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Purificação, caracterização e atividade imunomodulatória da lectina presente no soro do peixe beijupirá (*Rachycentron canadum*).

Marília Cavalcanti Coriolano

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Purificação, caracterização e atividade imunomodulatória da lectina presente no soro do peixe beijupirá (*Rachycentron canadum*).

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Orientadora: Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho

Banca Examinadora: Dra. Patrícia Maria Guedes Paiva (UFPE)

Dra. Maria Tereza dos Santos Correia (UFPE)

Dra. Michele Dalvina Correia da Silva (UFERSA, RN)

Dr. Roberto Araújo Sá (UFPE-CAA)

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BANCA EXAMINADORA

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

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Dra. Patrícia Maria Guedes Paiva (UFPE)

_____

Dra. Maria Tereza dos Santos Correia (UFPE)

_____

Dra. Michele Dalvina Correia da Silva (UFERSA, RN)

_____

Dr. Roberto Araújo Sá (UFPE-CAA)

"*Procuro semear otimismo e plantar sementes de paz e justiça. Digo o que penso, com esperança. Penso no que faço, com fé. Faço o que devo fazer, com amor. Eu me esforço para ser cada dia melhor, pois bondade também se aprende. Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida, que o mais importante é o decidir*." (Cora Coralina)

Dedico aos meus pais

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RESUMO

Lectinas constituem um grupo heterogêneo de proteínas e glicoproteínas que se ligam especificamente a carboidratos com alta afinidade. O beijupirá, *Rachycentron canadum*, pertence à família Rachycentridae, e é uma espécie que reune as melhores condições para o cultivo de peixe marinho. Uma lectina foi purificada do soro do peixe *Rachycentron canadum* (RcaL) através de cromatografia de afinidade com uma coluna Concanavalina A-Sepharose 4B. Um pico com atividade dessa lectina foi Ca^{2+} (20 mM) dependente. RcaL é uma proteína com atividade em pH 7.0-8.0 e resistente a 40 ºC por 10 min. A lectina mostrou maior especificidade pelos açúcares metil-α-D-manopiranosídeo e D-manose (200 mM); frações cromatografadas de RcaL eluídas aglutinaram eritrócitos de coelho (AH: 128^{-1}), mantiveram 66% da atividade da lectina purificada e o fator de purificação obtido foi 1.14. Sob condições redutoras, uma banda de 19.2 kDa foi revelada em SDS-PAGE. PAGE confirmou que RcaL é uma proteína ácida revelada em um única banda. Ensaios citotóxicos e imunomodulatórios com RcaL em culturas de esplenócitos de camundongos foram realizados e mostraram que a lectina não foi citotóxica e induziu alta produção de IFN-γ e óxido nítrico. Além disso, também foi avaliada a resposta proliferativa e a produção de citocinas em esplenócitos de camundongos *in vitro* estimulados com as lectinas RcaL e Con A. Os resultados demonstraram altos índices de proliferação induzidos por RcaL em relação às células controles e a Con A. RcaL induziu alta produção de IL-2 e IL-6 em relação ao controle. Somente apoptose tardia foi promovida pelo tratamento com RcaL em relação ao controle, em 24 horas de ensaio; RcaL e Con A promoveram também apoptose tardia em 48 horas de ensaio. No entanto, a viabilidade celular foi superior a 90% em esplenócitos tratados com RcaL. Os resultados mostraram que a lectina RcaL induz preferencialmente resposta imune Th1 sugerindo que ela atua como um composto imunomodulador e também induz resposta proliferativa, revelando que esta lectina pode ser usada como agente mitogênico em ensaios imunoestimulatórios.

Palavras-chave: *Rachycentron canadum*, RcaL, apoptose, atividade imunomodulatória, resposta proliferativa.

ABSTRACT

Lectins are a heterogeneus group of proteins and glycoproteins that specifically bind to carbohydrates with high affinity. The beijupirá, *Rachycentron canadum*, belongs to the family Rachycentridae, and it is a species with best conditions for marine fish cultivation. A lectin, named RcaL, was purified from the serum of *Rachycentron canadum* through of affinity chromatography with a Concanavalin A-Sepharose 4B column. A peak with activity of this serum lectin was Ca^{2+} (20 mM) dependent. RcaL is a protein with activity at pH 7.0-8.0 and resistant to 40 ºC for 10 min. The lectin showed greater specificity for sugar methyl-α-D-mannopyranoside and D-mannose (200 mM); eluted fractions of RcaL agglutinated rabbit erythrocytes (titer: 128^{-1}), retained 66% of chromatographed lectin activity and the obtained purification factor was 1.14. Under reducing conditions a band of 19.2 kDa was revealed in SDS-PAGE. PAGE confirmed RcaL as an acidic protein revealed in a single band. Cytotoxic and immunomodulatory assays with RcaL in mice splenocyte cultures was performed and showed that the lectin was not cytotoxic and induced higher IFN-γ and nitric oxide. In addition, was also evaluated the proliferative response of cytokine production in splenocytes of mice stimulated *in vitro* with RcaL and Con A lectins. Results demonstrated higher and statistical indices of proliferation indexes induced by RcaL lectin in relation to control cells and Con A. RcaL induced higher IL-2 and IL-6 production in relation to control. It could observe that only late apoptosis was promoted by RcaL treatment at 24 hours of assay in relation to control; RcaL and Con A promoted also apoptosis at 48 hours of assay. However, the cell viability was superior to 90% in splenocytes treated with RcaL. Results showed that RcaL lectin induces preferential Th1 immune response suggesting that it acts as an immunomodulatory compound and also induces proliferative response, revealing that this lectin can be used as a mitogenic agent in immunostimulatory assays.

Keywords: *Rachycentron canadum*, RcaL, apoptosis, immunomodulatory activity, proliferative response.

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LISTA DE ABREVIATURAS

AH	Atividade Hemaglutinante
AAG	Lectina Abrina, isolada de *Abrus precatorius*
APAF-1	Fator Ativador de Apoptose-1
ATP	Adenosina Trifosfato
Ca^{2+}	Cálcio
Con A	Concanavalina A
Cramoll-1,4	Lectina isolada de *Cratylia mollis*
CRD	Domínio de Reconhecimento de Carboidratos
DIFBL	Lectina isolada de *Dicentrarchus labrax*
EDTA	Ácido etilenodiaminotetracético
FBS	Soro Fetal Bovino
FITC	Isotiocianato de Fluoresceína
F3	Fração 40-60%
GANL	Lectina isolada de *Aristichthys nobilis*
GlcNAc	N-acetil-D-glicosamina
^{3}[H]TdR	[3H]-timidina
HAI	Inibição da Atividade Hemaglutinante
IP	Iodeto de propídeo
IL(1,2,4,5,6,10,12, 13, 15,18,23)	Interleucina
INF-γ	Interferon-γ
IFN-β	Interferon-β
Jacalina	Lectina isolada de *Artocarpus heterophyllus*
MBL	Lectina que liga manose

MLI	Lectina isolada de *Viscum album* Var. aglutinina
MLII	Lectina isolada de *Viscum album* Var. *coloratum* aglutinina
MLIII	Lectina isolada de *Viscum album*
MMP	Permeabilidade da Membrana Mitocondrial
NADPH oxidase	Nicotinamida Adenina Dinucleotídeo Fosfato reduzido
NK	Natural Killer
NO	Óxido nítrico
NOS	Óxido nítrico sintase
PAGE	Gel de poliacrilamida
PAMPs	Patógenos não próprios associados a padrões moleculares
PBS	Tampão Fosfato Salino
PHA	Lectina isolada de *Phaseolus vulgaris*
PHA-M	Lectina isolada de *Phaseolus vulgaris*, a mucoproteína
PHA-P	Lectina isolada de *Phaseolus vulgaris* , a glicoproteína
PMNs	Polimorfonucleares
RcaL	Lectina isolada de *Rachycentron canadum*
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecilsulfato de sódio
SI	Índices de estimulação
SIDA/AIDS	Síndrome da Imunodeficiência Adquirida
SFL	Lectina isolada de *Sophora flavescens*
SHA	Atividade Hemaglutinante Específica
SINAU	Sistema de informação das organizações de uso de águas de domínio da união
SPL	Lectina isolada de *Setcreasea purpúrea*
STL2	Lectina isolada de *Oncorhynchus mykiss*
TBS	Tampão Salino Tris

TCR	Receptor de células T
TNF-α	Fator de necrose tumoral-α
TGF-β	Fator de crescimento transformante-β
Th0,Th1,Th2, Th3 e T γ/δ	Linfócitos T auxiliares
WGA	Lectina isolada de *Triticum vulgares*

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1 INTRODUÇÃO

Lectinas constituem um grupo heterogêneo de proteínas e glicoproteínas que se ligam especificamente a carboidratos e com alta afinidade (DIMITRIJEVIC et al., 2010), desempenham um importante papel na identificação de glicoconjugados da superfície celular (XIE et al., 2009) e estão amplamente distribuídas na natureza (LAM et al., 2010).

Segundo Sharon (2008), a descoberta das lectinas em tecidos animais foi um dos maiores avanços da glicobiologia e vêm sendo muito estudadas nas últimas décadas, uma vez que essas proteínas são ferramentas muito úteis como moléculas de reconhecimento em interações de célula-molécula e célula-célula em uma diversidade de sistemas biológicos (SHARON e LIS, 2004). Em peixes, uma nova dimensão têm sido adicionada ao estudo dessas proteínas (DUTTA et al., 2005), as quais estão presentes em seus tecidos e fluidos biológicos (SUZUKI et al., 2003).

Do ponto de vista funcional, a interação específica das lectinas a glicoconjugados em solução ou na superfície celular concede a estas moléculas diversas atividades biológicas e as tornam ferramentas valiosas em diferentes aplicações biotecnológicas (CORREIA et al., 2008). Nesse contexto, as lectinas são utilizadas em diversos estudos das funções do sistema imune, tais como produção e proliferação celular e produção de citocinas, efeitos imunoestimulatórios (CARLINI e GROSSI-DE-SÁ, 2002; STAUDER e KREUSER, 2002) e produção de óxido nítrico (ANDRADE et al., 1999). Além disso, a interação lectina-carboidrato também pode causar citoaglutinação, citotoxicidade e indução da apoptose (SOBRAL, 2010; LAM e NG, 2010; YAN et al., 2010; ZHANG et al., 2010).

O beijupirá é um peixe marinho nativo do Brasil de significativo potencial econômico devido às principais qualidades biológicas que apresenta, além do alto potencial no aumento de sua produção ao redor do mundo, que estão despertando a atenção dos psicultores. O Brasil possui a maior e mais variada ictiofauna do planeta. Muitas espécies brasileiras são de extrema importância em aplicações biológicas e médicas e nenhum estudo foi realizado com lectinas de peixes do litoral brasileiro (Região Tropical). As lectinas, por suas propriedades características, são importantes ferramentas de pesquisa na área de Bioquímica, Biologia Celular, Medicina, Imunologia e áreas relacionadas.

Com base nessas considerações, o objetivo da presente tese foi realizar a purificação, caracterização e atividade imunomodulatória da lectina presente no soro do peixe beijupirá

(*Rachycentron canadum*), RcaL. Adicionalmente, foi descrito na forma de capítulo isolado uma revisão sobre o beijupirá abordando suas principais características biológicas. Finalmente, foi avaliada a atividade citotóxica e imunomodulatória da lectina isolada, através da produção de citocinas e óxido nítrico, como também foi avaliada a resposta proliferativa e produção de citocinas estimuladas por RcaL. A lectina presente no soro do beijupirá constitui um potencial novo agente biológico.

2 OBJETIVOS

2.1 Objetivo Geral

Purificar, caracterizar e avaliar a atividade imunomodulatória da lectina RcaL presente no soro do peixe beijupirá (*Rachycentron canadum*).

2.2 Objetivos Específicos

- ✓ Purificar e caracterizar a lectina presente no soro do peixe beijupirá (*Rachycentron canadum*);
- ✓ Avaliar o efeito da lectina na liberação de citocinas Th1 e Th2, bem como do mediador químico NO, em culturas de esplenócitos murinos;
- ✓ Investigar a citotoxicidade da lectina RcaL em culturas de esplenócitos murinos;
- ✓ Investigar a atividade proliferativa da lectina RcaL em culturas de esplenócitos murinos.

3 REVISÃO BIBLIOGRÁFICA

3.1 Lectinas: Breve Histórico, Conceito e Detecção

Lectinas são biomateriais de uso potencial, cujo estudo teve início em 1888, quando Stillmark, a partir de uma preparação protéica parcialmente pura, obtida de *Ricinus communis* (i.e. mamona), a qual foi denominada Ricina, testou seu efeito em sangue e observou que ao adicionar esta lectina à amostra sanguínea, as células vermelhas se agrupavam (STILLMARK, 1888). Em 1889, Hellin obteve um resultado de hemaglutinação similar, utilizando o extrato de *Abrus precatorius*, chamando a proteína de Abrina – AAG (SHARON e LIS, 1987). Na década de 60, houve relatos sobre a descoberta da mitogenicidade da lectina de *Phaseolus vulgaris* - PHA (NOWELL, 1960), como também da intensa aglutinação de células transformadas pela lectina de germe de trigo, *Triticum vulgares* - WGA (AUB et al., 1963). Estes relatos aumentaram o interesse pela atividade biológica das lectinas e, principalmente pelo estudo da Concanavalina A (Con A), lectina obtida de extrato de sementes da planta *Canavalia ensiformes* por Inbar e Sanches em 1969, os quais verificaram também a aglutinação preferencial de células malignas. Com essas descobertas iniciais houve um maior estímulo nas pesquisas básicas e aplicadas sobre as lectinas.

Primeiramente, Sharon e Lis (1972), incluíram no conceito de lectina todas as proteínas obtidas de plantas, microorganismos ou animais, de origem não imunológica, que se ligam a carboidratos, sendo específicas ou não para um determinado grupo sangüíneo. Goldstein et al. em 1980, definiram as lectinas como proteínas ou glicoproteínas de origem não imunológica, que apresentam dois ou mais sítios de ligação a carboidratos, através dos quais interagem com carboidratos, aglutinando células vegetais e/ou animais e precipitando polissacarídeos, glicoproteínas e glicolipídeos. Logo, o termo aglutinina é utilizado como sinônimo para lectina, porque se refere à habilidade de aglutinar eritrócitos ou outras células (PEUMANS e VAN DAMME, 1995). Nos últimos anos, com a nova descrição, lectinas foram definidas como proteínas ou glicoproteínas que ligam reversivelmente a mono, oligo ou polissacarídeos com alta especificidade e sem alterar sua estrutura através de sítios de reconhecimento a carboidrato (SITOHY et al., 2007; CORREIA et al., 2008).

As lectinas apresentam uma ampla distribuição na natureza e muitas dessas moléculas têm sido isoladas de plantas (YAN et al., 2010; YAO et al., 2010), diferentes microrganismos (SINGH et al., 2010) e animais (BATTISON e SUMMERFIELD, 2009; CHEN et al., 2010). Além disso, desempenham papéis importantes em eventos celulares como a aglutinação, proliferação celular,

opsonização, transdução de sinal e apoptose (TASUMI et al., 2002; NAUTA et al., 2004; TATENO et al., 2002; TSUTSUI et al., 2006b; LITMAN et al., 2007).

As lectinas podem se ligar a açúcares livres ou resíduos de carboidratos de polissacarídeos, glicoproteínas ou glicolipídeos, onde podem estar livres ou ligados à membrana da célula (MONZO et al., 2007). A presença de lectinas é principalmente revelada através de um ensaio de hemaglutinação, como ilustrado na Figura 1, que utiliza uma diluição seriada da lectina antes da incubação com eritrócitos (COELHO e SILVA, 2000; PAJIC et al., 2002).

Os carboidratos específicos ou grupo de carboidratos em oligossacarídeos ou glicoproteínas, através dos seus sítios de ligação que tendem a se localizar na superfície da molécula protéica, podem se ligar às lectinas por pontes de hidrogênio, interações de Van der Walls e interações hidrofóbicas (COMINETTI et al., 2002; SHARON e LIS, 2002).

