores para classificá-las. Elas podem ser

classificadas com base na especificidade de ligação a monossacarídeos (LIS & SHARON 1998) ou em famílias estruturais que podem ainda, ser divididas em subfamílias. De maneira geral, as lectinas estão divididas em duas categorias que inclui as "famílias de lectinas com homologias de sequências e/ou relações evolutivas" e as proteínas semelhantes às lectinas "*lectin-like proteins*", sem classificação evolutiva estabelecida (figura 3) (VARKI *et al*., 2009, VASTA *et al*., 2011).

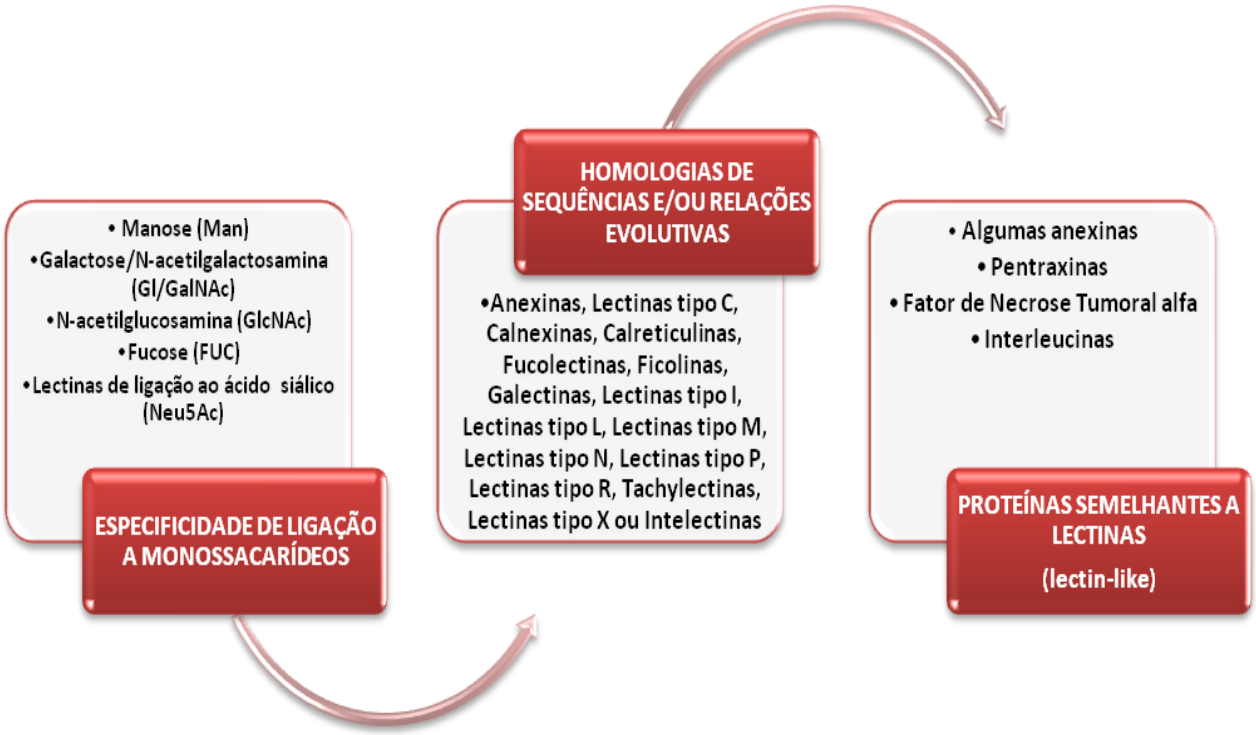

Figura 3. Critérios de classificação das lectinas animais.

Segundo Vasta *et al*. (2011), a fundamentação genética para essa elevada diversidade de lectinas inclui as duplicações de genes em tandem, o desenvolvimento de famílias multigênicas complexas (conjunto de genes originados por duplicação e variação de algum gene ancestral), a formação de estruturas quiméricas por embaralhamento de exons e a variabilidade adicional por *splicing* alternativo (processo que remove os íntrons e junta os éxons depois da transcrição do RNA). Embora o processo de replicação do DNA seja fidedigno, as mutações são conhecidas como fontes primárias de variabilidade genética, compondo a base de toda a biodiversidade. De acordo com Vasta & Ahmed (2008), a base estrutural para essa potencial "plasticidade" dos sítios de ligação a carboidratos é uma área de grande importância para os pesquisadores.

1.4 Tilápia do Nilo o "carro chefe" da piscicultura brasileira

Os peixes são fonte de proteínas essenciais para alimentação humana e são igualmente importantes para produção de farinhas, óleos, ornamentação e até mesmo, como fonte de moléculas bioativas para múltiplos fins farmacêuticos e industriais (FAO, 2008; FAO, 2010).

Dados recentes fornecidos pela Organização das Nações Unidas para a Agricultura e Alimentação (FAO, 2012), revelam que a produção mundial de pescado, no ano de 2011, ficou estimada em torno de 154 milhões de toneladas, das quais 130,8 milhões se destinaram ao consumo humano. De acordo com o relatório, a pesca extrativa e a aquicultura forneceram juntas, cerca de 90,4 e 63,6 milhões de toneladas de pescado, respectivamente.

As estatísticas mostram que a pesca extrativa tem se mantido estabilizada, por ter alcançado seus limites sustentáveis e a aquicultura se desenvolve aceleradamente, colaborando para suprir a demanda mundial por pescados. Estima-se que até o ano de 2013, a produção originada pela aquicultura superará a produção advinda da pesca extrativa. A aquicultura contribui para produção de alimentos, ajudando ao mesmo tempo, a resguardar populações naturais de organismos aquáticos de ocasionais ameaças de extinção (FAO, 2010; FAO, 2012).

A tilápia do Nilo, *Oreochromis niloticus* (L.), uma espécie exótica de origem africana (figura 4), introduzida no Brasil na década de 70, pelo Departamento Nacional de Obras Contra as Secas (DNOCS) em um programa oficial para produção de alevinos e peixamento dos reservatórios públicos no Nordeste, rapidamente adquiriu elevado valor econômico e social, sendo considerada atualmente a principal espécia da piscicultura brasileira (FIGUEIREDO JÚNIOR & VALENTE JÚNIOR, 2008; GUERRA *et al.,* 2012).

Segundo Popma & Masser (1999), as tilápias são os primeiros peixes cultivados no mundo, e as evidências, ilustradas em tumbas no Egito, sugerem que tais ciclídeos vêm sendo criados há mais de três mil anos. Os dados atuais reportam que a quota de peixes dulciaquícolas produzidos pela aquicultura mundial aumentou significativamente nas duas últimas décadas, impulsionado especialmente pelo rápido desenvolvimento da tilápia do Nilo (FAO, 2012).

Bastante apreciada pelo mercado consumidor brasileiro e internacional (BORGHETTI & SILVA, 2008) e por proporcionar excelentes características zootécnicas (POPMA & MASSER, 1999), a tilápia é cultivada em diversos países, como Egito, Indonésia, Filipinas e Tailândia e na China, considerada como principal país produtor e consumidor de tilápias no mundo. Algumas projeções sugerem que o Brasil poderá se tornar um grande rival da China, na produção da espécie, nas próximas décadas (MJOUN & ROSENTRATER, 2010).

Figura 4. Tilápia do Nilo, *Oreochromis niloticus* (Linnaeus, 1758).

A piscicultura brasileira avança a passos seguros se constituindo numa das principais atividades produtivas de pescado. O avanço acelerado da tilapicultura no país ocorreu a partir da década de 1990 (MPA, 2010), como ilustrado na figura 5.

Figura 5. Produção Nacional de tilápia do Nilo (Período 1995 a 2009). *Fonte: MPA, 2010.*

O cultivo da tilápia ocorre nas regiões Nordeste, Sudeste e Sul, sendo a maior produção verificada na região Nordeste do Brasil (BOSCARDIN, 2008). Os peixes geralmente são cultivados

em viveiros escavados (produção semi-intensiva), em tanques-rede (produção intensiva ou super-intensiva) instalados em grandes reservatórios e em sistemas de fluxo continuo ou raceway (super-intensivo) com geração de empregos diretos e indiretos (KUBITZA, 2000; BORGHETTI & SILVA, 2008; KUBITZA, 2011).

A intensificação e a diversificação dos cultivos, no entanto, são desafios para o setor aquícola (FAO, 2010). A ocorrência de doenças pode originar impactos sobre a oferta, demanda e comércio do pescado, tanto nos mercados internos como internacionais, cujas restrições comerciais decorrentes podem alterar tais negócios por períodos prolongados. No ano de 2010, por exemplo, a aquicultura na China sofreu perdas na produção de aproximadamente 1,7 milhões de toneladas (cerca de 3,3 bilhões de dólares americanos) causadas por doenças (295 mil toneladas) (FAO, 2012).

Apesar de não existirem dados oficiais indicando os prejuízos econômicos advindos de enfermidades nas pisciculturas brasileiras (BOSCARDIN, 2008), a presença desses agentes patogênicos no ambiente de cultivo pode se tornar uma ameaça real à medida que o setor se expande e os sistemas de cultivo se intensificam (SHOEMAKER *et al.*, 2000; LIMA, 2007; FIGUEIREDO & LEAL, 2008; EVANS, 2009; DELLA FLORA, *et al.*, 2009).

O vasto repertório de doenças (virais, bacterianas, fúngicas e parasitárias), representa uma ameaça tanto para os peixes cultivados e selvagens, para o ambiente de cultivo em si, demais ecossistemas e pessoas que trabalham diretamente com tais organismos. As doenças infecciosas estão incluídas na lista das principais preocupações dos produtores e gestores da aquicultura mundial (KUBITZA, 2005; FAO, 2010; KURCHEVSKI *et al.*, 2010; ARAUJO *et al.*, 2009; OIE, 2011).

Um exemplo comum são as bacterioses, tais como Streptococcose, *Aeromonas* móveis, Edwardisiellose, Vibriose, Columnariose e Rickettsiose, responsáveis por graves prejuízos nas pisciculturas comerciais. Os gêneros *Streptococcus* spp ou *Enterococcus* spp geram perdas econômicas nas tilapiculturas no Japão, Israel, Estados Unidos da América, Taiwan, Filipinas e Brasil, especialmente em criações intensivas. *Streptococcus iniae*, *S. agalactiae* e *Mycobacterium.* Tais bactérias podem igualmente, infectar as pessoas que lidam direta ou frequentemente com os peixes (POPMA & MASSER, 1999; SURESH, 1998; KUBITZA, 2005; SHOEMAKER *et al.*, 2006; KUBITZA, 2008).

Considerando-se também que a identificação e o estabelecimento de diagnósticos eficazes para determinadas enfermidades em peixes é difícil, e na maioria das vezes consiste em simples observações físicas e/ou alterações comportamentais, e que o uso de medicamentos veterinários (antibióticos, quimioterápicos, desinfetantes ou vacinas), muitas vezes é restrito ou proíbido pela legislação, a adoção de medidas preventivas são as únicas alternativas utilizadas para redução dos

problemas arrolados a doenças (ROTTA & QUEIROZ, 2003; KUBITZA, 2005; FIGUEIREDO & LEAL, 2008; FAO, 2010).

Apesar de todos os esforços para produção de peixes saudáveis e redução dos impactos indesejáveis na aquicultura ainda existe uma necessidade de maiores informações sobre as funções imunológicas destes vertebrados. A prevenção das doenças é importante tanto para os piscicultores, como para a indústria, comércio e o meio ambiente. Assim, à medida que a aquicultura se desenvolve, os estudos sobre o sistema imunológico dos peixes se tornam fundamentais.

Segundo Ewart *et al.* (2001), além da maior compreensão dos mecanismos que envolvem a defesa dos organimos, o estudo das lectinas pode se consistir em poderosas ferramentas utilizadas para a prevenção de doenças em programas de saúde dos peixes ou no desenvolvimento transgênicos que expressem níveis elevados de lectinas. Nos anos recentes, com desenvolvimento de novas tecnologias moleculares, como sequenciamento de DNA, clonagem e análise da expressão gênica, incorporadas às técnicas de purificação de proteínas com elevado rendimento (CORREIA & COELHO, 1995; KANG *et al.*, 2004; COELHO *et al*., 2012), entre outros métodos aplicados ao estudo e caracterização das lectinas, uma explosão de informações continuam sendo geradas (FRANK & SCHLOISSNIG, 2010; KUMAR & MITTAL, 2011). Diversos relatos tem demonstrado que as lectinas protegem os peixes contra o ataque de diferentes patógenos como fungos, bactérias e vírus. Portanto, a identificação e caracterização dos genes e suas proteínas correspondentes podem fundamentar futuros programas de melhoramento genético ou geração de vacinas que aumentem a resistência dos peixes frente a determinadas doenças, ou mesmo na produção de novas drogas e muitas outras aplicações biológicas.

2 OBJETIVOS

2.1 Objetivo Geral

Estudar a diversidade de lectinas presentes em peixes, particularmente na tilápia do Nilo *Oreochromis niloticus* e verificar a atividade imunomoduladora, *in vitro* de OniL em esplenócitos de camundongos.

2.2 Objetivos específicos

I- Reunir as informações sobre a diversidade de lectinas em peixes e produzir uma revisão geral sobre o assunto

II – Estudar a diversidade molecular de lectinas presentes nos tecidos de tilápia do Nilo

- Desenhar *primers* de lectinas por homologia com outros peixes;
- Extrair os tecidos (baço e rim) de tilápia e isolar o DNA genômico total;
- Amplificar (PCR), isolar (eletroforese em gel de agarose), purificar e sequenciar o DNA genômico;
- Analisar o produto gênico através de diversas ferramentas da bioinformática: algoritmos para clusterização de sequências, alinhamento de sequências de nucleotídeos e proteínas, predição de genes;
- Analisar as funções moleculares dos produtos gênicos codificados (relação entre sequências, estruturas e função das proteínas) em bancos de dados públicos;
- Avaliar os padrões evolutivos das frequências de aminoácidos, conteúdos de GC;

III - Identificar e caracterizar lectinas a partir do soro de tilápia do Nilo e avaliar atividade imunomoduladora em esplenócitos de camundongos

- Obter e pré-purificar o soro da tilápia (extrato bruto) por fracionamento salino com sulfato de amônio;
- Detectar a lectina através da atividade hemaglutinante (AH) das diferentes frações obtidas;
- Purificar a lectina da fração de maior rendimento por processos cromatográficos;

- Caracterizar a fração eluída através da inibição da atividade hemaglutinante (IAH) por carboidratos, estabilidade térmica, influência de íons e eletroforese em gel de poliacrilamida;
    - Avaliar a citotoxidade em esplenócitos de camundongos induzida pela lectina purificada em diferentes concentrações;
    - Investigar atividade imunomoduladora induzida pela lectina, observando os níveis de produção de citocinas (IL-10, IFN-γ) e óxido nítrico;
    - Identificar o tipo de resposta imune celular (Th1 – Th2).

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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...

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Aimee Himelfarb
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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).

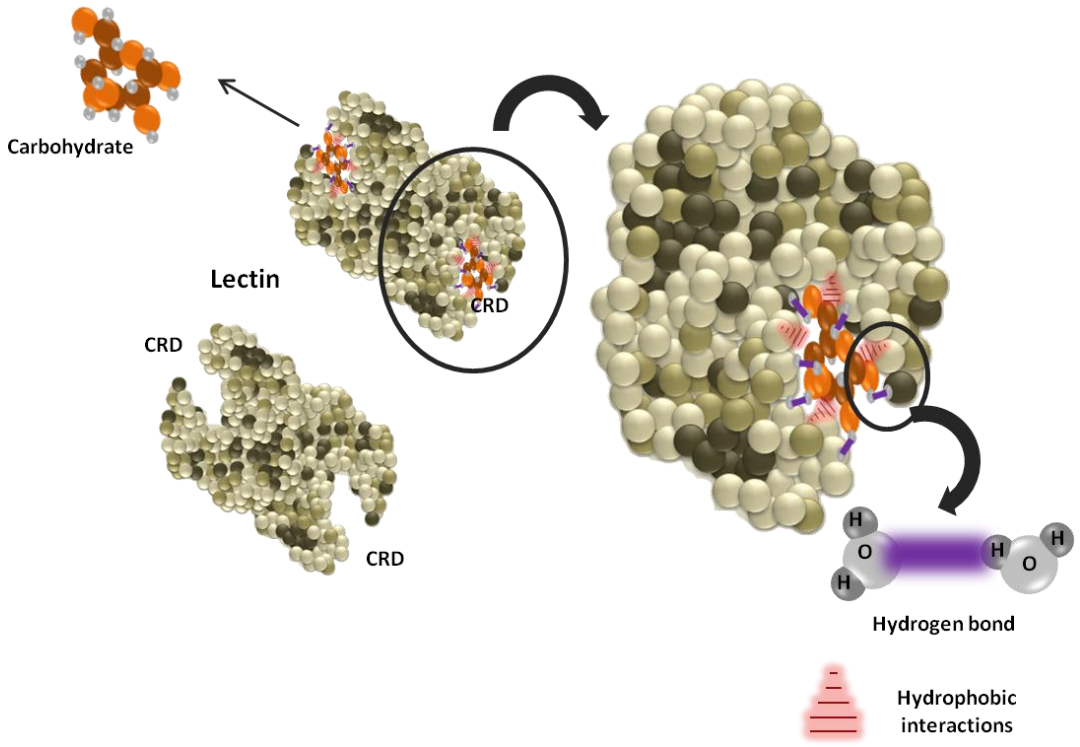

Figure 1. Scheme illustrating the binding of lectin to the carbohydrate through the Carbohydrate Recognition Domain. The carbohydrate-lectin interaction involves, among other non-covalent forces, the formation of hydrogen bonds and hydrophobic interactions.

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 (Ca_2CO_3), 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).

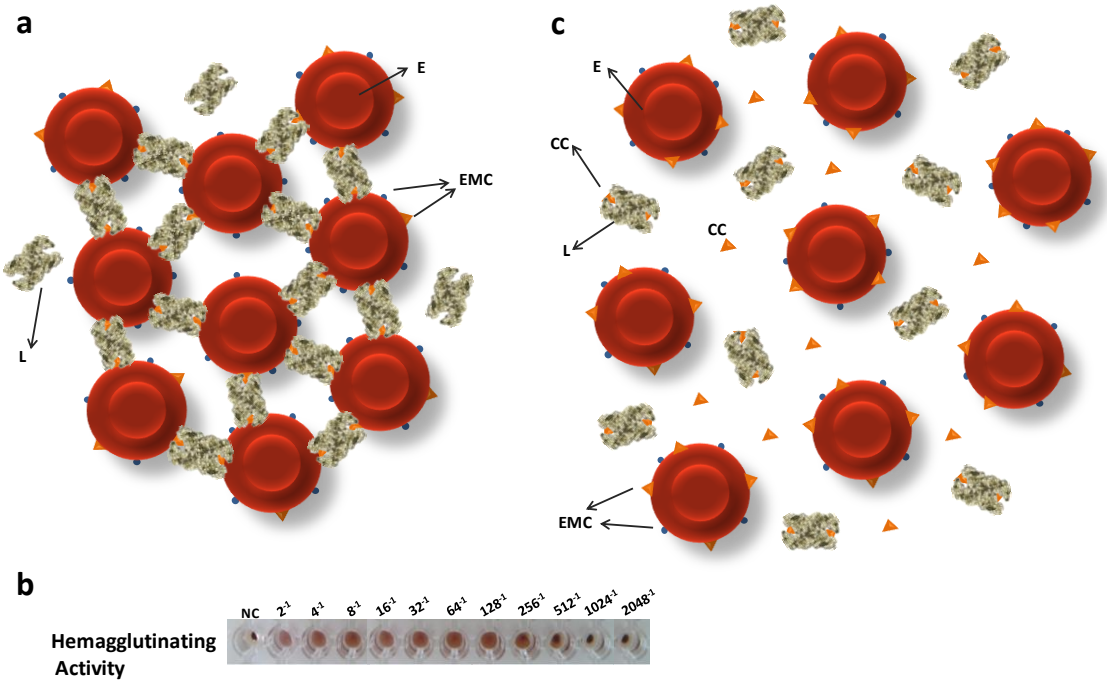

Figure 2. Hemagglutinating and inhibition of hemagglutinating activity assay (HA). The presence of lectin is revealed by the formation of a hemagglutination net due to lectin binding to erythrocyte surface carbohydrate (**a**). HA is performed in 96-wells microtiter plates. Lectin preparations (50 µL) 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

Identification and Characterization of C-Type Lectin Receptors in *Oreochromis niloticus*

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Identification and characterization of a C-type lectin receptor in *Oreochromis niloticus*

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Abstract

A gene was identified whose DNA sequence suggests it codes for a novel protein containing a C-type domain lectin. The sequence DNA obtained from kidney tissue of Nile tilapia was denominated *Oreochromis niloticus* C-type lectin receptor OnilCLR with unconventional patterns that recognize small fragments of DNA sequences, without traditional homology (low identity). It exhibits similarity to several genes related to the immune system and different functional aspects such as key, signaling, precursors, up regulation, supports high mutation and alternative splicing. The homologous sequences are involved in activities such as adhesion, migration, proliferation, differentiation, and apoptosis. The study also revealed in the sequence, a region with 47% homology for predicted C-type lectin domain family 10 member A-like, *O. niloticus*, and a region with 25% homology for predicted low-density lipoprotein receptor-related protein 1, *Danio rerio*, both belonging to the family of C-type lectin receptors; the protein identified may be involved in host defense.

Key words: *Oreochromis niloticus*, Nile tilapia, C-type lectin receptor, Innate immune system.

Introduction

Lectins are glycan binding proteins that exhibit a variety of interactions, being recognized as key components of innate immunity in vertebrates and invertebrates (Vasta et al., 2011). Innate immunity is fundamental in disease resistance in fishes (Magnadóttir, 2010) and their responses are mediated for a diversified group of pattern recognition receptors (PRR). The C-type lectin receptors, Toll-like receptors and others, that recognize broad molecular patterns found in pathogens, alert the innate and adaptive components to assemble the most appropriate response in any perceived risk signals (Fujita, 2002; Iwasaki and Medzhitov, 2004; Mogensen, 2009).