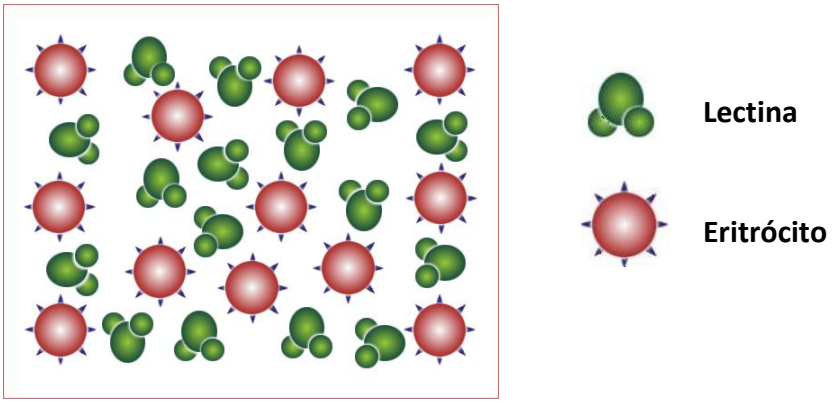

Figura 1: Atividade hemaglutinante (AH) mostrando a formação da malha de aglutinação. Fonte: BEZERRA, 2007.

A especificidade de uma lectina tem sido analisada através de ensaios de inibição da atividade hemaglutinante, utilizando para isto diferentes carboidratos (GABOR et al., 2001; OTTA et al., 2002). Os eritrócitos utilizados para este ensaio podem ser humanos ou de animais, onde estes podem ser tratados enzimaticamente (tripsina, papaína, entre outras) ou quimicamente (glutaraldeído ou formaldeído) aumentando ou não a sensibilidade das células a lectina (CORREIA e COELHO, 1995; COELHO e SILVA, 2000; MO et al., 2000).

3.2 Lectina Animal e sua Classificação

Segundo relatos de ainda 1960, Sillas Weir Mitchell foi o primeiro pesquisador a observar a atividade de uma lectina animal isolada a partir do veneno da cascavel (*Crotalus durissus*),

(KILPATRICK, 2002; ZELENSKY e GREADLY, 2005). Entretanto em 1974, pesquisadores afirmaram que Ashwell observou pela primeira vez em células animais, uma lectina animal do tipo-C (VARKI, 1999; SHARON, 2008; VARKI et al., 2009).

A primeira classificação, com base na estrutura molecular das lectinas animais, foi proposta pelo pesquisador Kurt Drickamer em 1988, utilizando ao menos, um sítio específico de ligação a carboidrato, denominados Domínios de Reconhecimento a Carboidratos (CRDs); o qual se liga a carboidratos ou glicoconjugados em solução ou que estejam conectados ao envoltório celular (WEIS e DRICKAMER, 1996). Os CRDs encontrados em cada tipo particular de lectina compartilham um padrão de resíduos de aminoácidos altamente conservados e invariáveis (KISHORE et al., 1997; RINI e LOBSANOV, 1999; EWART et al., 2001; LORIS, 2002; SUZUKI et al., 2003). Esses CRDs foram então designados do tipo-C e do tipo-S (DRICKAMER, 1988; KILPATRICK, 2002). Posteriormente, receptores de manose-6-fosfato indicaram que os CRDs nestas proteínas formam outro grupo distinto de domínios de ligação ao açúcar, designado como tipo-P (DRICKAMER, 1995). De acordo com Kilpatrick (2002), a capacidade de ligação a carboidratos pode ter evoluído de forma casual e independente, em um grande número de famílias não relacionadas, sendo provável que cada família tenha evoluído de uma estrutura que foi conservada para realizar outras atividades e funções. Assim, as famílias das lectinas animais cresceram, e variam de acordo com o seu ligante de carboidrato e suas atividades biológicas.

Lectinas do tipo-C são proteínas que ligam uma variedade de carboidratos contendo um ou dois sítios de ligação ao cálcio, sendo este(s) sítio(s) também o mesmo sítio de ligação a carboidrato (EWART et al., 1999; RICHARDS et al., 2003). As lectinas do tipo-C podem constituir homodímeros, homotrímeros e oligômeros, aumentando assim a sua afinidade por ligantes. Ainda que as lectinas do tipo-C compartilhem uma homologia estrutural, diferem significativamente entre os tipos de glicanos que se ligam com alta afinidade. De acordo com a seqüência do CRD, lectinas animais tipo-C podem ser classificadas dentro de 17 subgrupos (I ao XVII) (ZELENSKY e GREADY, 2005), onde cada subgrupo tem um CRD em diferente arranjo estrutural (DRICKAMER e TAYLOR, 1993, EWART et al., 2001).

As lectinas do subgrupo III são denominadas colectinas. A estrutura básica de cada colectina é composta pelo segmento N-terminal, seguida pela região colagenosa, depois região α-hélice e CRD tipo-C na extremidade C-terminal, e geralmente constitui um trímero (HOLMSKOV et al., 1994), como ilustrado na Figura 2. Lectina que liga manose (MBL) é um tipo clássico deste subgrupo de lectinas do tipo-C, uma colectina plasmática dependente de cálcio, secretada pelo fígado e que parece ser um importante componente da imunidade inata (KILPATRICK, 2003). As MBLs também podem estimular a fagocitose *in vitro*, através do reconhecimento a carboidratos da

superfície de patógenos, quimiotaxia e produção de oxigênio reativo, além de regularem a liberação de citocinas por células do sistema imunológico (CUMMINGS, 1999). Em mamíferos, essas proteínas desempenham muitas funções como apoptose, receptores de sinalização e adesão celular; e muitas funções imunológicas como inflamação e imunidade a tumores e células infectadas por vírus (DRICKAMER, 1999; CUMMINGS, 1999; ZELENSKY e GREADY 2005; CUMMINGS e MCEVER, 2009). Esse subgrupo de lectinas é muito estudado em teleósteos marinhos, peixes cartilaginosos e de água doce. As MBLs podem ter sua capacidade de se ligar a carboidratos inibida por açúcares simples como a fucose, manose e N-acetilglicosamina, porém sua maior afinidade de ligação é a manose (VITVED et al, 2000; KILPATRICK, 2002; NIKOLAKOPOULOU e ZARKADIS, 2006).

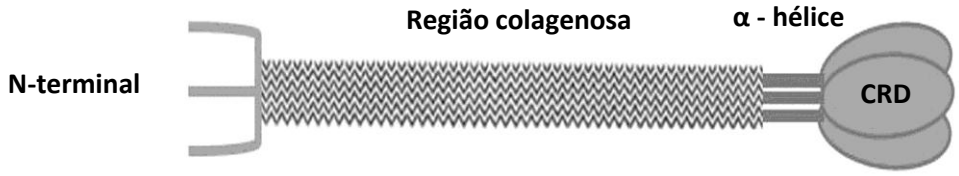

Figura 2: Representação esquemática da estrutura básica de colectinas. Composta pelo segmento N-terminal, seguido pela região colagenosa, depois região α-hélice e CRD tipo-C na extremidade C-terminal. Fonte: M. C. CORIOLANO.

Lectinas do tipo P reconhecem como ligante principal manose 6-fosfato e podem ser ou não cálcio dependentes (KISHORE et al., 1997; PROBSTMEIER e PESHEVA, 1999).

Lectinas do tipo S são proteínas intra e extracelulares, não são cálcio dependentes e possuem pontes dissulfeto. Esse tipo de lectina reconhece predominantemente o carboidrato galactose. De acordo com Fukumori et al. (2007) galectina é um membro de lectina animal do tipo S caracterizada pela sua afinidade por β-galactose.

3.3 Purificação e Caracterização de Lectinas

A partir do extrato bruto, as proteínas podem ser isoladas por alguns métodos, tais como o fracionamento de proteínas com sais. A purificação parcial de lectinas através de fracionamento salino, utilizando o sulfato de amônio tornou-se um dos procedimentos mais utilizados, no qual a sua solubilidade depende da concentração dos sais dissolvidos (LEHNINGER, 2006); a solubilidade aumenta com o acréscimo de sais (*salting in*) e volta a decrescer à medida que mais sal é adicionado (*salting out*). O sulfato de amônio, altamente hidrofílico, remove a camada de solvatação das

proteínas fazendo com que as mesmas se precipitem (DELATORRE et al., 2006), mas mantendo sua conformação nativa (COELHO e SILVA, 2000).

As lectinas parcialmente purificadas pelo tratamento salino são geralmente submetidas ao processo de diálise em membranas semipermeáveis, método baseado na separação de moléculas por diferenças de peso molecular. Nesse caso, as proteínas ficam retidas dentro da membrana enquanto moléculas menores (como carboidratos ou sais), presentes na amostra, passam para a solução solvente (THAKUR et al., 2007).

Em seguida, são utilizadas técnicas cromatográficas que purificam as lectinas de acordo com a massa molecular, carga e afinidade específica de ligação a carboidratos A cromatografia de afinidade (Figura 3), técnica mais comumente utilizada, baseia-se na habilidade das lectinas se ligarem especificamente a suportes polissacarídicos através dos seus sítios específicos para ligações não-covalentes. A proteína desejada pode ser obtida com alto grau de pureza (YE e NG, 2002) pela eluição com uma solução contendo um competidor (OLIVEIRA et al., 2002). As matrizes de afinidade podem ser selecionadas de acordo com a especificidade da lectina a carboidratos.

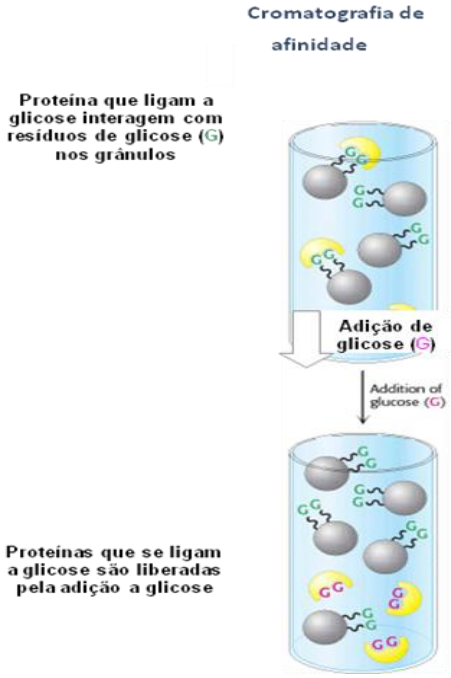

Figura 3: Cromatografia de afinidade para purificação de proteínas. Fonte: STRYER et al., 2004.

A caracterização é realizada por meio da determinação de diferentes propriedades físico-químicas da lectina e envolve métodos diversos como avaliação da AH com eritrócitos de diferentes espécies de animais (por exemplo: coelho, galinha, sistema sangüíneo humano A, B, AB e O), em presença de íons e em diferentes valores de pH e temperatura (SANTOS et al., 2009), e inibição da AH por carboidratos e/ou glicoconjugados (YANG et al., 2007). Métodos eletroforéticos são

utilizados para caracterizar estruturalmente as lectinas, bem como para estabelecer o grau de pureza das mesmas pode ser realizada através de eletroforese em gel de poliacrilamida (PAGE), sob condições desnaturantes na presença de dodecilsulfato de sódio (SDS-PAGE), e redutoras (na presença de ß-mercaptoetanol). O grau de pureza pode ser determinado por PAGE para proteínas nativas (PAIVA e COELHO, 1992; KENNEDY et al., 1995; COELHO e SILVA, 2000; PAIVA et al., 2006).

Um grande número de pesquisas foi realizado com lectinas isoladas e caracterizadas do muco da pele (MURAMOTO e KAMIYA, 1992; SUZUKI et al., 2003; CHONG et al., 2006; TSUTSUI et al., 2007), do soro (FOCK et al., 2000), de ovos (HOSONO et al., 2005) e tecidos de peixes. Hoje, existem muitas lectinas animais descritas como conseqüência dos avanços da engenharia de proteínas, biologia molecular, proteômica e genômica, além do conhecimento sobre as propriedades das lectinas animais já relatadas por pesquisadores no mundo inteiro (TSUTSUI et al., 2003; TSUTSUI et al., 2006).

3.4 Lectinas como Agentes Imunomoduladores

Lectinas são proteínas amplamente versáteis e quando purificadas, devido a sua especificidade de ligação a carboidratos, têm demonstrado um papel interessante em modelos médicos e biológicos, sendo assim consideradas importantes ferramentas na compreensão da sinalização e modulação da resposta biológica (GOSH et al., 1999; CECHINEL et al., 2001; LOPES et al., 2005; DAS et al., 2007; GOSH e MAITI 2007; KHIL et al., 2007; SONG, et al., 2007; SÁ et al., 2009).

A Jacalina, lectina isolada da planta *Artocarpus heterophyllus*, exibe ligação específica ao antígeno de células T associado a células tumorais (JEYAPRAKASH et al., 2002; BENOIST et al., 2009) e ativa os linfócitos T, especialmente $CD4^+$ e Natural Killer (NK), além de ser usada para investigar a proliferação de células mononucleares do sangue periférico em pacientes portadores da Síndrome da Imunodeficiência Adquirida (SIDA/AIDS) (BUNN-MORENO e CAMPOS-NETO, 1981; PINEAU et al., 1989, 1990; KAY et al., 1990; LAFONT et al., 1994; TAMMA et al., 1996, 2003).

A Abrina (AAG) é uma lectina com ligação específica à galactose considerada uma imunoadjuvante (GHOSH e MAITI, 2007). Ensaios de atividade antitumoral com AAG têm apresentado citotoxicidade e atividade antitumoral pelo seu potencial de indução de imunidade antitumoral (resposta Th1 e ativação de linfócitos NK), como também a inibição do sarcoma 180

em modelos animais (TUNG et al., 1979, 1981; HEGDE et al., 1991; OHBA et al., 2004; BHUTIA et al.2008a,b; BHUTIA et al., 2009).

A partir de extratos de plantas conhecidas como Visco ou Mistletoe (ML) foram identificadas três lectinas, MLI (*Viscum album* Var. aglutinina – VAA), MLII (*Viscum album* Var. *coloratum* aglutinina – VCA) e MLIII (FRANZ et al., 1981; HOLTSKOG et al., 1988; STAUDER e KREUSER, 2002; MENGS e GOTHEL, 2002), as quais são usadas como agentes imunomodulatórios e modificadores da resposta biológica (JUNG et al., 1990; STEIN, 2000; THIES et al., 2005). Lectinas Mistletoe induzem ainda a liberação do fator de necrose tumoral-α (TNF-α), interferon-γ (IFN-γ), interleucinas 1, 2, 3, 5, 6, 10, 23 (HEINY e BEUTH, 1994; BAXEVANIS et al., 1998) e modulam a resposta imunológica diferenciando as respostas Th1 e Th2 (YOON et al., 2003; LYU e PARK, 2006, 2009; LEE et al., 2007).

A lectina Con A tem sido bastante utilizada no estudo da função linfocitária (MOHD e KHAN, 2003). Tal lectina reconhece glicoproteínas de superfície de leucócitos (PINK et al., 1983; PESCHKE et al., 1990), de linhagem de células transformadas e não-transformadas (OZANNE e SAMBROOK, 1971; CLINE e LIVINGSTON, 1971) e ainda induz uma alta resposta mitogênica associada a expressão e secreção de citocinas específicas por se ligar ao receptor de células T (CD3) e outras moléculas co-receptoras da superfície celular de células imunes, (DISABATO et al., 1989; PANI et al., 2000; TRIPATHI e MAITI, 2005).

Dois compostos ativos são isolados do extrato de *Phaseolus vulgaris*, a mucoproteína PHA-M e a glicoproteína PHA-P, o componente mais potente e mais extensivamente investigado devido a suas propriedades hemaglutinantes e leucoaglutinantes (RIGAS e TISDALE, 1969a,b). A lectina PHA-P atua como um poderoso mitógeno induzindo proliferação linfocitária, ativando as funções efetoras citotóxicas das células NK e macrófagos, e estimulando a secreção de citocinas e quimiocinas específicas (WIMER, 1990; WIMER e MANN, 2002); possui ainda atividades antivirais e antifúngicas (D'COSTA e HURWITZ, 2003). Estudos relacionados à terapia antitumoral têm demonstrado a eficácia do tratamento com esta lectina (CUMMING e KORNFELD, 1982; RAEDLER e SHREIBER, 1988; WIMER, 1997; KASUYA et al., 2008).

A partir das sementes de *Cratylia mollis* foram realizados experimentos envolvendo Cramoll livre e encapsulada em lipossomas, com atividade antitumoral (ANDRADE et al., 2004), atividade mitogênica de linfócitos (MACIEL et al. 2004), alta proliferação de interleucina-2 (IL-2) e liberação de IL-6 sobre esplenócitos de rato pela Cramoll 1,4 (MELO et al., 2010), mostraram um alto desempenho da lectina de *C. mollis* na imunomodulação.

Atualmente, ainda são poucos os estudos realizados com lectinas de peixes em ensaios de imunomodulação. Segundo Silva et al. (2012), OniL, uma lectina que reconhece manose, isolada do soro de *Oreochromis niloticus*, é um potencial imunomodulador que apresenta preferencialmente resposta imunológica do tipo Th1. Watanabe et al. (2009), isolaram uma lectina de *Oncorhynchus keta* que se liga a L-ramnose e induz a produção de citocinas pró-inflamatórias. O efeito da atividade mitogênica de lectina de *Cyprinus carpio* é evidenciado pela indução de IL-2 e INF-γ em esplenócitos de rato (ROITT et al., 1986; LAM e NG, 2002); Ng et al. (2003), mostraram também que a lectina de carpa exerce um efeito mitogênico sobre esplenócitos de ratos e uma ação estimulante sobre a atividade fagocítica de macrófagos sobrenadantes. Dutta et al. (2005), analizando uma lectina de *Clarias batrachus*, observou que esta lectina foi capaz de induzir a proliferação de linfócitos na cabeça do rim. E Wang et al. (2001), revelaram que lectinas de ovas dos peixes *Coregonus clupeoides*, *Rutilus rutilus* e *Perca flavescens* apresentam atividade mitogênica ou citotoxicidade indireta mediada por macrófagos ou citotoxinas.

3.5 Resposta Imune

Segundo Sikkeland et al. (2007), a resposta imune é complexa e envolve muitos componentes plasmáticos como citocinas, fatores de crescimento, fatores do complemento, proteínas quinase e receptores celulares.

No momento em que um organismo animal entra em contato com um determinado antígeno, inicia-se a imunidade inata, que utiliza mecanismos de reconhecimento molecular para detectar a presença desses antígenos, não levando necessariamente à imunidade prolongada. Na resposta imune inata são recrutadas células polimorfonucleares (PMNs), citocinas e proteínas plasmáticas (quimiocinas e sistema complemento) (GOLDMAN e PRABHAKAR, 2000) para o local onde se encontra o antígeno. A resposta imune adaptativa é mais tardia e específica ao invasor em questão, gerando memória imunológica para o mesmo, através da seleção clonal de células imunes para esse agente oportunista.

As células responsáveis por ambas as respostas imunológicas são, principalmente, os granulócitos, os linfócitos e as células teciduais relacionadas a eles (BENJAMINE et al., 2002; PARHAM, 2001). Os linfócitos são as únicas células capazes de reconhecimento especializado, e distinguem diferentes determinantes antigênicos, sendo responsáveis pela especificidade e memória imunológica (GOLDMAN e PRABHAKAR, 2000; PARHAM, 2001). Essas células são divididas em subpopulações de acordo com suas moléculas de superfície, por meio do sistema de designação de “cluster” CD (linfócitos B/CD19; linfócitos NK/CD16; linfócios CD3/CD3; linfócitos T

auxiliares (Th1 e Th2)/CD4; linfócitos T citotóxicos/CD8 e linfócitos supressores/reguladores/CD8) sendo, portanto, classificadas pelo seu fenótipo (GOLDMAN e PRABHAKAR, 2000; PARHAM, 2001). Os linfócitos T auxiliares apresentam ainda comportamentos distintos entre si. Este comportamento que está relacionado aos tipos de atividade das citocinas específicas produzidas por estas células T, dividiu os linfócitos T auxiliares em Th0, Th1, Th2, Th3 e linfócitos T γ/δ, sendo as respostas Th1 e Th2 amplamente estudadas na imunologia (KOURILSKY e TRUFFA-BACHI, 2001).

De acordo com Chtanova e Mackay (2001), os linfócitos Th0 na presença da interleucina-4 (IL-4) transformam-se em Th2 e na presença de interleucina-12 (IL-12) tornam-se Th1. A resposta Th1 é definida pela produção de INF-γ e está associada com a imunidade mediada por células, incluindo reações de hipersensibilidade tardia, recrutamento e ativação de macrófagos inflamatórios e leucócitos, e respostas citotóxicas que levam a proteção contra microrganismos celulares. Além disso, os linfócitos Th1 respondem bem aos antígenos apresentados pelas células B, e produzem ainda IL-2, linfotoxinas e fator de necrose tumoral. Por outro lado, a resposta Th2 é caracterizada pela produção de interleucinas, IL-4, IL-5, IL-6, IL-10, IL-13 (YANG et al., 2005) e está associada com a imunidade humoral, levando a proteção contra microrganismos extracelulares. Assim como as células T $CD4^+$ que deram origem ao paradigma Th1/Th2, outros tipos celulares como linfócitos T $CD8^+$, células dendríticas, macrófagos e células NK também produzem citocinas de ambos os tipos, ou seja, tipos 1 e 2 (KIM et al., 2002). Isso levou a divisão do estudo das respostas imunológicas em resposta do tipo 1 ou tipo 2 peculiarmente relacionadas aos tipos de citocinas produzidas pelas diferentes populações celulares (KOURILSKY e TRUFFA-BACHI, 2001).

Nestes subtipos celulares ocorre o processo de regulação autócrina e inibição recíproca de crescimento e função. As citocinas IL-2 e IL-4 são produzidas por células Th1 e Th2, respectivamente, e favorecem o crescimento das mesmas (BOOTHBY et al., 2001). Nesse contexto, a produção de IFN-γ por células Th1 inibe a proliferação de células Th2, limitando o campo de difusão das citocinas do tipo 2. O contrário também ocorre e citocinas Th2 (IL-4 e IL-10) diminuem a produção de IFN-γ e o desenvolvimento do campo de difusão das citocinas do tipo 1, limitando a produção de IL-12 (MUPHY e REINER, 2002). As citocinas são polipeptídeos e glicoproteínas produzidos por diversos tipos celulares que atuam diferentemente nas imunidades inata e adquirida, por meio de interação de alta afinidade com os receptores de superfície de diferentes células (ESTAQUIER e AMEISEN, 1997), como demonstrado nas Tabelas 1 e 2, respectivamente.

Tabela 1: Caracterização das citocinas da imunidade inata, seus alvos e mecanismos de ação.

Citocina	Principais células produtoras	Principais células alvo e efeitos biológicos
Fator de Necrose Tumoral (TNF)	Macrófagos, células T	Células endoteliais: ativação (inflamação, coagulação). Neutrófilos: ativação. Muitos tipos celulares: apoptose.
IL-1	Macrófagos, células endoteliais e algumas células epiteliais	Células endoteliais: ativação (inflamação, coagulação).
Quimiocinas	Macrófagos, células endoteliais, células T, fibroblastos e plaquetas	Leucócitos: quimiotaxia, ativação e migração nos tecidos.
IL-12	Macrófagos, dendríticas células	Células T: diferenciação em Th1. Células NK e células T: síntese de IFN-γ, aumento da atividade citotóxica.
IFN-α, IFN-β	IFNα: macrófagos IFNβ: fibroblastos	Todas as células: estado antiviral, aumento da expressão de MHC I . Células NK: ativação.
IL-10	Macrófagos, células T (manutenção de Th2)	Macrófagos, células dendríticas: inibição da produção de IL-12 e expressão de moléculas co-estimuladoras MHC.
IL-6	Macrófagos, endoteliais células, T células	Proliferação celular e produção de anticorpos pela célula B.
IL-15	Macrófagos, outras	Células NK: proliferação de células T (especialmente linfócitos T $CD8^+$).
IL-18	Macrófagos	Células NK e células T: síntese de IFN-γ.

Modificado a partir de ABBAS e LICHTMAN, 2005.

Tabela 2: Caracterização das citocinas da imunidade adquirida, seus alvos e mecanismos de ação.

Citocina	Principais células produtoras	Principais células alvo e efeitos biológicos
IL-2	Células T	Células T: proliferação, aumento da síntese de citocinas. Células NK: proliferação e ativação. Células B: proliferação e síntese de anticorpos *in vitro.*
IL-4	Células T $CD4^+$ (Th2)	Células B: regula a expressão de IgE. Células T: diferenciação e proliferação de células Th2. Macrófagos: ativação mediada por IFN-γ.
IL-5	Células T $CD4^+$ (Th2)	Eosinófilos: ativação e aumento na produção. Células B: proliferação e produção de IgA.
IFN-γ	Células T $CD8^+$ (Th1) e Células NK	Macrófagos: ativação e aumento das funções efetoras. Células B: regula a expressão de IgG. Células T: diferenciação em Th1. Outras células: aumento da expressão de moléculas MHC de classe I e II, aumento do processamento de antígeno e apresentação para as células T.
TGF-β	Células T, macrófagos e outros tipos celulares	Células T: inibição da proliferação e funções efetoras. Células B: inibição da proliferação e produção de IgA. Macrófagos: inibição das funções efetoras.
IL-13	Células T $CD4^+$ (Th2)	Células B: regula a produção de IgE. Macrófagos: inibição das funções efetoras.

Modificado a partir de ABBAS e LICHTMAN, 2005.

Assim como as citocinas, o óxido nítrico (NO) é um importante mediador da resposta imune indireta. Este composto, produzido por macrófagos, é um radical, gasoso, instável, altamente reativo, derivado da oxidação do átomo de nitrogênio pela ação catalítica da enzima óxido nítrico sintase (NOS), na presença do oxigênio molecular (O_2) (PALMER et al., 1988; RYU et al., 1999).

Estudos mostram que a regulação da produção do NO dentro de macrófagos ativados se dá através da produção de duas citocinas de efeitos antagônicos, o TNF-α e o fator de crescimento

transformante-β (TGF-β). Segundo Green et al. (1994), um parasito pode ativar o gene do TNF-α e este atuar como sinal autócrino para a produção de IFN-γ, que irá induzir a produção do NO. Em contrapartida, a indução da produção do TGF-β atuaria como bloqueador da produção deste radical. Pesquisadores observaram também que o NO produzido em altos níveis por macrófagos ativados tem ação citotóxica contra bactérias, parasitas, tumores e vírus, exercendo, assim importante função na modulação do sistema imune (MONCADA et al., 1991; KIM et al., 1999).

3.6 Morte Celular

O equilíbrio entre a morte celular e proliferação celular regula e controla o número de células no organismo. A cascata de eventos, bioquímicos e fisiológicos, que leva a mudança na síntese de macromoléculas, na homeostase e volume celular, assim como na perda da viabilidade celular estão relacionadas às alterações morfológicas características de cada tipo de morte celular (TINARI et al., 2008).

A morte celular por apoptose difere da necrose em diversos aspectos bioquímicos e morfológicos (McCONKEY, 1998; ELMORE, 2007; KROEMER et al., 2009). A apoptose é considerada um mecanismo vital em diversos processos, tais como homeostase dos tecidos, apropriado funcionamento do sistema imune e desenvolvimento embrionário (BRAS et al., 2005: ELMORE, 2007). Por outro lado, a desregulação da apoptose pode afetar o balanço entre proliferação celular e morte celular, resultando no aparecimento de várias doenças humanas, incluindo o câncer (ZORNING et al., 2001; DANIAL e KORSMEYER, 2004). A apoptose está relacionada a insultos celulares mais amenos, por não resultarem em inflamação, e sua ativação depende da produção de energia na forma de ATP (Adenosina Trifosfato), ativação de caspases e outros fatores pró-apoptóticos. Além disso, possui características morfológicas e bioquímicas como a integridade das organelas celulares, condensação da cromatina, fragmentação do DNA nuclear, formação de corpos apoptóticos (McCONKEY, 1998; ELMORE, 2007; KROEMER et al., 2009), exposição da fosfatidilserina (ZIEGLER e GROSCRURTH, 2004) e mudanças na permeabilidade de membrana mitocondrial com perda do potencial de membrana (RICCI e ZONG, 2006). Em contrapartida, a necrose está relacionada a intensas agressões nas células associadas com a inflamação, processo que resulta na queda da produção de ATP e ou lesão da membrana celular, morfologicamente caracterizada por: tumefação, rompimento celular e das organelas, aparecimento de vacúolos, acidofilia citoplasmática e, em suas etapas finais, a necrose é responsável pela degradação total das células (McCONKEY, 1998; ELMORE, 2007; KROEMER et al., 2009).

Em condições normais, os fosfolipídios são assimetricamente distribuídos, com o fosfolipídio fosfatidilserina normalmente confinado na face citoplasmática da membrana plasmática. A distribuição assimétrica pode ser mudada principalmente durante o processo de apoptose, na qual serve como um primeiro sinal para a remoção fagocítica de células apoptóticas (BALASUBRAMANIAN e SCHROIT, 2003). Poucas horas após o estímulo apoptótico, ocorre a externalização da fosfatidilserina, a qual corresponde a um evento quase universal da apoptose, sendo facilmente acessível na face externa da membrana (BOERSMA et al., 2005; BLANKENBERG, 2008).

A alteração da membrana plasmática levou Koopman et al. (1994) a esboçar um ensaio de detecção da fosfatidilserina por coloração com isotiocianato fluoresceína (FITC)-conjugado com Anexina V, uma proteína com forte afinidade natural para fosfatidilserina (MARTIM et al., 1995; OZGEN et al., 2000; BRUMATI et al., 2008). O iodeto de propídeo (IP) também foi adicionado por ser capaz de distinguir células apoptóticas de necróticas, as quais têm comprometida a integridade da membrana. Nesse ensaio, células viáveis, apoptóticas e necróticas podem ser discriminadas por microscopia de fluorescência ou citômetro de fluxo (VERMES et al., 1995; BOERSMA et al., 2005; GROSSE et al., 2009).

Ainda hoje, pesquisas indicam que os mecanismos de apoptose são divididos em duas vias principais, a *extrínseca* ou via dependente de receptores de morte e a *intrínseca* ou via mitocondrial (Figura 4). Estas duas vias ocorrem independentes, sendo que a interação de ambas também pode ocorrer (IGNEY e KRAMMER, 2002; TAKAHASHI et al., 2004).

A apoptose pode ser causada pela via *intrínseca* ou mitocondrial, a qual envolve alteração no potencial de membrana mitocondrial, levando a permeabilização da membrana mitocondrial (MMP), e seguida por liberação do citocromo c (CHIPUK et al., 2006; KROEMER et al., 2007). Essa via é induzida pela resposta a estímulos pró-apoptóticos com a ativação de receptores de morte celular (REED, 2006), como a proteína Bid da família Bcl-2 (Bax, Bid e Bad) que se liga a membrana mitocondrial (BUDIHARDJO et al., 1999; POLSTER e FISKUM, 2004). Esta união promove a permeabilização da membrana mitocondrial e formação de poros (DATTA et al., 1999; GROSS et al., 1999). Dessa forma há efluxo mitocondrial de citocromo c e da proteína Apaf-1 para o citosol (GROSS et al., 1999; JOZA et al., 2001; ALIROL e MARTINOU, 2006). No citosol, citocromo c e Apaf-1 se ligam, desencadeando a formação de um complexo protéico chamado apoptossomo (complexo de alto peso molecular responsável pela ativação de várias pró-caspases), o qual permite a ativação da pró-caspase-9 (SCORRANO e KORSMEYER, 2003; KADENBACH et al., 2004; POLSTER e FISKUM, 2004; GREEN, 2005; GARRIDO et al., 2006). Uma vez ativada,

a caspase-9 ativa a caspase-3, culminando então na morte celular programada (SCHULER et al., 2003; RUPNARAIN et al., 2004).