C-type lectin receptors (CLR) superfamily includes proteins with at least one C-type lectin-like domain (CTLD) and C-type lectins (CTL) that bind to carbohydrates in a calcium-dependent manner, mediated by conserved carbohydrate-recognition domains (CRD) (Geijtenbeek and Gringhuis, 2009; Zelensky and Gready, 2005). They consist of different members, such as, tetranectin (CLEC3B) a receptor that binds plasminogen and complex sulphated polysaccharides including heparin, suggested to be a useful prognostic biomarker for metastatic oral squamous cell carcinoma (Nielsen et al., 1997; Graversen et al., 2000), Dectin-1 (CLEC7A) beta-D-glucan binding, involved in the antifungal innate immunity (Brown et al., 2007), mannose binding-lectin (MBL) that binds to repetitive mannose and/or N-acetylglucosamine residues on microorganisms, leading to opsonization and activation of the lectin complement pathway (Van Asbeck et al., 2008).

The fish genomes encode various lectins, including CLR (Vasta et al., 2011; Ogawa, et al., 2011). Recently two novel lectins were identified from the serum of Nile tilapia, *Oreochromis niloticus* (L.) an African cichlid, of high economic value for tropical aquaculture: a lectin that recognizes mannose-(methyl-alpha-D-mannopyranoside) and D-mannose, named (OniL) inducing cytokine, purified in our laboratory by Silva et al. (2012) and fucose-binding proteins (TFBP) specific for L-fucose with hemagglutinating activity against bacteria or parasites (Argayosa and Lee, 2009).

The Nile tilapia culture in Brazil occurs often in semi-intensive systems (ponds) or intensive (cages), generating approximately three jobs direct and indirect per hectare of cultivated water depth (in the property, industry inputs, equipment and processing, distribution of fish, etc.) (Borghetti and Silva, 2008). The intensive aquaculture is a lucrative business however, possess a great challenge since increase the chances of biological risks for farmed animals, people and ecosystems (FAO, 2010). Despite all efforts to the production of healthy fishes and reduction of undesirable impacts of diseases, more knowledge is needed about the immunologic functions of fishes (Ewart et al., 2001; Magnadóttir, 2010). Then, we decided to focus our research in type-C

lectins potentially involved in immune defense of the tilapia, considered to be the flagship of the Brazilian fish-farming.

Distortions of the guanine cytosine (GC) base contents and the genome homogenization process indicates high levels of rearrangements DNA sequences (Oda et al., 2008). This information allied to the data of GC3 (GC content of the third codon position) evolution show the genome as a selection unit. Grantham et al., (1980) proposed that each gene in a genome tends to have a GC3 value according to its species what can explain the molecular selection mechanisms (Li, 1987). Recently, new lectins have been described, but generally the information about quantitative variations in codon usage bias of these lectins are not well known. In this paper, it was identified and characterized a gene encoding a C-type lectin receptor and other proteins related to immune system; their quantitative relationships of amino acid content were also evaluated.

Material and Methods

Samples and DNA Sequencing

Juveniles of *Oreochromis niloticus* (chitralada strain) regardless of sex were provided by Estação de Aquacultura Continental Professor Jokey Koike of Universidade Federal Rural de Pernambuco (Recife, Brazil). Animals were sacrificed using the technique of spinal cord section (Pedrazzani et al., 2007); total DNA was extracted from spleen and kidney tissues samples using a Protocol CTAB 2% (Ferreira and Grattapaglia, 1998). The reaction mixture contained 20 µL: template DNA (50 ng), 2 µL (10X) reaction buffer, 0.8 µL $MgCl_2$ (2.0 mM), 1 µL (8.0 pmol) each primer, 2 µL dNTP (50 mM) and 0.24µL Taq DNA polymerase (0.08 U). Amplification was started at 94°C for 4 min followed by 30 cycles at 95°C for 50 s, 61°C for 50 s, 72°C for 1 min; a final extension at 72ºC for 8 min was performed to extend completely the amplified product. Electrophoresis of 6.0 µl of the polymerase chain reaction (PCR) product was performed in 1X TAE buffer (tris-acetate-EDTA pH 8.0) for constant voltage of 65 V for 50 min, in 0.8% agarose gel (w/v) stained with 0.02% ethidium bromide. The size of the PCR products was checked against a 1kb plus DNA ladder (Invitrogen). The resulting amplified fragments were visualized by UV transilumination and photographed. Subsequently the amplicons were purified, according to the protocol supplied by the manufacturer (Promega™). The PCR products were sequenced using the Applied Biosystems Automated 3730 DNA Analyzer.

Design and synthesis of primers

Primers were designed by homology with different organisms and used for the amplification of the gene in Nile tilapia. The sequence *Danio rerio* (Zebrafish) mannose binding-like lectin

precursor (mbl) mRNA, complete cds, access number [AF227738], obtained from the GenBank/NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/genbank/) was used as the query to search homologous applying Blast/NCBI. Subsequently the obtained sequences were clustered in the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) and the generated contigs were submitted to ORF Finder/NCBI (Open Reading Frame) and evaluated in the program Conserved Domains/NCBI. The synthesis of primers was performed by the Primer3 (http://frodo.wi.mit.edu/primer3) and the evaluation by Netprimer (http://www.premierbiosoft.com/netprimer/index.html).

Data Analysis

From the sequence produced it was performed a search of homologies with other genes already published, using bioinformatics tools available in public databases. Blast/NCBI (Blastn, somewhat similar sequences; Blastp database Reference Proteins and Blastx, non-redundant protein sequences) was used, all optimized for teleost fishes (taxid: 32443). The amino acid sequences were deduced by ORF Finder/NCBI. Then, the sequence of the gene encoding the target protein was evaluated by the database Bioinformatics Toolkit (HHpred, http://toolkit.tuebingen.mpg.de/sections/tertstruct). The analysis of multiple alignments used ClustalW software, implemented in MEGA v.4.1 software (Tamura et al., 2007). The sequences, except those related lectins, had their mRNAs like product sequenced were localized and FASTA compiled in a local database. Subsequently, the files containing these sequences were submitted to INCA v. 2.1 software (Supek and Vlahovicek, 2004), to obtain the matrices of codon usage bias (refers to differences in the frequency of synonymous codons occurrence in coding DNA), aminoacids and GC3 frequencies of each ORF with more than 10 codons.

Results

The PCR using primers (*sense* 5'-CCTGTAGCTCTCCAGGCATC-3' and *antisense* 5'-AGGCCTGGAACTTGACACAC-3') designed by homology with the sequence access [AF227738] and the program for amplification allowed to obtain a single band of high purity 40.5 ng (A260/A280=1.8) (Figure 1). The sequenced product was named *O. niloticus* C-type lectin receptor (OniLCLR).

The OniLCLR nucleotide sequence analysis using Blastn/NCBI produced twenty-three significant alignments whose annotations of indexed proteins are shown in Figure 2. In some cases, were high identity was obtained, for example, clone DKEY-237J10 Zebrafish with identities of 83%, 188292pb, no annotation of cds consequently offered low *query* coverage. In other cases, the

return of the query was complex, such as, the clone CH211-199M3 Zebrafish (locus BX571790/NCBI), 200944bp, containing various cds: the 5' end of the *copg2* gene for coatomer protein complex, subunit gamma 2, the gene for a novel protein similar to vertebrate urocortin 3 (stresscopin) (UCN3), two genes for a novel protein similar to vertebrate collagen family, three novel genes and a CG island, complete sequence.

When submitted to ORF Finder/NCBI, OniLCLR nucleotide sequence revealed about 30 frameshift (not shown data). We selected a peptide length with 32 amino acids (RXRXXXAREIXAXSPICLLLENASLXWNEARS) Frame -3, located in terminal sequence region. The analysis of the peptide through the Blastp/NCBI revealed homology (47% of amino acid identity, e-value of 0.031) with the predicted: C-type lectin domain family 10 member A-like, *O. niloticus* [access number XP_003446092.1] (Figure 2). Members of this family contains a domain similar to DC-SIGN-like, length of 186 aminoacids, that are type II membrane proteins with a CTLD ectodomain. The peptide was analyzed for the presence of functional domains using the program HHpred/Bioinformatic Toolkit platform. The peptide sequence had 40% identity with CLECT 1 Subgroup 1 (C-type lectin/C-type lectin-like domain,) superfamily and related subfamilies (CLECT DC-SIGN-like, 27% identity). The peptide identified was called OniLCLEC10A. We identified in OniLCLEC10A seven amino acid residues (Cys-Ser-Asn-Trp-Asn-Glu-Arg) conserved in all domains of the superfamily (Table 1).

The OniLCLR nucleotide sequence analysis using Blastx/NCBI revealed homology (25% amino acid identity, e-value of 0.004) to predicted: low-density lipoprotein receptor-related protein 1, *Danio rerio* [access number XP_001920591.2]/LOC565797 similar to alpha-2-macroglobulin receptor, *D. rerio* [Gene ID 565797], length of 4547 aminoacids (Figure 3). The amino acid sequence of deduced protein contains an Open Reading Frame (Frame -2) encoding a peptide of 85 aminoacids, named OniLORL1 (Figure 3); however, his return from the HHpred/Bioinformatic Toolkit platform, does not match with the same protein.

The codon usage bias for homologous sequences to OniLCLR nucleotide sequence (Figure 2 and 4) indicate diverse mutations of nucleotides, but the amino acid frequency of the evaluated gene products, tend to homogenization patterns. However, when observed the biochemical properties of the amino acids homogenization patterns have not been identified (Figure 4). The GC3 contents were lower for the code sequences of the gene for tumor necrosis factor alpha-2 precursor and for human immunodeficiency virus type I-HIVEP2 (± 0.42%), while higher values were found to Rho GTPase-activating protein 18-like and for vesicular inhibitory amino acid transporter (± 0.67%). The other gene products similar to OniLCLR showed intermediate GC3 content.

Discussion

In this study, with the OniLCLR nucleotide sequence isolated from kidney tissue of Nile tilapia, we have identified and analyzed protein sequences that shared a homology, to sequence found, related to the immune system of teleost. The fact that OniLCLR have less than 50% homology may indicate a direct relationship with the sample population of tilapia, or the diversity of lectins and their origin. Recently, a study of the lectin gene, *Drosophila* 24A, which apparently developed functionally important to resistance against infection by parasites, ortholog sequence revealed no other organism. According to Keebaugh and Schlenke (2012), even with different genomes of the Melanogaster group available in the databases, the Blast/NCBI also failed to identify homologous domains the returns of the very poor *query* sequence.

The naked cuticle homolog 1 gene, initially identified in *Drosophila* (Figure 1a and 1b), was listed as a negative regulator of system *Wnt* signaling pathway, which is a network of proteins known for playing essential roles in signaling during embryogenesis, cancer and other normal physiological processes in adult animals (Lie et al., 2005; Van Raay et al., 2007; Zhang et al., 2011). *Wnt* signals control cell fate decisions and orchestrate cell behavior in metazoan animals (Wharton et al., 2001). The monocarboxylate transporter 1 gene is related to cancer cell invasion in humans (Izumi et al., 2011). Thus, we evaluated OniLCLR as *query*; the sequences producing significant alignments (Bast/NCBI) also had products with functions related to defense mechanism of different living beings, even when the DNA sequences showed low homology and identity with novel, hypothetical or predicted proteins.