A via *extrínseca* tem início com a ativação dos receptores de morte (*death domains*), tais como Fas e TNF (fator de necrose tumoral), segue com a ativação da pró-caspase-8 (KADENBACH et al., 2004) e culmina com a ativação de caspases efetoras, como a caspase-3 (TAKAHASHI et al., 2004; POLSTER e FISKUM, 2004). A interação entre ambas as vias pode ocorrer quando a proteína citosólica Bid, uma proteína da família Bcl-2, é clivada e translocada à mitocôndria, onde interage com a membrana e permite a liberação de citocromo c (TAKAHASHI et al., 2004).

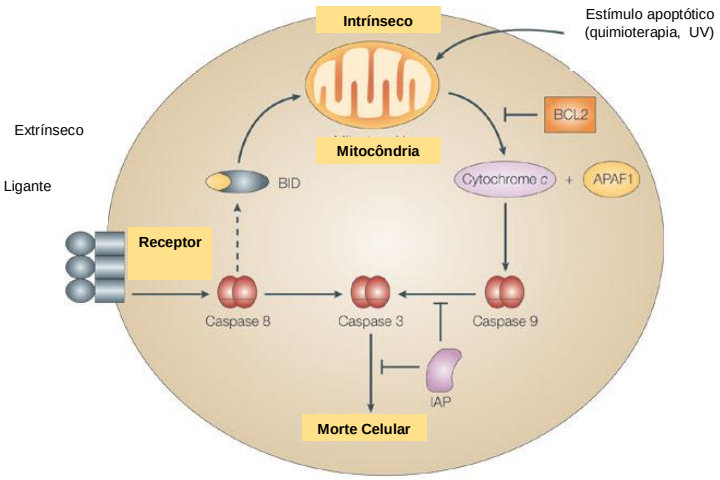

Figura 4: Vias *intrínseca* e *extrínseca* da apoptose. Fonte: ANDERSEN et al., 2005.

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5. CAPÍTULO I

Cobia (*Rachycentron canadum*): A MARINE FISH NATIVE TO BRAZIL WITH BIOLOGICAL CHARACTERISTICS TO CAPTIVE ENVIRONMENT

Artigo de Revisão *aceito* para publicação no periódico: **Advances in Environmetal Research**

Chapter

Cobia (*Rachycentron canadum*): A Marine Fish Native to Brazil with Biological Characteristics to Captive Environment

Marília C. Coriolano
and Luana Cassandra B. B. Coelho*
[1]Departamento de Bioquímica, Centro de Ciências Biológicas,
Universidade Federal de Pernambuco, Cidade Universitária,
Recife, Pernambuco, Brazil

ABSTRACT

In this review *Rachycentron canadum* will be approached, a species with best biological characteristics for marine fish cultivation. Aquaculture is recognized as an important activity to promote commercial increase in Americas from cobia, a marine fish native to Brazil, neritic and with migratory behavior. The species is widely distributed in tropical and subtropical waters of all oceans, except the Central and Eastern Pacific. Cobia does have favorable characteristics to be qualified such as high rates of growth, good feed conversion and adaptation to environmental captivity, low mortality, high market value as well as excellent meat quality. The external morphological appearance of this species is characterized by a dorsoventral flattening in the head, an elongated body and the skin covered with tiny scales. Along the sides of the body are stretched two silver bands, contrasting with a dark-brown body and yellowish color of womb; cobia can reach up to 68 kg and 2 m in length. Cobia has carnivorous feeding habits of preferably fish and crabs, although it can consume shellfish; in its diet is also included zoobenthos and nekton. Cobia has already been successfully cultivated in several countries such as China, Taiwan, Puerto Rico, Vietnam, United States, Bahamas, Thailand, Iran, Dominican Republic, Martinique, Panama, Mexico, Philippines, Belize, Japan, Indonesia, Reunion Island, Mayotte and Brazil. It is a very strong fish, but occasionally needs the control of diseases mainly caused by parasites, bacteria and virus. Economic indicators, such as production cost and return on investment, have shown the viability of cobia cultivation. A lectin has been detected in the serum of cobia with hemmagglutinating activity inhibited by methyl-α-D-mannopyranoside, *R. canadum* lectin, RcaL. Lectins have been described with potential function in the immune defense of fish. This paper evaluates the perspectives of cobia, with favorable biological characteristics, to succeed in environmental captivity cultivation and reveals that this species may contribute to unravel immune and biotechnological purposes.

Keywords: *Rachycentron canadum*; cobia.

* Corresponding Author: Luana Cassandra Breitenbach Barroso Coelho; E-mail address: lcbbcoelho@gmail.com Tel: +558121268540. Fax: +558121268541.

1. INTRODUCTION

Aquaculture is a farming activity for production of organisms in captivity with predominantly aquatic habitat such as fish, crustaceans, molluscs and aquatic plants (Bursztyn and Assad, 2000); it is based on profitable production, environmental conservation and social development (Valenti et al., 2000).

Aquaculture represents an alternative to the exploitation of natural resources and presents itself as the fastest-growing food production in the world. In assessing the state of world fisheries, the FAO (2009) - United Nations Food and Agriculture - considers likely that fishery has reached its maximum catch potential in the oceans, tending to capture stabilization of about 90 million tons, with 1.2% growth rates per year. This means that aquaculture continues to grow at a faster rate than other production sectors of animal production.

According to estimates, population growth by 2020 will result in increased fish consumption in the order of 30 million tons/year, and such increase in demand will have to be supplied by aquaculture (Chang, 2003). Thus, the various activities related to aquaculture have been perfected over the years including marine fish farming.

Aquaculture is recognized as materially important to the increased commercial activity of *Rachycentron canadum* (cobia species) in the Americas (Hernández-Rodriguez et al., 2001). In this context, with growing demand for fish originating products and the continued pressure on the finite wild stocks, the prognosis is the significant increase in aquaculture production of freshwater cobia for the next 10 years (Rojas and Wadsworth, 2007).

The seafood industry in several countries of Latin America, with extensive infrastructure already in place, benefits from recent technological advances. The introduction of commercial operations for growing cobia on commercial farms is due to increased market demand for high quality fish seen in the U.S. (Benetti et al., 2007) as well as Asian countries like China and Taiwan.

In Brazil, despite the growing research in this area in the last decade, aquaculture has never existed in practice as commercial activity, with no national official records of marine fish production (Ostrensky and Boerger, 2008; FAO, 2010).

On the other hand, although not all technological aspects of marine fish farming are as advanced as those of freshwater fish farming, this activity is considered very promising (Tsuzuki, 2006).

According to Cavalli and Hamilton (2007), to be considered ideal for growing, fish needs to dispose of animal husbandry and biological characteristics that justify the investment in research to knowledge of the species and development of appropriate technology to farming.

Characteristics are related to weight gain, feeding habit, adaptation to artificial food, disease resistance, ease eggs and larvae production, good acceptance by market and economic conditions. Cobia in Brazil occupies the first place ranking in this classification, followed by snook (*Centropomus undecimalis* and *C. parallelus*). Cobia also stands out among the more cultured marine fish worldwide (Table 1).

Despite the obvious interest in the cultivation of *R. canadum* species, there are few scientific studies about the fish (Cavalli and Hamilton, 2007; Ostrensky and Boerger, 2008). This paper will address biological characteristics and capability for farming in captivity as well as potential biotechnological applications of this species.

2. *RACHYCENTRON CANADUM*

Rachycentron canadum is a fish species historically appreciated in Brazil. Reports of the work "Descriptive Treaty from Brazil in 1587" reveals some peculiar features of cobia, as the most esteemed Brazilian fish, brown in color, large head covered with scales, very tasty meat; head formed by very delicate bones; females have yellow eggs (Sousa, 1987).

The species *R. canadum* (Linnaeus, 1766), the sole representative of the family Rachycentridae, receives various names around the world, commonly called beijupira or bijupira in Portuguese; cobia, black kingfish or ling, in English; mafou in French; cabi, in Spanish; suji, in Japanese; runner on Italy and bacalao, medregal or esmedregal, in Central and South Americas.

Cobia is a neritic fish of active swimming habit; it has migratory behavior and can be found in shallow waters with rocky or reef bottoms, as well as in estuaries and bays (Figueiredo and Menezes, 2000). The species is widely distributed in tropical and subtropical waters of all oceans, except for the central and eastern Pacific. Cobia in the wild does not form large schools (Shaffer and Nakamura, 1989). Figure 1 represents cobia's taxonomic classification (Fishbase, 2010).

Among the favorable characteristics that qualify cobia as a potential candidate for marine aquaculture are included the high growth rates from 4 to 6 kg/year, compensatory growth when grown at lower temperatures (18° C), good feed conversion in open-ocean growing systems, adaptation to confinement, transport tolerance, ease of spawning in captivity, high market value and excellent meat quality (Chou et al., 2001; Arnold et al., 2002; Liao et al., 2004; Kaiser and Holt, 2005; Wang et al, 2005; Faulk and Holt, 2006; Sun et al., 2006; Schwarz et al., 2007; Benetti et al., 2010). In addition, cobia easily accepts extruded diets (Craig et al., 2006).

According to Benetti et al. (2007) and Liao et al. (2004), many other features give the cobia, the quality of excellent species for farming as well as acceptance to artificial food (Kilduff et al., 2002; Liao et al., 2004) low mortality rate (Benetti et al., 2007), excellent use for human consumption, once 60% can be used for this purpose (Kilduff et al., 2002); easily adaptation to captive environment (Holt et al., 2007) in addition to tolerating variations of water quality (Kilduff et al., 2002).

2.1. Morphology

The external morphological appearance of this species is characterized by a dorsoventral flattening in the head and elongated body, the skin covered with tiny scales. Two silver bands stretch along the body sides, contrasting with its dark brown and yellowish ventral color. This species has seven to nine spines and 31 dorsal-fin rays as well as and two spines and 24 rays in the anal fin. It can reach up to 68 kg and over 2 m in length (Froese and Pauly, 2009). It is known as "scale dogfish" among fishermen due to its resemblance to a small shark or dogfish. The cobia's appearance is similar to a remora (Echeneididae), but morphological analyses on the larvae indicate a closer phylogenetic relationship with the goldfish (Coryphaenidae) (Ditty and Shaw, 1992). Figure 2 (A and B) shows cobia specimens in breeding ponds.

2.2. Feeding Habit

Cobia, carnivorous feeding habit, includes zoobenthos and nekton on the diet, feeding preferably on fish and crabs, although it can consume shellfish. During larval stages, their food is made preferably by copepods (Shaffer and Nakamura, 1989). On the other hand, cobia has feeding behavior associated with food availability from where they live in, which characterizes the species as fast swimmer and aggressive predator able to catching its prey in full motion, usually feeding in the water column (Chou et al., 2001).

Phylum
Cordata

Class
Actinopterygii

Subclass
Neopterigii

Infraclass
Teleostei

Superorder
Acanthopterygii

Order
Perciformes

Suborder
Percoidei

Family
Rachycentridae

Genus
Rachycentron

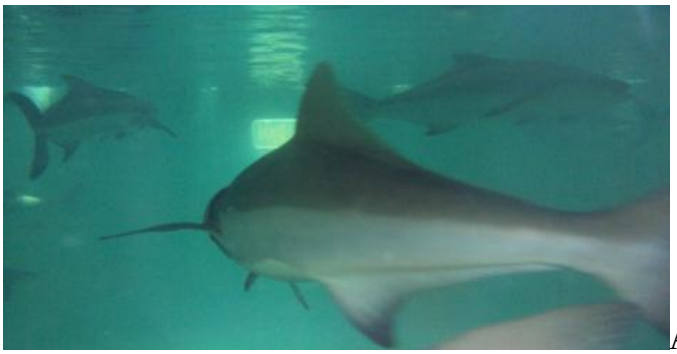
Species
Rachycentron canadum

Figure 1. Taxonomic classification of cobia (Linnaeus, 1766). Modified from Fishbase (2010).

Table 1. Major marine fish species grown in the world

Species	Major producing countries
Rachycentron canadum	** Bahamas, Belize, United States, Belize, Puerto Rico, Dominican Republic, Mexico, Philippines, Vietnam, China, Taiwan; and Brazil.
Lates calcarifer	*Malaysia, Indonesia.
Dicentrarchus labrax	*Italy, Greece, Egypt.
Salmo salar	*Norway, Chile.
Oncorhyncus keta	*Japan, Chile.
Seriola quinqueradiata	*Japan.
Pagrus pagrus	*Japan.
Mugi cephalus	*Egypt, Italy, Israel.
Paralichthys orbignyanus	*Japan, Korea, Spain, France.
Epinephelus sp	*Thailand, Malaysia.

*(Duarte, 2008) and **(FAO, 2010).


A


B

Figure 2. Cobia specimens, *Rachycentron canadum*. Source: Laboratory of Aquaculture from the Aqualider Company in the State of Pernambuco, Northeastern Brazil (author's photos, *M. C. Coriolano*). A: Side view of cobia specimens. B: Cobia specimens seen from above.

2.3. Farming

The first report on the cobia farming occurred in 1975 on the coast of North Carolina (USA) (Hassler and Rainville, 1975). Despite the good initial results, it was only in late 1980 and early 1990 that researches on the farming were conducted in the United States and Taiwan. Since then, cobia has been grown successfully, especially in China and Taiwan (Liao et al., 2004); other countries such as Puerto Rico and Vietnam are also listed as producers of cobia (Benetti et al., 2006; Nhu, 2009). Years later the growing of cobia were reported in the United States, Bahamas, Thailand, Iran, Dominican Republic, Martinique and Panama Benetti et al, 2006), Mexico (Segovia-Valle et al., 2006), Belize (Sampaio, 2006), Japan and Indonesia (Liao and Leaño, 2007), Reunion Island and Mayotte (Gaumet et al., 2007). The first report of *R. canadum* in Brazil occurred in 2006 in the State of Bahia. In recent years, the commercial and experimental farming of cobia has been reported in the States of Pernambuco, Sao Paulo, Rio de Janeiro, Bahia, Espírito Santo, Rio Grande do Norte and Paraná. According to the Information System for Authorizations of Union-Domain Water Use for Aquaculture - SINAU (2009), there were ongoing requests of projects with cobia's fattening for the States of Bahia, Parana, Rio de Janeiro and São Paulo.

Food is responsible for more than 50% of costs in intensive fish farming (Lovell, 2002). The cost of food may be even higher due to the eating habits of the species and ingredients used in the diet. Overall, carnivorous fish such as cobia have high protein requirements. Brazil uses one or two daily feeding in commercial farming in the open ocean (Cavalli et al., 2011). The same feeding frequency is used in farming at ponds set in the State of Bahia (Carvalho, 2010).

According to Chang (2003), the ideal temperature range for cobia growth is between 22 and 32° C. The Brazilian coast has favorable conditions of surface water temperature for cobia farming; in particular, the Northeast Brazilian Region has average temperature ranging among 25.3 and 29.5° C over the year (Medeiros et al., 2009). Researchers found decrease in growth and high mortality rates, when water temperature goes below 16° C (Liao and Leaño, 2005). According to Faulk and Holt (2006), some studies also showed that cobia have tolerance to variations of salinity in larval and juvenile stages. The cobia farming not necessarily needs coastal areas with high salinities. Studies have shown that juvenile cobia can be farmed in salinities between 5 to 30 ppt (Resley et al., 2006). However, the survival rates observed in 5 ppt salinity were lower than the others.

Brazil has excellent conditions for cobia farming development. Since 2003 studies have been conducted, being the Northeast a pioneer with the States of Bahia, Pernambuco and São Paulo (Carvalho, 2006). Research has shown that the species moves to reproduce in the open ocean at the natural environment (Carvalho, 1999). In this context, the necessary conditions for cobia farming in captivity will certainly have a decisive role in the development of aquaculture of this species.

2.4. Diseases

Pavanelli et al. (2000) warn of risks associated with the onset of diseases when promoting the intensification of cropping systems. Pavanelli et al. (2008) stated that regarding the increment of aquaculture in these environments, parasite species considered as problems for fish health can emerge as promoters of disease. The increased prevalence and intensity of parasitism establishes a direct relationship with high stocking densities.