The coding sequence is homologous Rho GTPase-activating protein, an activator to DC-SIGN, a C-type lectin expressed on dendritic cells (DC), can sequester human immunodeficiency virus (HIV) virions in multivesicular bodies. According to Hodges et al. (2007) and Liu et al. (2012) Rho GTPase regulating post-transcriptional regulation of unconventional processing adds to the complexity of Rho GTPase signaling network. Consequently OniLCLR can also be related to mechanism of virus isolation during the infectious process in tilapia.

The OniLCLR nucleotide sequence is related to proteins which play different biological roles, such as: key; precursor; upregulation. These multiplicities of features are not conventionally described for a gene, mainly because the common regions are small fragments of DNA or "small signs" that show no traditional homology. However, the set of sequences maintain a common evolutionary relationship with the immune system. It is important to remember that even without DNA gene high identity of the immune system all eukaryotes probably evolved from a common ancestor. It should be considered the discoveries about genes of toll-like receptors, classes of

proteins that play a key role in the innate immune system in both genomes of *Drosophila* and in human (Gay et al., 1991; Iwasaki and Medzhitov, 2004; Shen et al., 2012).

The roles of cortactin-binding protein 2-like gene, remain to be defined, but it is involved in diverse cellular processes, including cell motility, invasiveness, endocytosis, intercellular contact assembly, and host-pathogen interactions (Cosen-Binker and Kapus, 2006). Homologue fragment of the gene for cortactin-binding protein also has homologous two genes for a novel protein similar to vertebrate collagen family of innate immune reinforcing the potential of multifunction OniLCLR.

The homologous sequence of the activation transcription factor 6 (ATF6a) from *Oryzias latipes* has been little studied in fish. According Amyot et al. (2011). ATF6 in humans is directly linked to the insulin gene promoter and contributes to transcriptional repression. Other homologues are sequences of four genes for novel proteins similar to vitellogenin-1 of Zebrafish. Also it is used to measuring a molecular marker for estrogenicity in developing fish (Muncke and Eggen, 2006). Thus, these OniLCLR can be tested as a marker for development of tilapia populations.

The current information about the sequence homologous *Dicentrarchus labrax*, to Beta-1 adrenergic receptors is that this protein is responsible to mediate activation of epinephrine and norepinephrine (noradrenaline) with approximately equal affinity (Ghosh et al., 2012). Epinephrine (adrenaline) reacts with both α- and β-adrenoreceptors, causing vasoconstriction and vasodilation, respectively. The homolog of OniLCLR sequence that encodes HIVEP2 (human immunodeficiency virus type I enhancer protein 2) has been reported by ability to neutralize many enveloped viruses, including HIV-1. This ability to neutralize HIV-1 has been studied cyanobacterial lectin (Keeffe et al., 2011).

Another OniLCLR homolog for thyroid hormone receptor interactor 12 (TRIP12). New types of evidence for association of functional work-related (http://string-db.org/) suggest that thyroid hormone receptor or TR are critical in regulating gene expression in normal physiological processes. Decreased expression and/or somatic mutations of TRs have been shown to be associated with several types of human cancers and innate immune system. The six cds associated DNA sequence from clone CH211-163B1 (Zebrafish), four are new, two could be related to membrane-bound transcription factor protease and protein serine kinase H1 not even having a relationship individually observed but with properties similar to ATF6a gene of *O. latipes*.

The TNF-alpha-2 gene (tumor necrosis factor alpha-2 precursor) of *Salmo salar* has alternative splicing and signal transmembrane. The primary role is in the regulation of immune cells; it is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages, although it can be also produced by other cell types. The TNF-alpha-2 gene induces apoptotic cell death, to induce sepsis (through IL1 and IL6 production), inflammation, and to inhibit tumorigenesis and

viral replication. Swardfager et al., (2010) recently reported deregulation of TNF production implicated in a variety of human neurodegenerative diseases.

The latest information about ddx39b gene and CYR61 gene, indicate that the first gene encodes a member of the DEAD box family of RNA-dependent ATPases that mediate ATP hydrolysis during pre-mRNA splicing. The second gene splicing variants, also named CCN1, is a secreted, extracellular matrix (ECM)-associated signaling protein of the CCN family (CCN intercellular signaling protein). CYR61/CCN1 is capable of regulating a broad range of cellular activities, including cell adhesion, migration, proliferation, differentiation, apoptosis, and senescence through interaction with cell surface integrin receptors and heparan sulfate proteoglycans (Jun and Lau, 2011; Lau, 2011; Linder and Jankowsky, 2011). Thus both genes also play an important role in mRNA export from the nucleus to the cytoplasm.

We identified in OniLCLR nucleotide sequence, two peptides homologous to the receptors of type C lectins: OniLCLEC10A (Figure 2) and OniLORL1 (Figure 3). OniLCLEC10A is homologous to C-type lectin domain family 10, member A (CLEC10A) a calcium-dependent (C-type) lectin. Although the C-type domain was not completely achieved, the OniLCLEC10A sequence show a pattern of amino acids highly conserved among different subfamilies (Table 1). According to Sattler et al., (2012) pattern recognition receptors often belong to families of structurally and evolutionarily related proteins. DECTIN-1 and ORL-1, for example (Kikuno et al., 2004), share significant homology and are considered to have arisen from subsequent gene duplications. Members of the CLEC10A family include type II membrane proteins and may function as a cell surface antigen. The type II integral membrane proteins contain: a C-terminal part of the extracellular, a transmembrane region and a tail of the N-terminal intracellular (cytoplasmic) (Weis et al., 1998). CLEC10A family are conserved in chimpanzee, dog, cow, mouse, rat, and zebrafish and binds to oligosaccharides present on human tissues, as well as, on pathogens including parasites, bacteria, and viruses. They have diverse functions, such as cell adhesion, cell-cell signalling, glycoprotein turnover, and roles in inflammation and innate immune response, endocytosis (Suzuki et al., 1996; Takada et al., 2004; Marchler-Bauer et al., 2011).

OniLORL1 encodes a peptide of 85 amino acids whose predicted molecular weight is 9.35 kDa. The primary structure of the protein containing (Asp-Ile-Ser-Leu) conserved residues is homologous to an endothelial receptor for lectin-like oxidized LDL receptor-1 (OLR1). It is a type II transmembrane receptor, that belong to the class of a lectin-like scavenger receptor, a subfamily of the C-type lectin family, that recognize modified low-density lipoprotein (LDL) by oxidation or acetylation and probably play a significant role in the pathology of atherosclerosis (Yamanaka et al., 1998; Park et al., 2005). This lectin mediates the recognition, internalization and degradation of oxidatively modified low density lipoprotein by vascular endothelial cells (Hayashida et al., 2002).

ORL1 may act as an oncogene by activation of nuclear factor kappa B (NF-kB) target genes responsible for proliferation, migration and inhibition of apoptosis and lipogenesis genes. According to Khaidakov et al., (2011) ORL1 has been suggested as a possible link between obesity, dyslipidemia and cancer.

Similar results with the present study were found by Kikuno et al. (2004) studying the genome of Nile tilapia. The authors identified a set of genes which encode group V C-type lectin, proteins homologous to the mammalian NKG2/CD94 family of natural killer (NK) cell receptors, a region that encodes various C-type lectins but do not function as NK cell receptors and a third region that encodes an assortment of proteins with different structures and functions. The authors also observed in the genome of tilapia, presence of stop codons and frameshift mutations, pseudogenes, incomplete and truncated. *O. niloticus* killer cell-like receptor, according to Kikuno et al. (2004), was derived from a single most recent common ancestor, which is estimated to have existed 7.7 million years ago.

Via mutations, the domains acquire modified functions that contribute to the adaptability of cells and organisms (Xie et al., 2011). Although the specific function of OniLCLR remains to be determined, the present study provides information that is useful for future study of Nile tilapia C-lectin receptors and their function in the fish immune system. The different alternative frameshifts OniLCLR while hampered the search for homologous sequences, allowed the evaluation of numerous gene products associated to the immune system. There is still poor information about the common origin of lectins during evolution. Ogawa et al., (2005) suggested that there was duplication of genes and subsequent Darwinian selection.

The DNA OniLCLR sequence has not being evaluated in relation to the frequency of amino acids and GC contents due to several frameshifts and internal stop codons. However, the values of the homologues of *O. niloticus* in GenBank (Figures 2 and 4) allow us to infer that the patterns of amino acids should be similar to the genes of the same species; the GC contents are 10-20% larger than average of total genome (39% GC). According to Henkel et al., (2012) the genome of *D. rerio* (36% GC) with GC genes tends to be more transcript rich.

In summary, this study demonstrated that the amplification of the PCR product of Nile tilapia with primers generated an OniLCLR sequence, which has a region containing similarity with various proteins related to the immune system and different functional aspects. The sequence contains ORF encoding at least two C-type lectin receptors (OniLORL1 and OniLCLEC10A), that plays essential roles in atherogenesis cell surface antigen, inflammation and innate immune response. During evolution, eukaryotic, genomic DNA sequences accumulated GC-rich genic in regions that indicate "signs transcription" for functional and structural genes (Zhang et al. 2004). Thus, the GC content of a genome varies locally and regionally. High GC content has also been

correlated with short introns, and elevated levels of gene transcription and recombination (Vinogradov 2003). However, we can not discard that there may be populations of *O. niloticus* that, in different environments, have this sequence as a pseudogene, due to the options for reading ORF. The hypothesis that OniLCLR DNA sequence could be part of a novel gene and involved in the immune system is high considering the functional and structural relations to homologues.

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FIGURES/LEGENDS

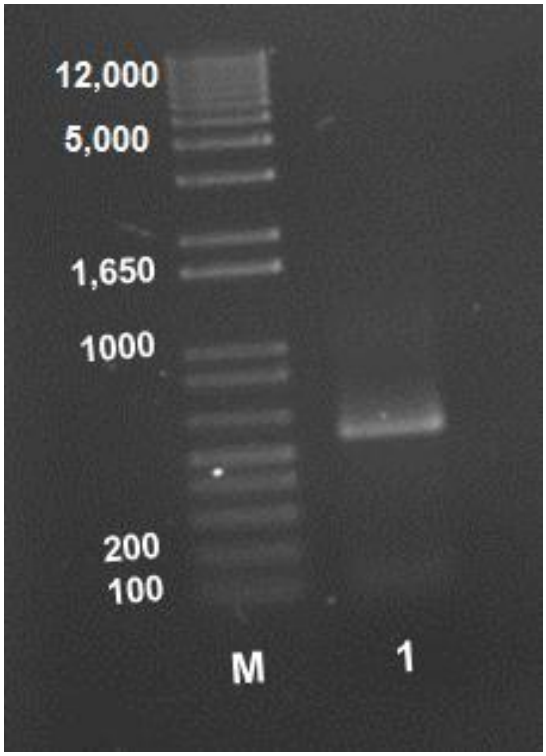
Figure 1. A single band of high purity sequenced product. Electrophoresis conditions: 0.8% gel, 1X TAE, 65 V/cm, 50 min, shows a single band and approximate length of 750 bp, called OniLCLR (1), compared with the length defined pattern (M=1 kb plus DNA Ladder Invitrogen).

Figure 2. (**a**) Homology between the nucleotide sequence OniLCLR and (**b**) products associated to the immune system (indexed proteins). Colored lines refer to homologous regions to gene products listed in the right hand. Dashed line refers to the homologous region to a C-type lectin domain.

Figure 3. Multiple alignments between sequences: OniLORL1 and LOC565797 similar to alpha-2-macroglobulin receptor, *Danio rerio*. Dark areas indicate identically conserved residues.

Figure 4. Variations of amino acid frequencies based on mRNA from homologous OniLCLR obtained at NCBI (INCA v. 2.1 software, Supek and Vlahovicek 2004).

Table 1. Similarity between OniLCLEC10A and C-type lectin/C-type lectin-like (CTL/CTLD) domain in terms of their tertiary structure–function relationships.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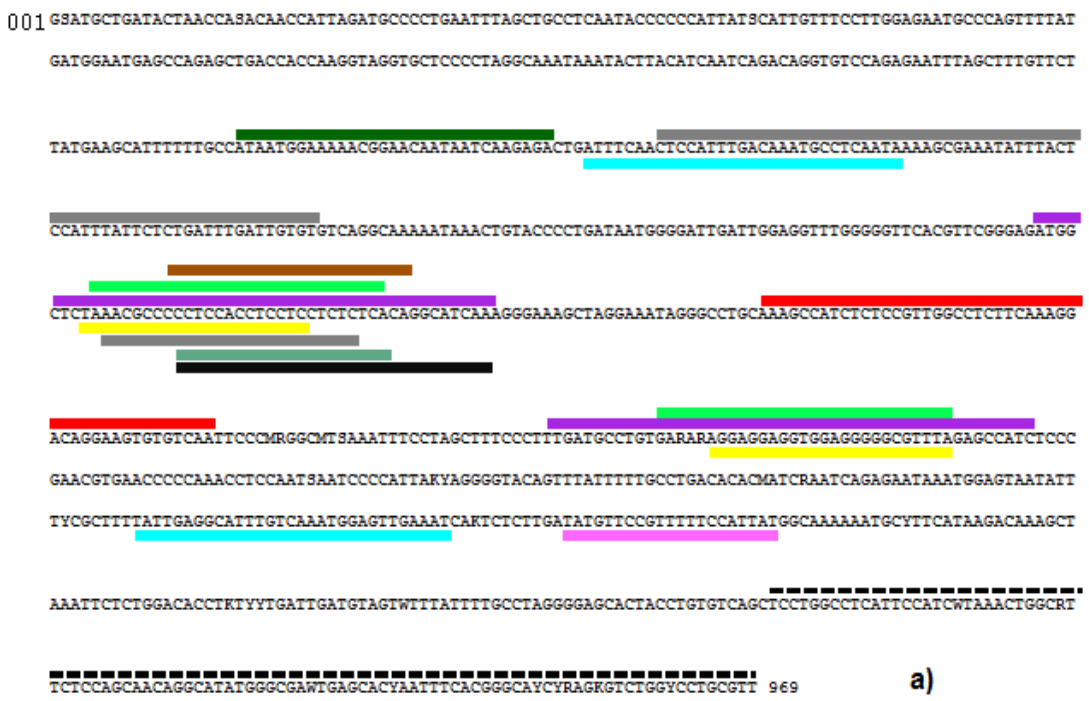

a)

	Gene	Organism	Accession/NCBI
	naked cuticle homolog 1	*Danio rerio*	BX664719
	monocarboxylate transporter 1, Protein FAM19A2	*Dicentrarchus labrax*	FQ310506
	Vesicular inhibitory amino acid transporter; Rho GTPase-activating protein 18-like	*Dicentrarchus labrax*	FQ310506
	cortactin-binding protein 2-like	*Oreochromis niloticus*	XM_003449207
	gene for coatomer protein complex; novel protein; collagen family	*Danio rerio*	BX571790
	beta-1 adrenergic receptor; NHL repeat-containing protein 2	*Dicentrarchus labrax*	FQ310507
	human immunodeficiency virus type I enhancer	*Oreochromis niloticus*	XM_003446649
	thyroid hormone receptor interactor 12	*Oreochromis niloticus*	XM_003441623
	membrane-bound transcription factor protease; serine kinase H1	*Danio rerio*	CT025908
	tumor necrosis factor alpha-2 precursor	*Salmo salar*	DQ787158
	ddx39b; angiogenic inducer, CYR61	*Danio rerio*	BX248081
--	C-type lectin domain family 10 member A-like	*Oreochromis niloticus*	XP_003446092