Relating to the attributes necessary for indication of a species for fish farming, it is of great importance to consider its ability to resist diseases. Holt et al. (2007) mentioned that cobia is a very resistant fish to captivity conditions, feeds voraciously and grows rapidly. However, diseases occasionally arise and need to be controlled. According to Pavanelli et al. (2008), the fish containment at high stocking densities, characteristic of intensive farming model, encourages the development of epidemics that have minimal expression under natural conditions. High population densities combined with the type of food, water quality degradation, treatment and management practices typical to intensive models, cause physiological stress in fish (chronic stress) which is reflected in its homeostasis, resulting in decreased immune responsiveness. Consequently, it generates lower resistance to infections of various etiologies that can cause serious threat to the farming success, foremost among them the parasites.

Currently, parasite species considered problems for health of marine fish are few in number; however, increased farming activity in marine environments worldwide, parasites emerge with great potential to cause damage to crops. The *Amyloodinium ocellatum* Brown, 1931, among the parasites that cause problems to marine fish health, stands out as the most important pathogen that can cause serious threats to marine aquaculture (Paperna, 1980; Noga et al., 1991; Eiras et al., 2006). The *Amyloodinium ocellatum* is a dinoflagellate, mandatory fish ectoparasit, commonly found in wild populations of marine environments; it has been a major obstacle in the development of marine fish, causing great economic losses (Reed and Francis-Floyd, 1994; Eiras et al., 2006). It is a parasite commonly found in wild populations of marine environments. At the first moment of parasitosis, *Amyloodinium* invades the gills, but shortly thereafter installs across the fish surface, giving it a velvety appearance (Eiras et al., 2006). The main signs of ectoparasite infestation are behavioral changes which include reduction or complete loss of appetite. The fish scraps against objects, walls or substrate in the tanks, water backflow through the gills (coughing), with rapid breathing, erratically swimming, crowding of fish near the aerators as well as on the water surface. The disease process evolution promotes congestion and erosion of fins, mucus hypersecretion, loss of scales, stomach dilation, in addition to affecting the eyes and may cause

depigmentation patches (Paperna, 1980; Reed and Francis-Floyd, 1994). The outbreaks resulting from this disease induce in high rates of morbidity and mortality (Sindermann, 1990).

The control of amyloodiniosis outbreaks represents a major barrier in the development of marine aquaculture. There is no chemical treatment registered such as parasiticide in the Food and Drug Administration - FDA for fish intended for consumption (Smith and Schwarz, 2009).

Chemical treatments have not been sufficient to control the *Amyloodinium* in species affected by the parasite. On the other hand, the copper sulfate is the most widely used form in controlling this disease (Reed and Francis-Floyd, 1994).

This substance has strong astringent effect on the gill epithelium and is hepatotoxic to fish (Scott, 2000). In addition to being a toxic chemical to the environment and requiring great care with the application, there is no knowledge as to ensure safety results and efficiency in reducing the infestation (Reed and Francis-Floyd, 1994).

Fish diseases are important factors limiting the development of aquaculture systems; bacteria and fungi, among the various pathogens, constitute other groups of economically significant etiologic agents. (Frerichs and Millar, 1993). In most cases, diseases related to bacteria and fungi are detected as secondary diseases, resulting from wounds caused by infestation with ectoparasites. The main symptom observed is the occurrence of red sores on the fish body.

Antibiotics have been the most common agents used to treat such diseases. However, the development of microbial pathogens with high resistance, with consequent food and environmental contamination are emerging problems that urgently need effective and environmentally safe solutions (Hameed et al., 2003).

Outbreaks of campylobacteriosis, mycobacteriosis, furunculosis and streptococcosis are commonly found (Liao et al., 2004) and a bacterial disease caused by *Photobacterium sp.* has been identified as one of the main problems emerging for cobia (Lopez et al., 2002; Rajan et al., 2003; Chen and Hsu, 2005).

Clinical signs of photobacteriosis or pasteurellosis or pseudotuberculosis include skin ulceration, whitish granuloma on the liver, kidney and spleen. Cobia nurseries when affected by this disease can cause up to 80% mortality, leading researchers to evaluate the usefulness of vaccines (Chen, 2001) and immunostimulant dietary to control or reduce in losses caused by outbreaks of *P. damselae* and *Streptococcus iniae* (Chang et al., 2006). Information about viral diseases in cobias is limited. However, Chi et al. (2003) reported deaths in cobia due to the β-nodavirus NNV (nervous necrosis virus). About 30% of the stocked animals succumb to the disease, which has stimulated interest in a recombinant vaccine. According to Rodgers and Furon (1998), nodavirus is overall associated with mass mortality of larvae and this infection is often accompanied by changes in fish behavior.

In Brazil, the great difficulty found when trying to apply treatment in fish is the few studies demonstrating the efficacy and mechanisms of action through which drugs are used to control the diseases act on the biochemical and physiological functions of animals (Pavenelli et al., 2008).

2.5. Economic importance

Economic indicators evaluated in studies by Sanches et al. (2008) demonstrated the feasibility of cobia farming in offshore system, being an attractive economic activity when considering the potential production of marine species. However, this type of system is not adapted to the small fisherman or family farming due to the high investments required for projecting implementation and funding the activity.

Luz (2001) warns that despite the various characteristics conferred on carnivorous species, the commercial production still faces difficulties related to the high rate of cannibalism, feeding difficulties, as well as high costs required to maintain this fish. On the other hand, studies indicate that cobia, with statistic records of little significant catches when compared to other fish production, still represents one of species with potential for marine aquaculture by holding several characteristics favorable to husbandry and market farming.

Benetti et al. (2008), Benetti et al. (2010), Benetti and O `Hanlon et al. (2010) claim that cobia is a species of great commercial interest in marine aquaculture worldwide by presenting various qualities and great market demand.

These factors have led to a rapid development of farming technology in the last decade in Taiwan and elsewhere. Sampaio et al. (2010) complemented informing that most production is still concentrated in China and Taiwan, with global production of farmed cobia approximately of 23 000 ton/year.

3. RCAL: A LECTIN DETECTED IN THE SERUM OF COBIA

The discovery of lectins in animal tissues was one of the greatest advances in glycobiology and these proteins have been much studied in recent decades (Sharon, 2008). These molecules are very useful tools to recognize carbohydrates in cell-molecule and cell-cell interactions in a variety of biological systems (Sharon and Lis, 2004).

A new dimension has been added in fish to the study of lectins (Dutta et al., 2005), which have been purified from tissues and biological fluids of various species. Watanabe et al. (2009) isolated a lectin from *Oncorhynchus keta* that binds to L-rhamnose and induces the production of proinflammatory cytokines.

The effect of mitogenic activity of *Cyprinus carpio* lectin is evidenced by the induction of IL-2 and INF-γ in mouse splenocytes (Roitt et al., 1986; Lam and Ng, 2002); Ng et al. (2003) carp lectin has mitogenic effect on splenocytes from mice and stimulating action on the phagocytic activity of supernatant macrophages. Dutta et al. (2005) analyzing a lectin of *Clarias batrachus*, noted that this protein was able to induce the proliferation of lymphocytes in head kidney.

Fish roe lectins from *Coregonus clupeoides*, *Rutilus rutilus* and *Perca flavescens* have mitogenic activity or indirect cytotoxity mediated by macrophages or cytotoxins (Krajhanzl et al., 1985).

Lectins are a heterogeneous group of proteins, widely distributed in nature; these proteins bind specifically to carbohydrates inducing cell agglutination. Hemagglutination is a simple method to demonstrate the lectin activity and can be performed on microscope slides and plastic microtiter plates (Rudiger, 1993). Erythrocytes exhibit a wide variety of glycans on the cell surface that are recognized by lectins, causing cells agglutination (Khan et al., 2002).

In our research, a lectin was detected in serum of cobia fish (*R. canadum*) which was fractionated with ammonium sulphate saturation (40-60%; F3). Inhibition hemagglutinating activity of F3 was performed with methyl-α-D-mannopyranoside; F3 showed hemagglutinating activity for rabbit erythrocytes, as showed in Figure 3.

Currently, our research group has been dedicated to the purification of the lectin RcaL, also performing in parallel studies on immune system functions with lectin, such as immunomodulatory activity, cytokine release, nitric oxide production, cell proliferation and cytotoxicity. Similarly, another protein was isolated from serum of *Oreochromis niloticus*, OniL, lectin that recognizes mannose, with hemagglutinating activity for rabbit erythrocytes, a potential immunomodulator which has preferentially Th1-type immune response (Silva et al., 2012).

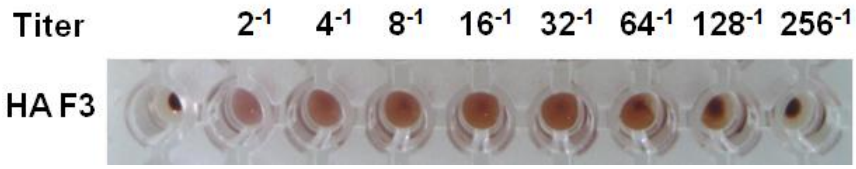


Figure 3. Hemagglutinating activity (HA) of F3. In the activity evaluated, each lectin molecule forms a network of lectin-cell-lectin interaction, maintaining the cells in suspension. The agglutinating assays were performed in 96-wells microtiter plates. Lectin preparations (50 μL) were serially two-fold diluted in 0.15 M NaCl, and an equal volume of rabbit erythrocyte (2.5%) suspension was added to each well. The plates were incubated at room temperature for 1 h.

CONCLUSIONS

This study addressed the biological characteristics of cobia, favorable to success at captivity environment; also, revealed that this species can contribute to unravel fish immune responses and be applied to biotechnological purposes.

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6. CAPÍTULO II

Immunomodulatory response of mice splenocytes induced by RcaL: a lectin isolated from cobia fish *(Rachycentron canadum)* serum

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Immunomodulatory response of mice splenocytes induced by RcaL: a lectin isolated from cobia fish *(Rachycentron canadum)* serum

Marília Cavalcanti Coriolano[1], Cynarha Dayse Cardoso da Silva[1], Cristiane Moutinho Lagos de Melo[2], Ranilson de Souza Bezerra[3], Athiê Jorge Guerra dos Santos[4], Valéria Rêgo Alves Pereira[2], Luana Cassandra Breitenbach Barroso Coelho[1,*].

[1]*Departamento de Bioquímica, Laboratório de Glicoproteínas, Universidade Federal de Pernambuco/UFPE, Pernambuco, Brazil.*

[2]*Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães – CPqAM/FIOCRUZ, Pernambuco, Brazil.*

[3]*Departamento de Bioquímica, Laboratório de Enzimologia, Universidade Federal de Pernambuco/UFPE, Pernambuco, Brazil*

[4]*Departamento de Engenharia de Pesca, Universidade Federal Rural de Pernambuco/UFRPE, Pernambuco, Brazil*

Corresponding author: Luana Cassandra Breitenbach Barroso Coelho, Departamento de Bioquímica, Laboratório de Glicoproteínas, UFPE, Av. Prof. Moraes Rego s/n, Cidade Universitária, CEP: 50670-420, Recife, Pernambuco, Brazil.

Phone: +55-81-21268540; Fax: +55-81-21268541.

e-mail: lcbbcoelho@gmail.com

Abstract

Was isolated and purified a serum lectin from cobia fish (*Rachycentron canadum*), named RcaL. Immunomodulatory activity on mice splenocyte experimental cultures through cytotoxic assays and cytokine production were also performed. RcaL was obtained through precipitation with ammonium sulfate and affinity chromatography with a Concanavalin A-Sepharose 4B column. The ammonium sulfate fraction F3 showed the highest specific hemagglutinating activity and was applied to affinity chromatography. The lectin was eluted with methyl-α-D-mannopyranoside. RcaL showed highest affinity for methyl-α-D-mannopyranoside and D-mannose; eluted fractions of RcaL agglutinated rabbit erythrocytes (titer: 128^{-1}), retained 66% of chromatographed lectin activity and the obtained purification factor was 1.14. Under reducing conditions a polypeptide band of 19.2 kDa was revealed in SDS-PAGE. PAGE confirmed RcaL as an acidic protein revealed in a single band. Cytotoxic and immunomodulatory assays with RcaL in mice splenocyte cultures showed that the lectin was not cytotoxic and induced higher IFN-γ and nitric oxide production in splenocytes cultures. Purified RcaL induced preferential Th1 response suggesting that it acts as an immunomodulatory compound.

Keywords: *Rachycentron canadum*, cobia, lectin purification, immunomodulatory activity.

1. Introduction

Cobia, *Rachycentron canadum*, is a migratory, pelagic fish which is widely distributed in the world and is found in tropical, subtropical and temperate waters in Asia and the Americas [1]. In Brazil, there is growing interest in *R. canadum*, also called cobia fish, and some projects are already being developed at various points along the coast. The State of Bahia is expanding the cultivation of cobia fish which has achieved the first spawn in captivity, producing, the first captive-bred fingerlings [2]. The State of Pernambuco also began investments in infrastructure and technology for the capture, breeding, nursery and fattening of fish. Cobia have high growth rates, high feed conversion efficiencies, good flesh quality and general hardiness in captivity and are cultivated throughout Asia [3, 4].

Lectins are a heterogeneous group of proteins and glycoproteins that specifically bind with high affinity to carbohydrates [5]. In fact, lectins have so far been isolated from serum, plasma, skin mucus, egg surfaces and egg components of fish [6, 7, 8]. Animal lectins, classified depending on their sequence similarities and sugar-binding properties as P-type, F-type, S-type (galectins) or C-type (calcium dependent), can play a variety of immunological roles. These proteins are important in the innate immune system particularly by increasing oxidative burst activities [9, 10, 11] and lectin-mediated cellular cytotoxicity [12, 13].

Mannose-binding lectin (MBL) is an important component of innate immunity in mammals [14, 15, 16, 17] and increases in levels in response to an infection or as part of an inflammatory response. Another example of animal lectins involved with immune response are galectins, which are proteins produced by different immune cell types and which have homologues existing in mammals, birds, fishes as well as in lower organisms; they are involved in the regulation of cell growth and survival inducing apoptosis in $CD8^+$ T cells [18, 19].

Proliferative and immune responses are mediated by specific cytokines and cells that determine effective functions of immune system compounds [20]. IFN-γ and nitric oxide (NO) are Th1 type cytokines secreted by activated T and natural killer cells which regulate host defense, inflammation, and autoimmunity [21, 22]. IL-10 is an important immunoregulatory Th2 cytokine that influences innate and adaptive immune responses [23]. These cytokines may induce a division of immune response between Th1 and Th2 and have a role in pro- and anti-inflammatory activities [20].

Recently, fish lectins have been purified, identified and characterized which show different carbohydrate recognitions [13]. In this paper, we also purified and characterized a fish lectin RcaL, extracted from the serum of cobia, *Rachycentron canadum*. Furthermore, we evaluated its cytotoxicity against mice splenocytes and immunomodulatory activity through cytokine and nitric oxide production.

2. Materials and methods

2.1 Blood collection and serum separation

Cobia blood collection was performed in the *Laboratório de Aquicultura da Empresa Aqualíder* in the State of Pernambuco in northeastern Brazil, in partnership with the *Laboratório de Fisiologia e Ecologia de Peixes* (LAFIPE) na *Estação de Aqüicultura Continental Prof. Johei Koiki do Departamento de Pesca e Aqüicultura da Universidade Federal Rural de Pernambuco* (UFRPE). The blood from a juvenile cobia was collected from its caudal vein using a 1ml syringe. Immediately after collection, the blood was kept in tubes and stored overnight at 4°C. After blood coagulation, the samples were centrifuged at 1300 x g for 5 min at 4ºC and the serum was removed

with a pipette. The serum was again centrifuged under the same conditions. Subsequently, the serum was kept at -20°C until the moment to be used.

2.2 Saline fractionation

The serum was precipitated with ammonium sulfate saturation (0-20%, F1; 20-40%, F2; 40-60%, F3; 60-80%, F4) according to Green and Hughens [24]. The evaluation of HA was performed in the presence of different buffers (sodium citrate, sodium phosphate, Tris buffered saline, NaCl) at different molarities and values of pH. The fraction with the highest hemagglutinating activity (HA) was dialyzed against Tris buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, 20 mM $CaCl_2$, at pH 8.0) overnight at 4 °C, obtaining F3. After dialysis, the specific HA (SHA) was detected from fraction.