b)

```
OniLORL1    332  PDTQSNQRINGVNISLLLRHLSNGVEISLLIIVPFF--HYGKKCFIRTKLNSLDTCLIDV  159
                 P+ +S      +NISL L H+   + I  L    +F  +   + F+ +K  S+    L+D+
LOC565797   269  PNAKSFTEEKTINISLSLHHMEQ-MAIDWLTGNFYIFVDNVDDRIFVCSKDGSICVILLDM  327

OniLORL1    158  SIYLPRGAPTLVVSSGSFHHKTGHSPR  78
                    +Y P+G          F    G +P+
LOC565797   328  ELYSPKGIALDPAMGKVFFTDYGQTPK  354
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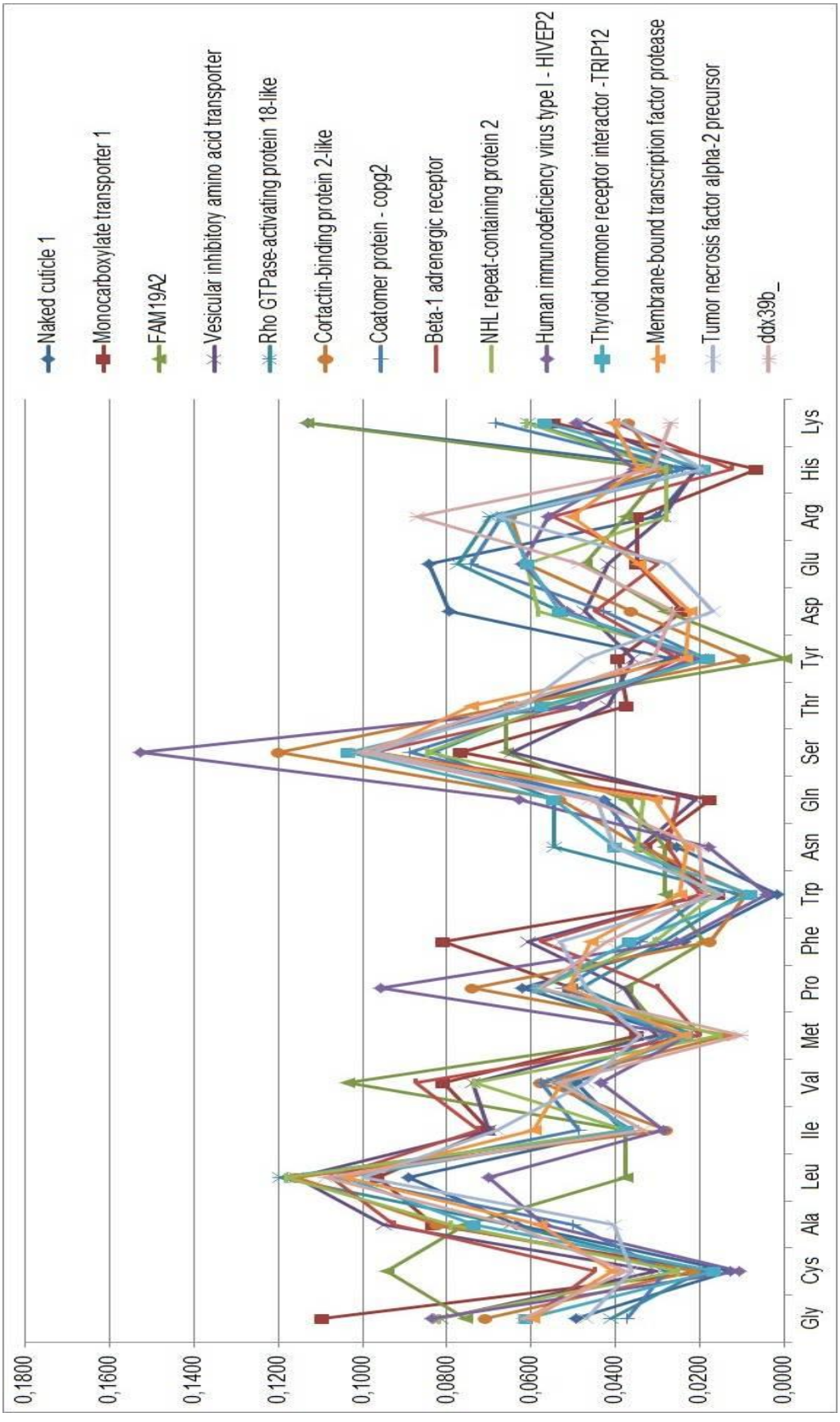
Naked cuticle 1
Monocarboxylate transporter 1
FAM19A2
Vesicular inhibitory amino acid transporter
Rho GTPase-activating protein 18-like
Cortactin-binding protein 2-like
Coatomer protein - copg2
Beta-1 adrenergic receptor
NHL repeat-containing protein 2
Human immunodeficiency virus type I - HIVEP2
Thyroid hormone receptor interactor -TRIP12
Membrane-bound transcription factor protease
Tumor necrosis factor alpha-2 precursor
ddx39b_
0,1800
0,1600
0,1400
0,1200
0,1000
0,0800
0,0600
0,0400
0,0200
0,0000
Gly Cys Ala Leu Ile Val Met Pro Phe Trp Asn Gln Ser Thr Tyr Asp Glu Arg His Lys

	Conserved Domain (Acesss)	Identities	Ligands	Biological Functions
1	CLECT-1 (cd03602)	40%	Mannose, N-acetylglucosamine, galactose, N-acetylgalactosamine, fucose) protein, lipids, $CaCO_3$, ice	Extracellular matrix organization, endocytosis, complement activation, pathogen recognition, cell-cell interactions
2	CEL-1-like (cd03589)	33%	Calcium ions; N-acetylgalactosamine	Cytotoxicity; toxin defense against predation; adhesive activity towards human cancer cells
3	DC-SIGN-like (cd03590)	27%	Oligosaccharides, galactose/N-acetylgalactosamine, pathogens parasites, bacteria, HIV, Ebola viruses	Mediate the initial contact between dendritic cells and resting T cells
4	REG-1-like (cd03594)	27%	Bacteria (intestinal flora)	Tissue regeneration (pancreatic beta-cell, intestinal mucosa, motor neurons, damaged heart), involved in the control of the intestinal bacterial ecosystem
5	NK-receptors-like (cd03593)	25%	Carbohydrate-binding (calcium ion independent)	Stimulate cytolysis by NK cells of virally infected or transformed cells
6	Tetranectin-like (cd03596)	25%	Calcium ions, plasminogen	Regulation of proteolytic processes, modulate angiogenesis and coagulation; play a role in myogenesis and in bone development
7	CSPGs C-type lectin-like (cd03588)	20%	-	Dermal bone development (zebrafish dermacan)
8	EMBP-like (cd03598)	13%	Proteoglycan heparin, (calcium ion independent)	Eosinophils and basophils carry out various functions in allergic, parasitic, inflammatory diseases
9	Collectin-like (cd03591)	7%	Carbohydrates (calcium dependent) pathogens, allergens	Apoptosis, phagocytosis, active the complement pathway, acts directly as an opsonin

*CLECT 1: C-type lectin/C-type lectin-like (CTL/CTLD) domain subgroup 1. Source: CDD Conserved Domain Database (Marchler-Bauer et al., 2011).

6 CAPÍTULO III

6.1 ARTIGO CIENTÍFICO 3

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

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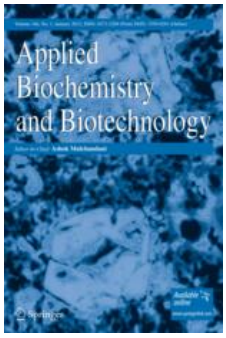

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Purification and Characterization of a Mannose Recognition Lectin from *Oreochromis niloticus* (Tilapia Fish): Cytokine Production in Mice Splenocytes

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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 OniL. OniL 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 (OniL) were eluted with methyl-α-D-mannopyranoside. OniL, a 17-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 OniL 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 husalmon 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 (OmiL) and submitted to dialysis against TBS at 4 °C. Protein was determined according to Bradford [25]; OmiL 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 OmiL 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 (OniL 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 OniL 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 OniL (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

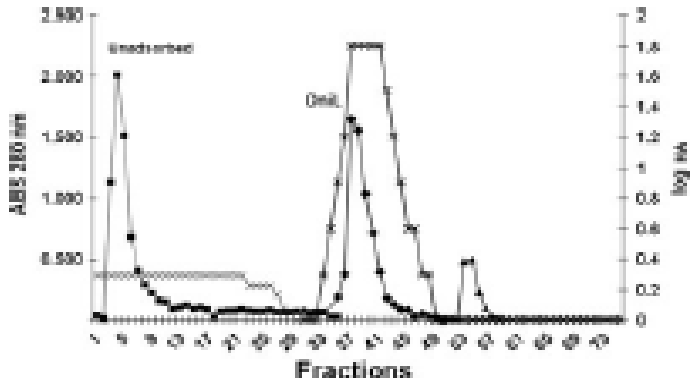

Fig. 1 Affinity chromatography of the tilapia serum on Con A–Sepharose 4B. The F2 was applied to the column (3 mL) and pre-equilibrated with Tris-buffered saline (TBS, 20 mM Tris–HCl containing 150 mM and NaCl 2.0 mM CaCl2, pH 8.0) at a constant flow rate of 20 mL/h. Bound protein was eluted with buffer 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 sign*) are represented

The molecular weight of OniL, 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, OniL 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).

OniL 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[a]
Galactose	100
N-acetylglucosamine	25
Mannose	6.25
Methyl-α-D-mannopyranoside	6.25
Methyl glycopyranoside	25
Asialofetuin	15.63
Azocasein	31.25
Casein	125
Fetuin	62.5
Ovalbumin	31.25

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

[a] Minimal inhibitory concentration corresponds to the lowest carbohydrate or glycoprotein concentration able to neutralize hemagglutinating activity of OniL. 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

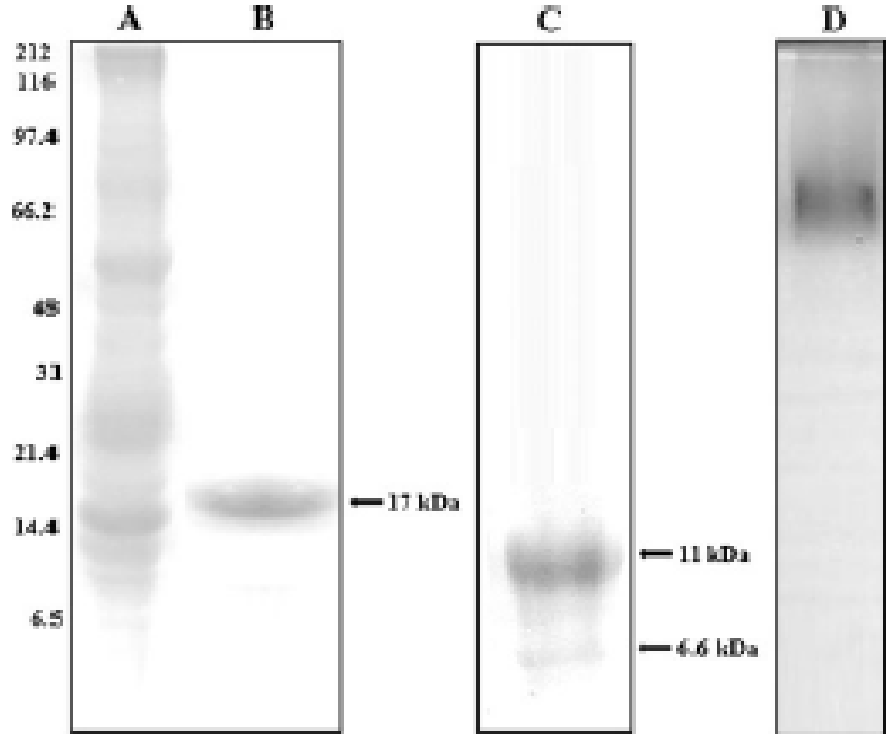

Fig. 2 Polyacrylamide gel electrophoresis (10%, w/v) in the presence of sodium dodecyl sulfate (SDS) of 20–40% ammonium sulfate fraction, F2 (a), and OniL (40 μg) from Con A–Sepharose 4B affinity chromatography in the absence (b) and presence of reducing agent β-mercaptoethanol (c). d PAGE for native acidic proteins of native OniL. 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 OniL was not cytotoxic at either experimental concentration (Table 3).

OniL Stimulated Higher IFN-γ in Mice Splenocytes

OniL induced higher IFN-γ in mice splenocyte cultures. Figure 3a–d shows that OniL, 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, OniL 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 OniL induce statistically different values for IL-10 (50.8±18 and 16.7±5.5 for OniL and control, respectively, $p<0.05$). Con A also induced IL-10 production and was superior to the control cultures, but similar to OniL, 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, OniL 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 (%)*					
OniL	–	–	–	–	–	–
Con A	58	–	–	–	–	–
Saponin*	94	91	90	89	88	88

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

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

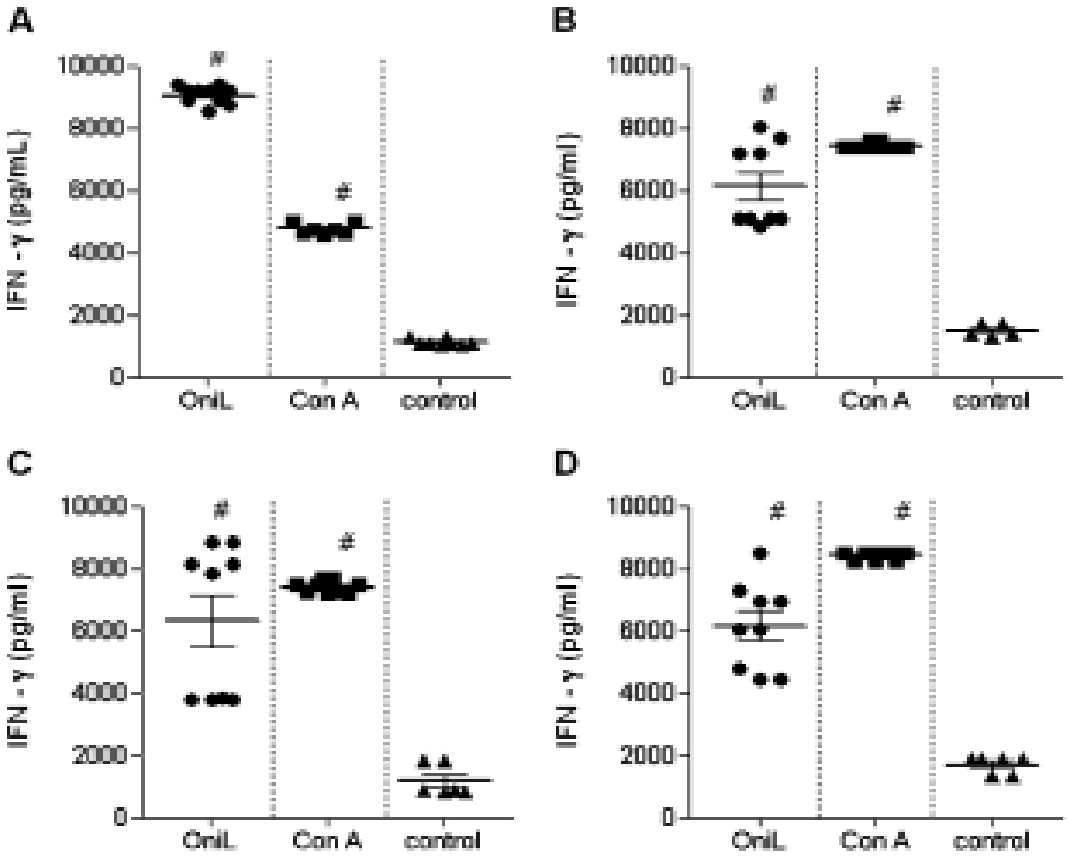

Fig. 3 IFN-γ production induced by OniL and Con A lectins on mice splenocyte cultures after 24 (a), 48 (b), 72 h (c) and 6 days (d). OniL and Con A induced higher and statistically different values of IFN-γ production at all experimental times in relation to control. At 24 h OniL stimulus was also superior 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, OniL, 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, OniL showed highest affinity for mannose derivatives. The inhibition by other carbohydrates and glycoproteins indicates that OniL 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 OniL probably because in the fraction there are other lectins. It is probable that the chromatography on Con A–Sepharose selected OniL 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 OniL 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].

OniL 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-s 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].

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

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7 CONCLUSÕES

- O diversificado repertório de lectinas (galectinas, pentraxinas, calnexinas, tipo C, tipo I, tipo F, L-ramnose, dentre outras) encontrado em fluidos corporais, células e tecidos aumentam a eficiência da imunidade inata conferindo aos peixes uma maior resistência a doenças;
- Através de sequências depositadas no *GenBank* foram desenhados *primers* que permitiram amplificar por PCR e sequenciar genes (OniLCLR) a partir de tecidos renais da tilápia do Nilo, que apresentaram similaridade para várias proteínas relacionadas com o sistema imune;
- OniLCLR contém ORF que codifica dois peptídios nomeados: receptor-1 de lipoproteína oxidada de baixa densidade de *Oreochromis niloticus* (OniLORL1) e lectina tipo C, membro da família 10 A de *Oreochromis niloticus* (OniLCLEC10A), ambos da família dos receptores de lectina tipo C, que desempenham papéis essenciais na arteriogênese, como antígeno de superfície celular, inflamação e resposta imune inata, sugerindo seu envolvimento no sistema imunitário da tilápia;
- A lectina OniL presente no soro da tilápia do Nilo, foi parcialmente purificada 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 caracterizando-se como uma lectina que reconhece manose;
- A atividade de OniL é potecializada na presença de Ca^{2+}, com pH ótimo 8,0 e abolida em temperatura acima de 70 °C;
- A massa molecular aparente de OniL é 17 kDa constituída por subunidades de 11 e 6,6 kDa, em condições redutoras, indicando a presença de pontes dissulfeto;
- Não foi atribuída citotoxidade para OniL em esplenócitos de camundongos; a lectina induz elevada produção de IFN-γ, baixa produção de IL-10 e não é eficiente na liberação de óxido nítrico (NO);
- A secreção de IFN-γ induzida por OniL revela diferenciação de células preferencialmente do tipo Th1, assim sugere-se que esta proteína seja um potencial imunomodulador em mamíferos.

8 ANEXOS

8.1 Normas do Periódico – Artigo 2

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Tables/Illustrations: up to 7

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This section should not be extensively descriptive but should contain sufficient detail so that, in conjunction with cited references, all experimental procedures can be reproduced by others. Essential technical detail or full descriptions of materials that are not of immediate importance for the understanding of the manuscript may be removed into Supplementary material, based upon the advice of peer-reviewers.

Laboratory chemical and biochemical supply firms should be indicated and commercial companies and institutions who may have provided analytical services should be included in this section.

Results and Discussion:

Results and Discussion can be presented under separate headings or as a combined heading. This can be further divided into subheadings. The presentation of experimental detail in the Results and Discussion section(s) should be kept to a minimum. Reiteration of information that is made obvious in tables, figures, or reaction schemes should be avoided. Within the discussion, brief speculation on the implications of the reported findings may be included if appropriate.

Conclusions:

If an optional conclusion section is used, its content should not substantially duplicate

the abstract.

2.1.2 Review Articles: Review articles are normally invited, but prospective authors are encouraged to contact the Editors-in-Chief or the Associate Editors to discuss possible contributions. Review articles will describe particular topics of current interest or controversy within the scope of 3 BIOTECH. Review articles should include an Abstract, an Introduction which describes the background to the article, the main text arranged under subheadings, and should end with a Conclusion section. Review articles should not contain an “Experimental” section.

Word count: up to 9,600 words (12 printed pages)

Structured abstract: maximum of 300 words

Tables/Illustrations: up to 7

References: up to 100

2.1.3 Short Research Reports: Short research report will describe important preliminary findings from innovative research that deserve immediate dissemination. Research reports should be of high scientific quality and should not present poorly elaborated research and scientific data.

Word count: up to 2,400 words (3 printed pages)

Structured Abstract: up to 200 words

Tables/Illustrations: up to 2

References: up to 20

2.1.4 Protocols and Methods: Protocols submitted to the journal are proven experimental procedures that authors have successfully used in their laboratories and reported as part of their research work in a peer-reviewed journal. The submitted protocol should not be in press or under consideration by any other journal. As protocols are constantly evolving, subsequent modifications made by the authors to improve the protocol are acceptable for submission as long as there is no direct repetition of text between this protocol and previous publications of the method, as this would constitute selfplagiarism.

Word count: up to 4,800 words (or 6 printed pages)

Structured Abstract: up to 200 words

Tables/Illustrations: up to 2

References: up to 20

2.1.5 Notes to the Editor: The journal will consider notes to the editor which Will provide further confirmatory information on a particular topic, or a novel aspect of a method, or an organism, or an application for which results are preliminary but the impact for biotechnology is deemed to be important and requires rapid publishing.

Word count: up to 1,600 words (2 printed pages)

Tables/Illustrations: 2

References: up to 15

2.1.6 Letters to the Editor: Letters which provide further debate on a particular topic arising from the publication of a paper in the Journal will be considered. The author(s) of the paper will be sent an edited copy of the letter and they will have the right of reply. Both letters (subject to editing) will be published in the Journal.

Word count: up to 800 words (1 printed page)

Tables/Illustrations: 1

References: up to 12

2.1.7 Book Reviews: 3 BIOTECH will publish reviews on books relevant to biotechnology. Book reviews should address and evaluate important developments and trends in scholarly work about biotechnology.

A potential reviewer may propose a book review by sending a one page proposal and a current CV to one of the two Editors-in-Chief via email who will review the proposal in light of reviews already commissioned and may suggest alterations accordingly.

Reviewers have the responsibility to summarise and assess authors‟ arguments fairly and accurately, within a broad scholarly context, and to emphasise the implications of any given work for future research in biotechnology. Reviewers have the right to make informative judgements about books under review, but personal attacks, ridicule, and distortion are not acceptable. The primary purpose of the book review

section is to foster a respectful and rigorous scholarly dialogue, rather than to deliver personal judgements or disagreements.

Word count: Up to 800 words (1 printed page)

2.2 Language

Manuscripts should be in English (consistent with either British or American spelling). Authors are strongly advised to ensure that the manuscript is written in clear and concise language, is intelligible to a broad readership and is of a publishable standard prior to submission. Manuscripts that are deficient in this respect may be returned to the author without peer review.

To help authors avoid receiving negative comments from referees or editors about the poor use of the English language in their manuscripts, and for authors who are unsure of correct English usage, at least one of the following steps should be considered:

•Have the manuscript reviewed for clarity by a colleague whose native

language is English.

•Use one of the many English language-editing services that are available

(e.g. Edanz, www.edanzediting.com).

Authors should note that the use of an editing service is at the author's own expense and risk and in no way implies that the article will be accepted by 3 BIOTECH. The decision of accepting a manuscript by 3 BIOTECH editors is based on the quality and suitability of a manuscript and is independent of whether that manuscript has been professionally edited with regard to the English language. 3 BIOTECH accepts no

responsibility for the interactions between the author and the service provider or for the quality of the work performed.

2.3 Nomenclature

Microbes

• The genus, species, and variety name should be in italics; strain number, and culture collection number and source of all strains under investigation should be given in the Methods section.

• The scientific names should be given in full (e.g., *Escherichia coli*) in the title, in the abstract, and when first mentioned in the body of the manuscript.

• Thereafter, the appropriate abbreviation of the full generic name of the microbe should be reduced to conform with the Rules of Nomenclature (except in tables and figure legends) as suggested in the International Code of Nomenclature of Bacteria (Lapage, S.P., Sneath, P.H.A., Less el, E.F., Skerman, V.B.D., Seeliger, H.P.R. and Clark, W.A. International code of nomenclature of bacteria (1990 Revision). American Society for Microbiology, Washington, D.C., 1992). Alternatively, use the validation lists published in the International Journal of Systematic and Evolutionary (IJSEM) and/or published in the Microbiology List of Prokaryotic names with Standing in Nomenclature at the URL http://www.bacterio.cict.fr/

• In case of usage of symbols that do not conform to those that have previously appeared in 12 the literature, their aliases may be obtained from the approved nomenclature in the Human Gene Nomenclature Database (Genew) [www.gene.ucl.ac.uk/nomenclature/guidelines.html] and LocusLink, to allow retrieval of all the information available for each gene.

Plants

• Guidance for naming plants correctly is given in The International Plant Names Index and in *The Plant Book: a Portable Dictionary of the Vascular Plants* (1997) by D.J. Mabberley (Cambridge: Cambridge University Press. ISBN 0521-414210-0).

• Scientific names of plants must be written out in italics except for the abbreviations “var.”, “subsp.”, “f.”, etc. which indicate rank at the infraspecific level (e.g., *Cedrus libani* subsp. *atlantica, Phytophthora parasitica* var. *nicotianae*).

• All names should be written full (Genus, species) in the abstract and again in the main text for every organism at first mention (but the genus is only needed for the first species in a list within the same genus, e.g. *Lolium annuum, L. arenarium*). After first mention, the generic name may be abbreviated to its

initial (e.g. *A. thaliana*) except where its use causes confusion.

• The authority (e.g. L., Mill., Benth.) is *not* required unless it is controversial or is helpful for historical or taxonomic reasons. In such cases, the name should be first mentioned in the body of the text (do not use author citation in the abstract or title). Author names are to abbreviated in accordance with the international standard provided by Brummitt, R.K., Powell, C.E., "Authors of Plant Names", Royal Botanic Gardens, Kew 1992. An on-line version of this work may be consulted via http://www.rbgkew.org.uk/data/authors.html.

• Once defined in full, plants may also be referred to using vernacular or quasiscientific names without italics or upper case letters (e.g. arabidopsis, dahlia, chrysanthemum, rumex, soybean, tomato). This is often more convenient.

• Cultivated varieties which are the product of selection and/or breeding are to be referred to as "cultivars" and not "varieties". Any cultivar should be added to the full scientific name and written in accordance with the *International Code of Nomenclature for Cultivated Plants*. The current (2009) edition is obtainable from ISHS via http://www.ishs.org/sci/icracpco.htm. In particular, the part of a name which denotes the cultivar is to be placed within single quotation marks. The abbreviation "cv." is not to be used within a name (e.g., *Malus* ×*domestica* „Golden Delicious", not *Malus* ×*domestica* cv. Golden Delicious).

• If indicating hybrid status, the multiplication symbol should be used before the name of the genus or the species epithet as appropriate (e.g., ×*Cupressocyparis leylandii, Mentha* ×*piperita*), or within the formula denoting the hybrid (e.g., *Mentha aquatica* × *M. spicata*). If the multiplication symbol is not available in your font set, use the letter "x" in lower case, but leave a space between it and the word to which it should be applied (e.g., x *Cupressocyparis leylandii, Mentha* x *piperita*). Neither the multiplication symbol nor the letter "x" are to be in italics.

• Use the letter "x" to indicate a cross such as "red x yellow" and for the term "by" in measurements (2 cm x 4 cm). Use italic *n* and *x* when indicating sporophytic or basic chromosome number (e.g., 2*n*=4*x* = 48)

Genetics

• Genes, mutations, genotypes, and alleles should also be indicated in italics but the protein product of a gene should be in Roman type; phenotypes should not be italicised.

• For human genetics nomenclature, use the HUGO database. (a) In case of usage of symbols that do not conform to those that have previously appeared in the literature, their aliases may be obtained from the approved nomenclature in the Human Gene Nomenclature Database (Genew) [www.gene.ucl.ac.uk/nomenclature/guidelines.html] and LocusLink, to allow retrieval of all the information available for each gene. (b) It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localisation

should be shown in Roman: v-fes, c-MYC.

• For bacterial genetics nomenclature follow Demerec et al (1966) Genetics 54: 61-76; J Bacteriol (first issue of each year); Microbiol Mol Biol Rev (1998) 62:814-984 (*Escherichia coli* K-12); Microbiol Rev (1988) 52:485-532 (*Salmonella typhimurium*); Microbiol Rev (1985) 49:158-179 (*Bacillus subtilis*); Annu Rev Microbiol (1986) 40:79-105 (Pseudomonas); Microbiol Rev (1982) 46:426-570 (*Neurospora crassa*); Nature (1997) 387 (6632 Suppl):67-73 (*Saccharomyces cerevisiae*).

• Nomenclature for plant genes should follow the recommendations of the International Society for Plant Molecular Biology Commission on Plant Gene Nomenclature, which are posted regularly on the public databases and published annually in Plant Molecular Biology Reporter, starting with the December 1993 issue.

Chemistry/Biochemistry

• For guidance in the use of biochemical terminology follow the recommendations issued by the International Union of Biochemistry and Molecular Biology (IUBMB; http://www.chem.qmw.ac.uk/iubmb/); International Union of Pure and Applied Chemistry (IUPAC; http://www.chem.qmw.ac.uk/iupac/index.html).

• For enzyme nomenclature use Enzyme Handbook (1990) Springer, Berlin

Heidelberg New York; Enzyme Nomenclature (1992) Academic Press, London

New York.

2.4 Taxonomy

2.4.1 Taxonomy Descriptions**:** Taxonomy papers relevant to any field of biotechnology will be accepted. Authors will have to provide evidence in the manuscripts of the relevance of the new taxon to biotechnological applications.

The proposal of new taxa should follow the guidelines described in the International Journal of Systematic and Evolutionary Microbiology (http://ijs.sgmjournals.org/). The new bacterial name should be approved by na international authority on nomenclature. Authors should consult the following publications for guidance in these matters:

o Tindall, B.J. et al. (2006) Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. IJSEM 56: 2715-2720 o Felis and Dellaglio. 2007. On species descriptions based on a single strain: proposal to introduce the status species proponenda (sp. pr.). IJSEM 57: 2185-2187

o Tindall, B.J. et al. (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. IJSEM International 60: 249-266

The micro-organisms must be deposited in two recognized culture collections in two different countries. If a database accession number for the gene sequences is not stated in the manuscript then the sequence data, separate from the manuscript, must be submitted for reviewing and the accession number provided before acceptance of the paper.

2.4.2 Taxonomic Requirements When Dealing With Biological Material

Manuscripts on studies of biological material (e.g. enzyme) will only be considered IF the organism producing the biological material has been identified to a species level and a brief taxonomic description is included in the manuscript. A voucher specimen of the organism should be deposited with a recognised taxonomist for the particular group of organisms in question. The taxonomist should then assign to specimen na identifying number unique to the organism so that any additional collections of the same organism would bear this same number. The number will be retained until the organism is completely identified.