2.3 Hemagglutination Assay

The evaluation of HA was performed in microtiter plates according to Correia and Coelho [25]. Lectin preparations (50 µL) were serially two-fold diluted in 0.15M NaCl before addition of 50 µL 2.5% (v/v) suspension of glutaraldehyde-treated rabbit erythrocytes or fresh human erythrocytes (types A, B, AB and O). The titer was expressed as the highest dilution exhibiting hemagglutination. Specific HA (SHA) was defined as the ratio between the titer and protein concentration (mg/mL).

2.4 Purification of lectin from serum from cobia fish

The 40-60% fraction (F3) was affinity chromatographed (0.5 mg of protein) in a Concanavalin A-Sepharose 4B (Sigma) column previously equilibrated with 10 mM $CaCl_2$ and 10 mM $MnCl_2$ in TBS buffer, at pH 8.0. The fraction was applied to a 3 mL column which was then washed with TBS to A 280 nm. 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. Fractions with the highest absorbance, which formed a peak, were pooled (RcaL) together and submitted to dialysis against TBS by 6 hours at 4 °C. The activity of RcaL lectin preparation was assayed using a 2.5% (v/v) suspension of glutaraldehyde treated rabbit erythrocytes.

2.5 Carbohydrate-binding specificity

The evaluation of HA inhibition (HAI) using RcaL and carbohydrates (D(+) glucose, D(+) mannose, galactose, methyl-glucopyranoside, methyl-mannopyranoside, methyl-α-D-galactopyranoside, N-acetylglucosamine) as well as glycoprotein solutions (fetuin, asialofetuin, ovalbumin, casein or azocasein) was performed according to Correia and Coelho [25] in microtiter plates with 96 wells. RcaL was assayed with 200 mM carbohydrates and 0.5 mg/mL glycoprotein solutions. The inhibitory assays were similar to the hemagglutinating assay with the exception of an incubation step (room temperature, 15 min) before erythrocyte addition. The HAI was established by the HA from the sample that showed reduction in the presence of carbohydrates.

2.6 Protein concentration assay

Protein concentration was estimated according to the Bradford method [26] and by absorbance at 280 nm.

2.7 Effects of pH, temperatures and Ca^{2+} on lectin HA

To determine pH stability, RcaL lectin was incubated in buffers for 12 h at 4 ºC. The solutions used were 10 mM glycine–HCl buffer (pH 2.0-3.0), 10 mM acetate buffer (pH 4.0-5.0), 10 mM sodium phosphate buffer (pH 6.0-7.0), 10 mM Tris–HCl buffer (pH 8.0-9.0), and 10 mM sodium bicarbonate buffer (pH 10-11). Thermal stability was evaluated with previously heated RcaL lectin for 10 min at various temperatures (25, 30, 40, 50, 60, 70, 80, 90, 100ºC). Assays for Ca^{2+} requirements were performed following dialysis in TBS (pH 8.0) containing 25 mM EDTA overnight at 4ºC and the lectin solution was incubated with different concentrations of Ca^{2+} (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 mM) at room temperature. HA was measured, for all experiments, as described previous using 50 µL of a 2.5% (v/v) suspension of glutaraldehyde treated rabbit erythrocytes.

2.8 Lectin characterization on eletrophoresis

PAGE (polyacrylamide gel electrophoresis) was performed for native and acidic proteins according to Davis [27]. RcaL lectin molecular weight was estimated at 7.5% (w/v) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [28] under non-reducing conditions and in the presence of β-mercaptoethanol using marker proteins (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 lysozime: 14.4 kDa); gels were stained for protein with Coomassie Brilliant Blue.

2.9 Animals

Male BALB/c mice (6 to 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).

2.10 Con A Lectin

Canavalia ensiformis (Concanavalin A) was purchased from Sigma Chemical Co., (St. Louis, MO, USA).

2.11 Preparation of splenocytes

Splenocytes were obtained according to Pereira et al. [29]. 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 individual Falcon tubes by spleen containing approximately 10 mL of incomplete medium. Spleen homogenates were overlaid onto a Ficoll-PaqueTM PLUS layer, with density adjusted to 1.076 g/mL, and centrifuged at 1000 x g at room temperature for 25 min. The interface cell layer

containing immune cells was recovered by Pasteur pipette, washed twice in PBS, and centrifuged two times at 500 x 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%

2.12 In vitro *cytotoxicity assays*

The cytotoxicity of the lectins (*R. canadum* – RcaL and Con A) were determined using BALB/c mice splenocytes ($6x10^5$ cells/well) cultured in 96-well plates in RPMI 1640 media (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil) and 50 μg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil). 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, Boston, MA) (1 μCi/well) for 24 h at 37 ºC and 5% CO_2. After this period, the content of the plate was harvested to determine the [3H]-thymidine ([^{3}H]TdR) incorporation using a beta-radiation counter (β-matrix 9600, Packard). The toxicity of the lectins was determined by comparing the percentage of ^{3}H-thymidine incorporation (as an indicator of cell viability) of lectin-treated cells in relation to untreated cells. Saponin (0.05%) and Concanavalin A (Con A) were used as positive controls. Non-cytotoxic concentrations were defined as those causing a reduction of [3H]-thymidine incorporation below 30% in relation to untreated controls.

2.13 Measurement of cytokine levels in splenocyte supernatants

Splenocytes were cultured in 24-well plates (TPP, St. Louis, Switzerland) at a density of 10^6 cells/well. Cytokines were quantified in 24, 48, 72 h and 6 day supernatants from cultures

stimulated with RcaL lectin at 10 µg/mL or Con A at 2.5 µg/mL or maintained only in culture medium (control). The levels of IL-10 and 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 (8000 pg/mL, for IL-10 and IFN-γ, respectively). After washes, 50 µl of all samples and standards were 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. Each lectin was evaluated in triplicate in four independent assays. Sample concentrations were calculated in the linear region of the titration curve of cytokine standards, and final concentrations were expressed in pg/mL, using the Microplate Manager Version 4.0 software (Bio-Rad laboratories).

2.14 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 RcaL (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 [30]. Each lectin was evaluated in triplicate in four independent assays. NO concentration was estimated by the standard curve (3.12 - 100 µmol.mL^{1}).

2.15 *Statistical analysis*

Data were analyzed using non-parametric tests. to detect differences between groups, the mann-whitney u test was used. all results were expressed as mean values of groups ±standard deviation and were analyzed considering the value of $p < 0.05$ as statistically significant.

3. Results

A lectin from the serum of *R. canadum*, named RcaL, was purified by affinity chromatography using a Concanavalin A-Sepharose 4B column. Subsequently, cytotoxic and immunomodulatory assays were performed to analyze the biological activity induced by this new purified fish lectin.

Was difference in Hemagglutinating Activity (HA) for F3 in different tested buffers with distinct molarities and pH. The buffer with highest HA was Tris buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, 20 mM $CaCl_2$, at pH 8.0); this buffer was chosen for analysis. The highest HA activities were detected in the serum from *R. canadum* and F3 fraction; this material was chosen for purification (Table 1).

Table 1

Hemagglutinating activities of serum and lectin preparations from *Rachycentron canadum* using rabbit erythrocytes.

Samples	Hemagglutination activity [a]
Serum	256^{-1}
0-20%, F1	0
20-40%, F2	8^{-1}
40-60%, F3	128^{-1}
60-80%, F4	0
FSupernatant	0

[a] Titer was expressed as the highest dilution exhibiting hemagglutination. The agglutinating assays were performed in 96-wells microtiter plates. Lectin preparations (50 μL) were serially two-fold diluted in 0.15 M NaCl, and an equal volume of rabbit erythrocyte suspension was added to each well. The plates were incubated at room temperature for 1 h.

When F3 (SHA: 20.3) of *R. canadum* serum was chromatographed in a Concanavalin A-Sepharose 4B column (as showed in Fig. 1), the HA was detected in the absorbed sample (SHA: 23.2). The methyl-α-D-mannopyranoside (200 mM) in TBS eluted fractions of RcaL retained 66% of the chromatographed lectin activity and the purification factor obtained was 1.14 (Table 2). RcaL showed HA to rabbit erythrocytes (titer: 128^{-1}) as well as HA to A, B, O and AB type fresh human erythrocytes (titer: 8^{-1}, 32^{-1}, 4^{-1}, 64^{-1}, respectively). RcaL is a protein active at pH 7.0-8.0 and resistant for 10 min at 40ºC. The presence of Ca^{2+} at a concentration of 20 mM increased RcaL HA but when the lectin was dialyzed against EDTA, the HA was lost and further addition of Ca^{2+} did not restore activity.

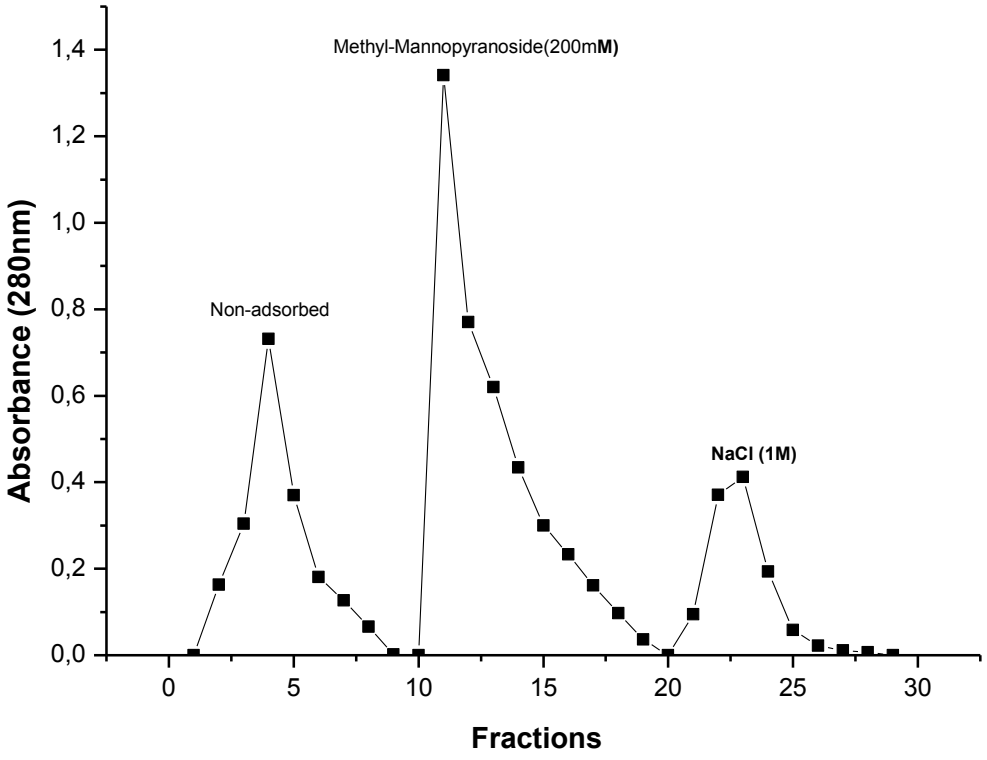

Fig. 1. RcaL (0.5 mg of F3 protein) purification by Concanavalin A-Sepharose 4B affinity chromatography. The F3 was applied to the column (3 ml) and previously equilibrated with Tris buffered saline (TBS, 20 mM Tris–HCl containing 150 mM NaCl, 20 mM $CaCl_2$) at pH 8.0. The lectin elution was performed with two-milliliter fractions which were collected at a flow rate of 20 mL/h. At the point indicated (arrow) elution buffer was changed to methyl-α-D-mannopyranoside (200 mM) in TBS. Absorbance at 280 nm is represented.

Table 2

Summary of steps of RcaL purification.

Samples	Total Protein (mg)	HA[a]	Total HA[b]	SHA[c]	Yield [d*] (%)	Purification* (folds)
Serum	13.7	256^{-1}	3328	18.6	-	-
F3	6.3	128^{-1}	384	20.3	100	1
RcaL	5.5	128^{-1}	1024	23.2	66	1.14

[a] Hemagglutinating activity (HA) was expressed as a titer. The activity was determined with rabbit erythrocytes.

[b] Total hemagglutination titer (HA×Volume).

[c] Specific HA (HA/total protein).

[d] Percentage of total activity recovered. More than 5 mg of RcaL were obtained from 13 mL of serum when submitted to 40-60% ammonium sulfate fractionation followed by Concanavalin A-Sepharose 4B affinity chromatography.

*Dashes indicate that the values were not determined.

The binding specificity of RcaL was evaluated using carbohydrates and glycoproteins. The monosaccharides methyl-α-D-mannopyranoside and D-mannose were the best inhibitors (Table 3). The glycoproteins (fetuin, asialofetuin, ovalbumin, casein or azocasein) showed no effect on HA of RcaL.

RcaL, purified after Con-A Sepharose 4B chromatography, was revealed as polypeptide band of 19.2 kDa in SDS-PAGE under reducing conditions (Fig. 2A); PAGE confirmed RcaL as an acidic protein with a single band (Fig. 2B).

Table 3

Inhibition assay of hemagglutinating activity of *RcaL* with carbohydrates.

Inhibitor (200 mM)	**RcaL[a]**
D (+)-Glucose	16^{-1}
D(+)-Mannose	2^{-1}
Galactose	8^{-1}
Methyl-glucopyranoside	4^{-1}
Methyl-mannopyranoside	0
Methyl-α-D-galactopyranoside	4^{-1}
N-acetylglucosamine	8^{-1}

[a]Were used rabbit erythrocytes. Inhibitory activity is expressed as the minimum inhibitory concentration that is required to completely inhibit the hemagglutinating activity of a titer. Carbohydrates (200 mM) was used.

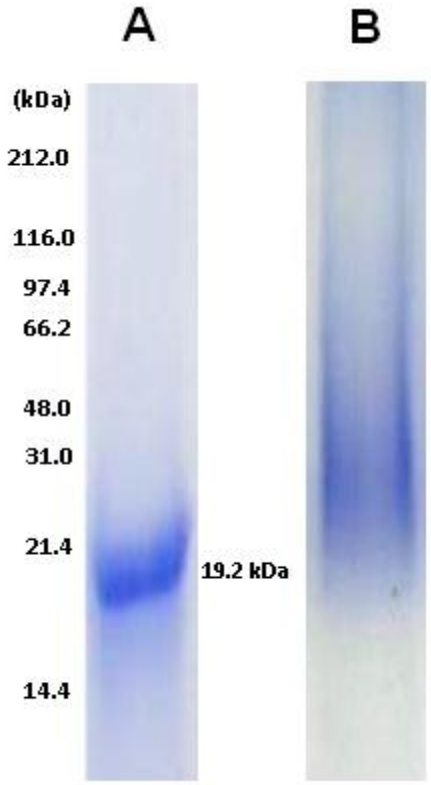

Fig. 2. The purification profile of the RcaL stained by Coomassie Brilliant Blue in 7.5% gels. (A) SDS-PAGE of lectin (40 µg) treated with β-mercapthoethanol and (B) PAGE showing purified native and acidic RcaL. Molecular weight markers: myosin (212.0 kDa), β-galactosidase (116.0 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (48.0 kDa), carbonic anydrase (31.0 kDa), soybean trypsin inhibitor (21.4 kDa) and lysozime (14.4 kDa).

RcaL did not show cytotoxicity against mice splenocytes. The cytotoxicity threshold was expressed as the highest tested concentration that was not cytotoxic to the splenocytes. Saponin and Con A were used as positive controls. Results showed that saponin (0.05%), known for its cytotoxicity, demonstrated higher inhibition. Con A, used a reference for immunological assays, did not show toxic activity below 50 µg/mL concentrations and RcaL did not reveal cytotoxicity at either experimental concentration (Table 4).

Table 4

Cytotoxicity evaluation induced by *Rachycentron canadum* (RcaL) serum lectin.

Compounds	**Concentrations (μg/mL)**					
	100	50	25	10	5	1
	Inhibition (%)*					
RcaL	-	-	-	-	-	-
Con A	58	-	-	-	-	-
Saponin*	94	91	90	89	88	88

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

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

Th1 preferential response was induced by RcaL. Immunological assays were performed to analyze the degree of immune response induced by RcaL fish serum lectin. Through ELISA assays, was observed that IFN-γ was the main cytokine produced by mice splenocytes treated, *in vitro*, with RcaL. Fig. 3A-D shows that RcaL was statistically superior to the control (untreated cells) at all experimental times. Con A also showed higher values which were greater than the control at all times and greater than RcaL at 24 and 48 h (Fig. 3A and B). RcaL showed similar results to Con A at 72 h and 6 days (Fig. 3C and D) being statistically higher than Con A at 72 h of assay (Fig. 3C).

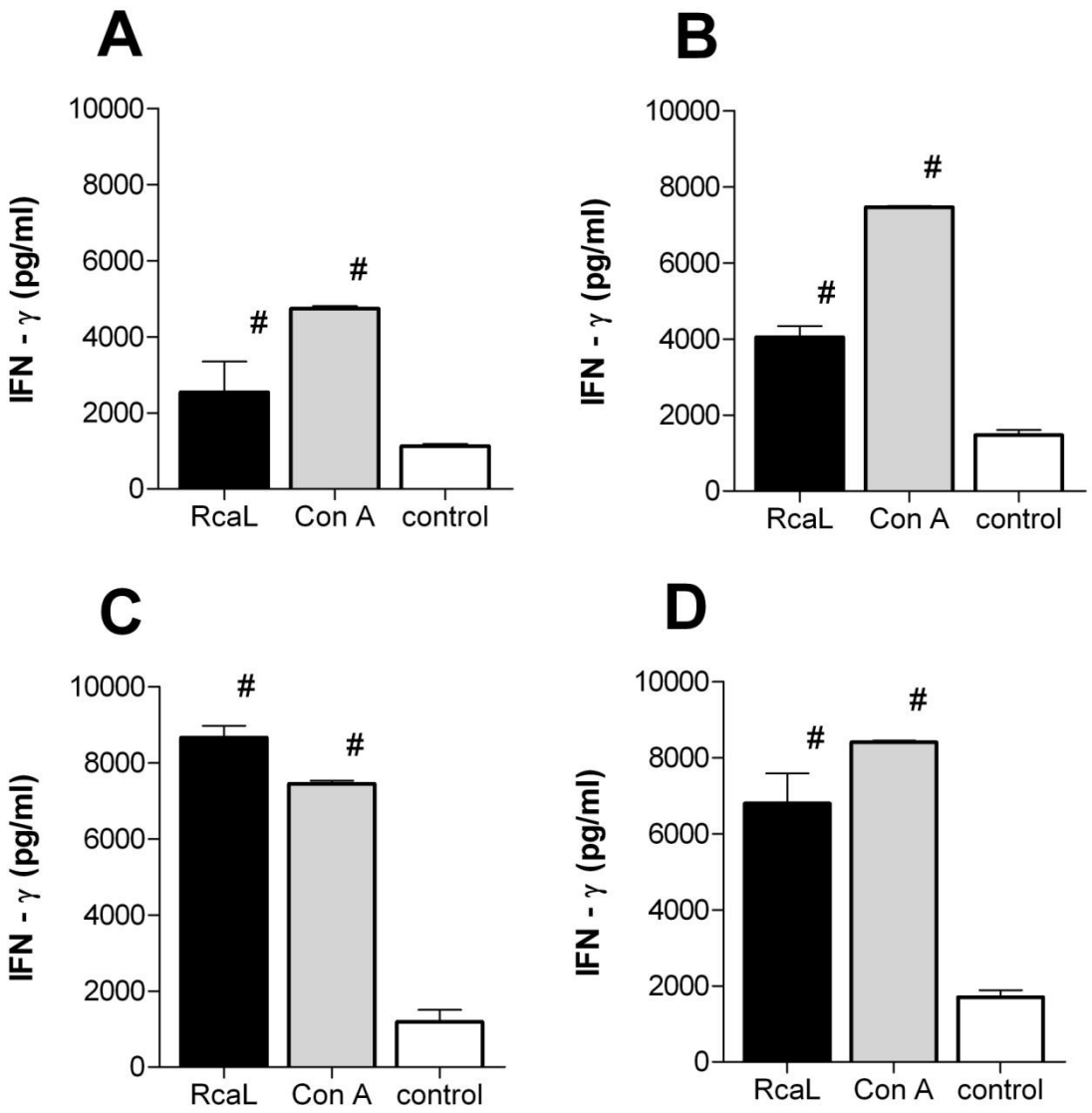

Fig. 3. IFN-γ production induced by RcaL and Con A lectins in mice splenocyte cultures. **A**, **B**, **C** and **D** – are 24, 48, 72 h and 6 days, respectively. RcaL and Con A induced higher and statistically significant values of IFN-γ production at all experimental times. Con A also showed higher values in relation to RcaL at 24 and 48 h (A and B, respectively) and RcaL lectin was statistically superior to Con A at 72 h of assay (C). Horizontal bars represent the average of four independent experiments per group. * $p < 0.05$.

IL-10 was also produced by splenocytes stimulated with RcaL and Con A lectins. At 48 and 72 h of assay, both lectins induced higher and statistically significant IL-10 production in relation to the control, but not between them (Fig. 4A and B). At 24 h RcaL (21 ± 9.6) and Con A (45 ± 5)

induced IL-10 release but not statistically different values in relation to the control (48 ± 15). Finally, at 6 days of assay only Con A induced higher and statistically significant IL-10 release in relation to the control (86 ± 6.6).

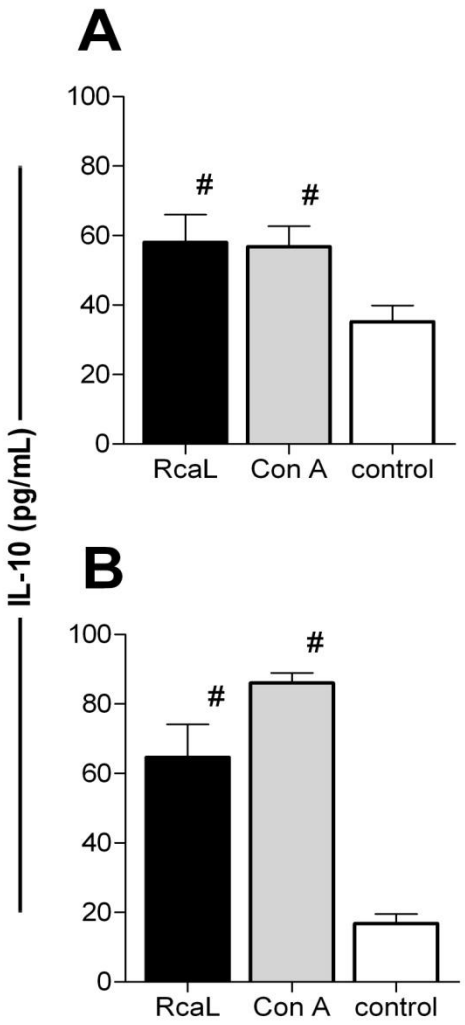

Fig. 4. IL-10 production induced by RcaL and Con A lectins in mice splenocyte cultures. **A** and **B** – are 48 and 72h of assay, respectively. Both lectins induced higher and statistically significant IL-10 production in relation to the control, but not between them at 48 and 72 h of assay. Horizontal bars represent the average of four independent experiments per group. * $p < 0.05$.

Higher IFN-γ may be responsible for statistically significant nitric oxide (NO) release observed in RcaL treated splenocytes. RcaL resulted in greater NO release than Con A and the control at 24 and 48 h (Fig. 5). Both lectins did not induce statistically significant values of NO at

72 h (34.75 ± 8.7 and 43.3 ± 13 for RcaL and Con A, respectively) and 6 days (not detectable –ND- and 40 ± 15 for RcaL and Con A, respectively) of assay.

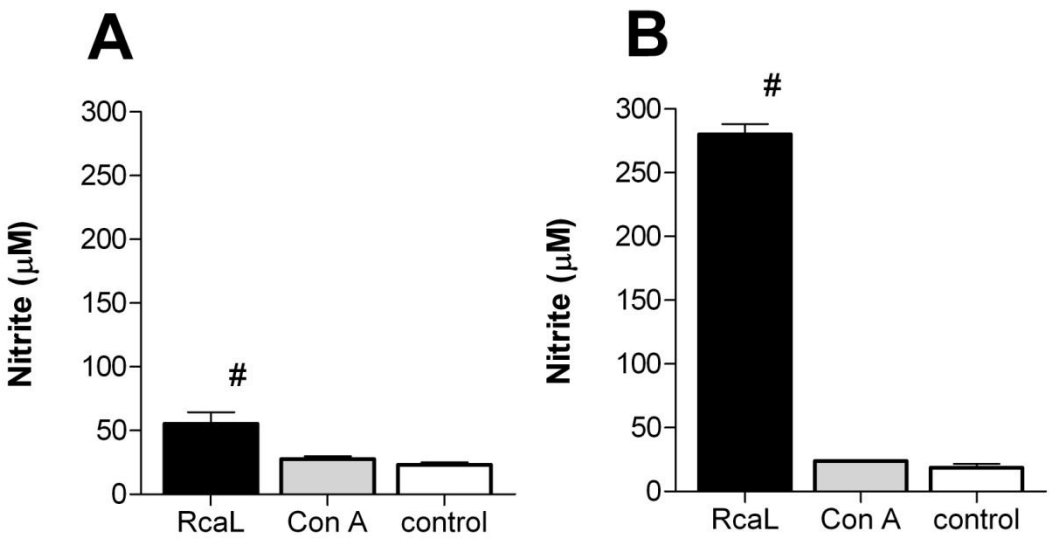

Fig. 5. Nitric oxide release induced by RcaL and Con A lectins in mice splenocyte cultures. **A** and **B** – 24 and 48 h of assay showing that RcaL induced higher and statistically significant NO release in relation to Con A and the control. Horizontal bars represent the average of four independent experiments per group. * $p < 0.05$.

4. Discussion

Numerous serum lectins have been identified in fish. Mannan (or mannose-binding) lectin is thought to be an important component of innate immunity. Some lectins prevent infection by binding to pathogens and promoting phagocytosis. In contrast, some lectins function as recognition and trafficking agents to promote extravasations of cells to sites of inflammation in the acquired immune system. In this context, many lectins may also be involved in modulating the immune response [31]. To clarify the biological function and to determine its immunomodulatory potential,

the present paper were investigated a lectin that recognizes mannose, obtained from *R. canadum* fish serum, RcaL.

Recently, a number of lectins have been purified from the plasma, mucus, and eggs of several species of fish [32, 13]. F-type lectins were identified and characterized in the serum from the fish *Anguilla japonica* [33] and *Anguilla anguilla* [34]. Was successfully purified and characterized a lectin from the serum of *R. canadum* efficiently using an affinity chromatographic method. This new and simple protocol purified a lectin different from others already published, such as the first reported case of the isolation and characterization of L-fucose-binding proteins from the serum of *O. niloticus*, popularly known as Nile tilapia, for which was used an affinity chromatographic method (L-Fuc-BSA-Sepharose CL 6B) effective for the isolation of the soluble serum fucose-binding proteins [35]. In this experiment, the support used had importance of the methyl group in the agglutinin-ligand interaction in the inhibition by methyl-α-D-mannopyranoside. The interaction of lectins with carbohydrates due to a combination of hydrogen bonds between sugar hydroxyl groups and the protein main-chain, as well as side-chain groups, water-mediated contacts, van der Waals packing of the hydrophobic sugar ring surface against an aromatic residue, and hydrophobic interactions [36].

A fucose-binding Ca^{2+} independent serum lectin was purified from the sea bass (*Dicentrarchus labrax*), named DIFBL [37], before the identification of the F-lectin family. On the other hand, similar to the results reported by Dutta et al. [11], the activity of RcaL is dependent on the availability of Ca^{2+} as evidenced by the complete loss in activity in the presence of a chelator. This suggests that loss of Ca^{2+} by calcium chelators probably causes irreversible damage to the lectin.

The hemagglutination property of RcaL was found to be stable over a wide pH 7-8. Likewise the HA of grass carp (*Ctenopharyngodon idellus*) lectin fell when subjected to high and low pH [12]. The purified lectin obtained from the gills of *Aristichthys nobilis*, GANL, is also stable

under alkaline conditions, where original activity was maintained following incubation at pH 11 for 1 h and pH 8 represented 100% activity [13]. The thermostability of various lectins is relatively stable while others are much less so. However, it is common for teleost lectins to be considered less stable. STL2 isolated from steelhead trout (*O. mykiss*) [38]: activity is completely inhibited at when maintained at 70ºC for 10 min. Moreover, catfish (*S. asotus*) lectin activity was completely inhibited after incubation at 100ºC for 5 min. A rhamnose-specific lectin, isolated from grass carp eggs, completely lost its activity after incubation at 80 ºC for 5 min [39].

Immunological assays have been performed to analyze the biological responses induced by new compounds or extracts [40, 41, 42]. Here, in addition to the isolation and purification of RcaL lectin, the aim was to analyze the potential immunomodulatory activity induced by this lectin, since it has sugar binding properties similar to Con A lectin (a known mitogen). Previous studies have used Con A at 2.5 µg/mL as positive control. Results showed that RcaL induced immune stimulation through higher cytokine and NO production. However, we could observe that RcaL, besides not being cytotoxitic to mice splenocytes, preferentially induced a Th1 response through the induction of higher levels of IFN-γ production. This result was similar to Con A and other plant lectins such as mistletoe lectins [43, 44], jacalin and abrin that promote a Th1 response and T cell activation [45]; Cramoll 1,4, a mannose binding lectin that induces stronger Th1 response [46]; and Con A that act as stimulant composites also promoting IFN-γ release [47]. Few studies using fish lectins in immunological assays have been performed. Similar to our study, Watanabe et al. [31] showed that L-rhamnose-binding lectins, isolated from chum salmon (*Oncorhynchus keta*), induce pro-inflammatory cytokine production; Ng et al. [12] showed that grass carp lectin exerts a mitogenic effect on mouse splenocytes and a stimulatory action on the phagocytic activity of seabream macrophages. Dutta et al. [11], analyzing a lectin of the Indian catfish (*Clarias batrachus*), showed that this lectin was able to induce the proliferation of head kidney lymphocytes.

Silva et al. [48] showed that a lectin isolated from serum of Nile tilapia (*Oreochromis niloticus*), OniL, is a potential immunomodulatory compound with preferential Th1 profile [48].

RcaL lectin was isolated and characterized in this study. It did not show cytotoxicity against mice splenocytes and showed a preferential Th1 response. Data suggest that this new purified lectin acts as an immunomodulatory compound and provide a preliminary understanding of the mechanism of the immune response RcaL lectin in animals.

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7. CAPÍTULO III

Cobia (*Rachycentron canadum*) mitogenic response in mice Balb/c splenocytes by RcaL lectin

Short title: ***Rachycentron canadum*** **lectin promoted mitogenic response in mice**

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Short title: ***Rachycentron canadum* lectin promoted mitogenic response in mice**

Marília Cavalcanti Coriolano[a], Cristiane Moutinho Lagos de Melo[b], Athiê Jorge Guerra dos Santos[d], Valéria Rêgo Alves Pereira[b], Luana Cassandra Breitenbach Barroso Coelho[a,*].

[a]Departamento de Bioquímica, Laboratório de Glicoproteínas da Universidade Federal de Pernambuco - UFPE, Recife, Pernambuco, Brazil, [b]Centro de Pesquisas Aggeu Magalhães - CPqAM/FIOCRUZ, Recife, Pernambuco, Brazil, [c]Departamento de Engenharia de Pesca, Universidade Federal Rural de Pernambuco - UFRPE, Recife, Pernambuco, Brazil.

***Corresponding author.** Luana Cassandra Breitenbach Barroso Coelho, Departamento de Bioquímica, Laboratório de Glicoproteínas da Universidade Federal de Pernambuco - UFPE, Av. Moraes Rego s/n, Cidade Universitária, 50670-420, Recife, PE, Brazil. Tel.: +55 81 21268540; fax: +55 81 21268576.

E-mail address: lcbbcoelho@gmail.com (L. Coelho).