Authors who purchase dried 'herbal remedies' or other materials from companies must make provision for their proper deposit in a herbarium, for access by future workers. When a commercially available extract is obtained, the extraction procedure from the organism of origin must be specified. The identification of the extract should be supported by an HPLC trace of known secondary metabolite constituents of the organism, which should be included in the manuscript.

2.5 Abbreviations

In general, use of abbreviations should be restricted to a minimum. Abbreviations should be restricted to SI symbols and those recommended by the IUPAC. Abbreviations must be defined in parentheses after their first mention in the text. Standard units of measurement and chemical symbols of elements may be used

without definition in the body of the paper.

3. SUBMISSION TO PUBLIC DATABASES

This section should be read in conjunction with section 1.7.

3.1 Sequence Data

Sequence data should be submitted in electronic form to any one of the three major collaborative databases given below:

- DNA Data Bank of Japan - DDBJ (http://www.ddbj.nig.ac.jp)
- European Bioinformatics Institute - EMBL (http://www.ebi.ac.uk)
- GenBank - (http://www.ncbi.nlm.nih.gov).

3.2 Structural Data

3 BIOTECH accepts and follows the recommendations of the International Union of Crystallography (IUCr) with regard to the deposition and release of macromolecular structural data. These guidelines are set out in the article by the IUCr Commission on Biological Macromolecules in Acta Crystallographica (2000), D56, 2.

Structures of biological macromolecules must be submitted to a publicly available and recognised database, such as Protein DataBank (http://www.rcsb.org./pdb/home/home.do), Biological Magnetic Resonance Databank (http://www.bmrb.wisc.edu/), NDB (http://ndbserver.rutgers.edu).

Manuscripts reporting new three-dimensional structures of small molecules from crystallographic analysis should include a .cif file and a structural figure with probability ellipsoids for publication as Supplementary material. These files must have been checked using the IUCR's CheckCIF routine (http://checkcif.iucr.org/) and a PDF copy of the output must be included at submission, together with a justification for any alerts reported. Crystallographic data for small molecules should be submitted to the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/). In the case of low-resolution structures for which only a chain trace is reported, a set of C-alpha positions and structure-factor amplitudes may be sufficient.

3.3 Microarray Data

Data from microarray experiments should be submitted to either the ArrayExpress (http://www.ebi.ac.uk/arrayexpress), GEO (http://www.ncbi.nlm.nih.gov/geo) or CIBEX (http://cibex.nig.ac.jp/index.jsp) databases. Microarray data should be described according to MIAME guidelines (http://www.mged.org/Workgroups/MIAME/miame.html).

3.4 Other Datasets

3 BIOTECH strongly recommends deposition of other types of data sets into appropriate public repositories that are at an earlier stage of development. Examples of such repositories that facilitate sharing large data sets, some of which can offer the option of anonymous referee access to data before publication, include:

Proteomics data:

PRIDE (http://www.ebi.ac.uk/pride/), PeptideAtlas (http://www.peptideatlas.org/), Tranche (http://www.proteomecommons.org)

Protein interaction data:

IMEx consortium of databases including DIP, IntAct and MINT (http://www.imexconsortium.org/)

Chemical compound screening and assay data:

PubChem (http://pubchem.ncbi.nlm.nih.gov/)

Cryoelectron microscopy:

Structures of biological macromolecules solved by electron microscopy must be submitted to the EMDB database at http://www.ebi.ac.uk/msd/Deposition.html. For a brief description of the database, see Tagari et al (2002) Trends Biochem Sci 27:589.

4. HOW TO SUBMIT

The corresponding author of the manuscript will need to register and submit the manuscript online via the Editorial Manager. Instructions on registering, manuscript submission and manuscript revision can be found at https://www.editorialmanager.com/btec/

4.1 Manuscript

The author should submit a complete manuscript including text, tables, graphics as word processor files (.doc or .rtf format) and not as pdf files.

4.2 Cover Letter

A cover letter must be uploaded with every manuscript in PDF format at the same time as the manuscript is uploaded. The cover letter should contain:

a. The corresponding author‟s name, postal and e-mail addresses, telephone and fax numbers.

b. The title of the manuscript and a brief paragraph explaining the significance of the work.

c. Type of manuscript.

d. A statement that the submitted manuscript is original, unpublished and not under simultaneous consideration by another journal.

e. The names, institutional affiliations, and postal and email addresses of up to 5 qualified reviewers.

5. REVISION

If a paper that is returned to the authors for amendment is not resubmitted in revised form within 8 weeks it will be regarded as withdrawn. Additional time for revision can be granted upon request, at the editors' discretion. Only a single round of revision Will be permitted.

Revised versions of the manuscripts must be submitted online at https://www.editorialmanager.com/btec/.

a. The revised manuscript including text, tables, graphics should be submitted as word processor files (.doc or .rtf format) and not as pdf files.

b. Clearly indicate in detail at the designated place on the web form and/or cover letter all changes/corrections that have been made to address the reviewers' recommendations/suggestions.

c. Revised manuscripts may be returned to authors for further modification of the scientific content and/or for shortening and language corrections.

6. PUBLICATION

6.1 Proofs

Authors will be sent an email with a link to download the proof. In the interest of speed, corrections should be returned within 48 hours. Only typographic corrections and other minor changes may be made in a galley proof and substantive changes Will require editorial approval and may delay publication of the article.

6.2 Page charges

Currently, there are no page charges for 3 BIOTECH.

6.3 Reprints

Hard copies of the paper are not published and therefore cannot be purchased. Papers are only published electronically online and can be downloaded as pdf free of charge.

6.4 Digital Object Identifier

Springer Publishing Group assigns a unique digital object identifier (DOI) to every article it publishes. The DOI initiative is an international effort for electronic content identification and is guided by the International DOI Foundation, composed primarily of academic publishers and societies. The DOI appears on the title page of the article. It is assigned after the article has been accepted for publication and persists throughout the lifetime of the article. It is important to include the article's DOI in the reference as volume and page information is not always available for articles published online. The DOI should be cited in a reference as follows: "Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086"

7. AUTHOR'S CHECK LIST

Please tick the appropriate check list box, fill in the required information (if applicable) and submit the check list (in pdf format) with the manuscript:

□ The selected manuscript category, a word count for the abstract and the text, and the numbers of tables and figures.

□ The paper conforms to the format of the selected manuscript category.

□ The manuscript has been checked for typographical errors.

□ References cited in the text are listed in the Reference List, and vice versa.

□ References conform to the journal's required format.

□ A brief statement of the relevance of the paper to biotechnology.

□ The submitted ms is original, unpublished and is not under consideration by another

journal.

□ The authors declare that they have no conflict of interest in the publication.

OR

□ The authors declare that they have a conflict of interest [in which please supply an appropriate statement describing the conflict of interest].

□ The data base accession number has been written in the manuscript and has been publicly released so that the editors can review the data during the review process.

□ The data base access number has been written in the text but has not yet been released publicly and therefore the data has been submitted for review in an electronic form.

□ Provide a list of up to 5 names of external expert reviewers together with their institutional affiliations, postal and email addresses. http://www.springer.com/journal/13205

8.2 Resumos apresentados em congressos

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]

[1]Departamento de Bioquímica, Laboratório de Glicoproteínas, UFPE;
[2]Departamento de Engenharia de Pesca, UFRPE – Brazil.

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].

1- Departamento de Bioquímica, Centro de Ciências Biológicas, UFPE, Recife-PE-50670-910.
2– Departamento de Engenharia de Pesca, UFRPE.

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

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].

1- Departamento de Bioquímica, Centro de Ciências Biológicas, UFPE, Recife-PE-50670-910.
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Financial support: CNPq.

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

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]; Fucks, 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]

1-Universidade Federal de Pernambuco, Departamento de Bioquímica, Laboratório de Glicoproteínas, UFPE
2-Universidade Federal Rural de Pernambuco, Departamento de Educação

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.
Supported: CNPq, CAPES, FACEPE, PRONEX/FACEPE.

Identification and Parcial Caracterization of MBL (mannose-binding lectin) 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]

[1]Departamento de Bioquímica, Laboratório de Glicoproteínas, UFPE;
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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.

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

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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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Identification of mannose binding lectin (MBL) in tissues of Nile tilapia (Oreochromis niloticus)

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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.

Keywords: Innate immunity; C-type lectin; mannose binding lectin; Oreochromis niloticus; aquaculture.

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Biochemical Models to Unravel Fish Protein Techniques for Fundamental Education Students

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This work was discussed at the National Week of Science and Technology, UFPE, an initiative of 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 presentation was started with posters describing 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 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. 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.

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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

Coriolano, M. C. [1], Silva, C.D.C.[1], Lino, M.A.S.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 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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Purification and Characterization of a Mannose-recognition Lectin from *Oreochromis niloticus* (Tilapia fish): Cytokine Production Induced in Mice Splenocytes

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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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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.

Keys words: Lectin, Oxidative Stress, Protective Effect.
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