Abstract

The mitogenic lectins are invaluable as tools to study the biochemical changes associated with lymphocyte activation and proliferation of various immune cells. RcaL is a lectin detected, isolated and purified from serum of cobia fish (*Rachycentron canadum*). The aim of this study was to evaluate the proliferative response and cytokine production in splenocytes of mice stimulated *in vitro* with RcaL lectin; Con A lectin was used as positive control. Results demonstrated higher proliferation indexes induced by RcaL statistical significant in relation to control cells. Futhermore, RcaL induced higher IL-2 and IL-6 production in relation to control. It could observe that only late apoptosis was promoted by RcaL treatment at 24 hours of assay in relation to control; RcaL and Con A promoted also apoptosis at 48 hours of assay. However, the cell viability was superior to 90% in splenocytes treated with RcaL. Results showed that RcaL induces proliferative response and suggested that this lectin can be used as a mitogenic agent in immunostimulatory assays.

Introduction

Animal lectins that have been isolated, recognizes carbohydrate moieties through specific carbohydrate recognition domain (CRDs) [1] and participate in various biological functions [2, 3, 4] including innate and adaptive immune responses [5,6]. Since most lectins binding to specific sugars like mannose [7,8], fucose [9], rhamnose [10] and galactose [11,12]. C-type lectins may display CRDs in combination with other domains. They not only recognizes carbohydrates on the surface of potential pathogens, but also mediate several effectors functions including agglutination, immobilization, opsonization of microbial pathogens, endowed with capacity to enhance respiratory burst and bactericidal activity of phagocytic cells [13-19].

One important function of animal lectins is to mediate recognition of surface polysaccharides on potentially pathogenic microorganisms [20,21]. Beyond, many animal lectins that recognizes mannose and N-acetyl-D-glycosamine (GlcNAc) residues in common on microorganisms and pathogens [22] are soluble and Ca^{2+}-dependent (C-type).

Lectins have been isolated and characterized from serum, skin mucus, and eggs from many fish species [9,19,23]. Mannose recognizes lectins have been reported in trout, salmon, carp, rohu fish, channel catfish, blue catfish and sea lamprey species [8, 11, 24-27].

Lectins are usually used to investigate the molecular basis and control of lymphocyte activation and proliferation of the immune system. In fact, mitogenic lectins are invaluable as tools to study the biochemical changes associated with lymphocyte activation and proliferation of various immune cells [28]. Con A, an example of plant lectin, has been enough used as a mitogenic agent, showing immunomodulatory effects in many studies [29,30].

Many lectins, especially mannose binding, are knows by their immunostimulatory properties, as cell proliferation and cytokine production [31]. A new lectin was detected in the serum of cobia fish (*Rachycentron canadum*), named RcaL [32]. The aim of this study was to

evaluate the proliferative response and cytokine production in splenocytes of mice stimulated *in vitro* with this isolated and purified lectin from serum of cobia fish.

Materials and methods

Animals. Experimental assays utilized mice (BALB/c, male, 30 days old, 5/group). The animals were raised at the animal facilities of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) and maintained at the animal facilities of the Aggeu Magalhães Research Center of the Oswaldo Cruz Foundation in Recife, Brazil. The guidelines of the Ethical Committee for the Use of Experimental Animals of the Oswaldo Cruz Foundation/FIOCRUZ (Ministry of Health, Brazil) were followed. All mice were killed and treated in accordance with the guidelines of the Oswaldo Cruz Foundation Commission for Experiments with Laboratory Animals (Ministry of Health, Brazil, 0266/05).

Lectins. Serum of cobia fish (*Rachycentron canadum*) was fractionated with ammonium sulphate saturation (40-60%) and the fraction obtained was affinity chromatographed on Concanavalin A-Sepharose 4B (Sigma) column previously equilibrated with 10 mM $CaCl_2$ and 10 mM $MnCl_2$ in buffer TBS, at pH 8.0. RcaL lectin elution was performed with methyl-α-D-mannopyranoside (200 mM) in TBS. Fractions with the highest absorbance, which formed a peak, were pooled together and submitted to dialysis against TBS, at 4 °C. Canavalia ensiformis (Concanavalin A – Con A) was purchased from Sigma Chemical Co., (St Louis, MO, USA)

Preparation of splenocytes. After killing the animals 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 from each spleen were transferred to Falcon tubes containing

approximately 10 ml of incomplete medium. Spleen homogenates were overlaid onto a Ficoll-PaqueTM PLUS layer, with the density adjusted to 1.076 g/mL, and centrifuged at 1000 x g at room temperature for 25 min. The interface cell layer containing immune cells was recovered by Pasteur pipette, washed twice in phosphate-buffered saline (PBS) and centrifuged twice at 500 x 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 >98%.

Cellular proliferation assay. Collected splenocytes of mice ($4x10^5$ cells/well) were cultured (at 37 ºC and 5% CO_2) in triplicate in 96-well culture plates (TPP, St. Louis, Switzerland) in RPMI 1640 media (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil) and 50 µg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil). Con A was used as positive control. Cells of each group were treated, *in vitro*, for 24 h with Con A (2.5 µg/mL) or RcaL (2.5, 5 and 10 µg/mL) to evaluate the proliferative activity of these lectins. The unstimulated culture plate was used as a negative control. Into each culture well, 0.5 µCi [3H]-TdR (Amersham Biosciences, Boston, MA, USA) was added and proliferation was assessed by [3H]-TdR incorporation. At the end of this period, the material was collected via automatic cell collector (Skatron Instruments, Sterling, VA, USA) and deposited on glassfibre paper (Whatman International Ltd., Maidstone, UK). The incorporation of [3H]-thymidine was determined through emitted β radiation, expressed in rate per minute (RPM). Results were expressed by the Proliferation Indices defined as the RPM arithmetic average of stimulated cultures, divided by the arithmetic average of unstimulated cultures, ±standard deviation. The cut-off was determined by the control group medium ±two standard deviations. PI values ≥ 3 were considered representative of positive proliferation.

Cytokine evaluation in culture supernatants. Mice were killed (at CO_2 chamber) and their splenocytes were isolated and cultured in 24-well plates (TPP) at a density of 10^6 cells/well for 24, 48, 72 h and 6 days. Each well received Con A (2.5 µg/mL) or RcaL (10 µg/mL) lectins, and supernatants from cultures stimulated *in vitro* with lectins were obtained at 24, 48, 72 h and 6 days. Cells maintained only in culture medium (unstimulated culture) were also obtained as a negative control. IL-2 and IL-6 cytokines were quantified by sandwich ELISA, according to the manufacturer's suggested protocols. The monoclonal antibodies used were from kit OptEIA (BDBiosciences, Mountain View, CA, USA), being previously titered. Plates with 96 wells (Nalge Nunc International Corporation, Roskilde, Denmark) were sensitized with specific anticytokine antibodies (according to the manufacturer's instructions) and incubated 'overnight' at 4 ºC. Cytokine standards were added after serial dilution from their initial concentrations (according to the manufacturer's instructions). After washes, 50 µL of all samples and standards were added in duplicate and the plate was incubated for 2 h at room temperature. Subsequently, the specific antibodies were combined with biotin (according to the manufacturer's instructions) and incubated for 1 h 30 min at room temperature. Revealer solution was added containing 2.2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS). The reaction was blocked with 1 M sulphuric acid, and the reading was carried out with a spectrophotometer (Bio-Rad 3550, Hercules, CA, USA) at 415 nm. Each lectin was evaluated in triplicate in five independent assays. Sample concentrations were calculated in the linear region of the titration curve of cytokine standards, and final concentrations were expressed in pg/ml, using the Microplate Manager Version 4.0 software (Bio-Rad laboratories).

Analysis of cell viability by annexin V-FITC and propidium iodide staining. Splenocytes (10^6 cells) stimulated with lectins were centrifuged at 4 ºC, 450 g for 10 min. After discarding the supernatant, 1 ml of PBS 1X was added to the precipitate and this was then centrifuged at 4 ºC, 450

g for 10 min. After discarding the supernatant, the pellet was resuspended in buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and annexin V conjugated with fluorescein isothiocyanate (FITC) (1:500) and propidium iodide (PI, 20 µg/ml; 10^6 cells) were added to each labelled cytometer tube. Flow cytometry was performed in a FACSCalibur (Becton Dickinson Biosciences) and analysed using Cell Quest Pro software (Becton Dickinson). Result analysis was performed in graphs by dot plot. Annexin-$FITC^+/PI^-$ represented splenocytes in apoptosis. Annexin-$FITC^-/PI^+$ were considered necrotic cells and Annexin-$FITC^+/PI^+$ represented splenocytes in the early stage of apoptosis. Double negatives were considered viable cells.

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

Results

RcaL showed higher mitogenic activity by [3H]-TdR incorporation in mice splenocytes

Mice splenocytes treated *in vitro* with RcaL lectin demonstrated higher and statistical Proliferation Indices (PI) in relation to control stimulated cells, for all analyzed concentrations (Fig. 1). RcaL at 5 and 10 µg/mL was also superior to Con A. Still, Con A stimulus, used as mitogenic standard, also induced statistical values of proliferation in relation to control.

RcaL also induced higher IL-2 and IL-6 production in cell cultures

Proliferative cytokines were also analyzed for measure how RcaL is capable of stimulates mice splenocytes. Results showed that RcaL and Con A showed similar behavior and induced higher IL-2 and IL-6 production in relation to control in all experimental times. RcaL induced higher IL-2 production in all experimental times in relation to control (Fig. 2A-D). Con A showed the same behavior and only at 24 hours of assay, Con A was also superior to RcaL lectin (Fig. 2A). On the other hand, RcaL induced also higher IL-6 production and results showed statistical values in relation to control (Fig. 3A-D). Con A was superior to RcaL only at 48 hours and 6 days of assay (Fig. 3B and D, respectively).

Cell viability was superior to 90% in splenocytes treated with RcaL lectin

Con A induced more cell damage than RcaL at 24 hours of assay (Fig. 4A). In fact, it could observe that only late apoptosis were promoted by RcaL treatment. In contrast, at this same time, Con A, treatment induced higher apoptosis, late apoptosis and necrosis in relation to control. Beyond, Con A also induced more apoptosis and necrosis than RcaL lectin (Fig. 4A). At 48 hours of assay (Fig. 4B) Con A and RcaL were similar between them and induced higher apoptosis in relation to control. RcaL was superior to Con A and control in relation to late apoptosis and necrosis. However, RcaL showed the same behavior of the control and also induced necrosis (Fig. 4B).

Discussion

Studies performed demonstrated the immunomodulatory potential from some fish lectins. Thus, here was investigated the mitogenic potential induced by RcaL and Con A lectins in Balb/c mice splenocytes stimulated *in vitro* with both lectins.

According Kourilsky et al. [33], proliferative and immune responses are mediated by specific cytokines and cells that determine effective functions of immune system compounds. In this context, the production of IL-2 by activated T-cells leads to the downstream proliferation of T-cells, B-cells and natural killer cells [34,35]. Besides, IL-6 is a pleiotropic cytokine which plays a central role in host defense being produced mainly *in vivo* by stimulated monocytes/macrophages, fibroblasts, vascular endothelial cells, T and B cells, glial cells and keratinocytes [36,37].

Lectins exhibit a wide variation with regard to their effect on murine splenocytes. In fact, Ng et al. [38] noted that grass carp (*Ctenopharyngodon idella*) lectin exerted immunostimulatory activity on murine splenocytes, fish macrophages and peritoneal exudates cells. Grass carp lectin exhibited weak mitogenic activity when compared to Con A. On the other hand, a D-galactose-binding lectin *Musca domestica pupae* was elicited with a mitogenic response from mouse splenocytes *in vitro*, with the maximal response at a concentration of 20 µg/mL [39].

To the best of our knowledge, this is the first report of a fish lectin that promotes IL-2 and IL-6 production. Similar to our study, a lectin isolated from *Cratyllia mollis*, Cramoll 1,4, (glucose⁄mannose binding), have shown high homology with Con A and also induced higher proliferative IL-2 and IL-6 cytokine release on murine splenocytes [40]. Fish roe lectins from powan (*Coregonus clupeoides*), roach (*Rutilus rutilus*) and perch (*Perca flavescens*) display mitogenic activity or indirect cytotoxity mediated by macrophages or cytotoxins, but *Silurus asotus* (catfish) roe lectin lacks mitogenic activity [41]. Grass carp lectin possesses weak mitogenic

activity when compared to Con A. The mitogenic effect of grass carp lectin is also evidenced by its induction of IL-2 and INF-γ in mouse splenocytes [42,43].

Apoptosis has since been recognized as a programmed or physiological cell death, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. In contrast, necrosis is referred to as degradative processes that occur after cell death [44]. Studies with mitogenic lectins have shown their immunomodulatory activities and proliferation of various immune cells. Study on cell viability (apoptosis and necrosis) of a fish lectin also to seems the first report; In this study realized with RcaL, apoptosis and late apoptosis were promoted by lectin treatment. Nevertheless, the cell viability was superior to 90% in splenocytes treated with RcaL lectin. SPL (*Setcreasea purpurea*), a new member of the chitin-binding lectin family, also possessed an obvious cytotoxic effect on CNE-1 cells (a well differentiated human nasopharyngeal carcinoma cells) and induced apoptosis in a time and dose-dependent manner; It is suggested that the sugar-binding site as well as the other regions of the lectin molecule is responsible for the apoptosis-inducing activity [45]. Con A in other study proved to be cytotoxic or strongly inhibitory to some tumor cells, which has been found to induce A375 cell death in a caspase-dependent manner or through a mitochondrial apoptotic pathway [46,47]. SFL (*Sophora flavescens*), a member of legume lectins, has a distinctive carbohydrate-binding site and three-dimensional structure. Thus, the anti-tumor mechanisms of them should be different and the apoptotic induction of SFL should be dependent on death-receptor pathway [48].

RcaL induces a proliferative response through cytokine production in splenocytes of Balb/c mice stimulated *in vitro* with RcaL lectin and we suggest that RcaL lectin can be used as a mitogenic agent in immunostimulatory assays.

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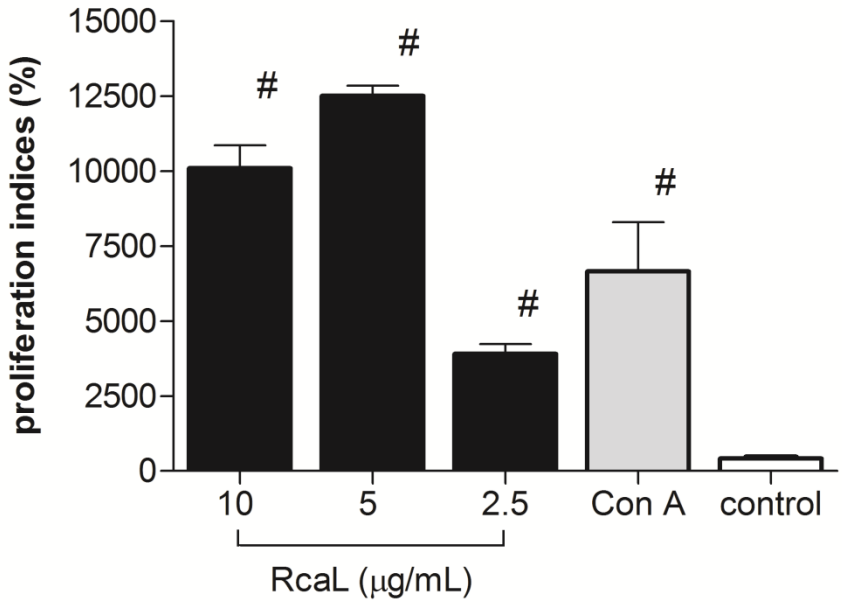

Figure 1 Proliferative activity induced by RcaL in splenocytes treated with different concentrations of fish lectin. Con A was used as positive control. RcaL at 10, 5 and 2.5 µg/mL concentrations demonstrated higher values in relation to control. RcaL at 5 and 10 µg/mL was also superior to Con A. Proliferation indices were evaluated by [3H]-thymidine incorporation. Results were expressed by the Proliferation Indices (PI) and PI greater than or equal to 3, were considered as positive for proliferation. ***** $p < 0.05$.

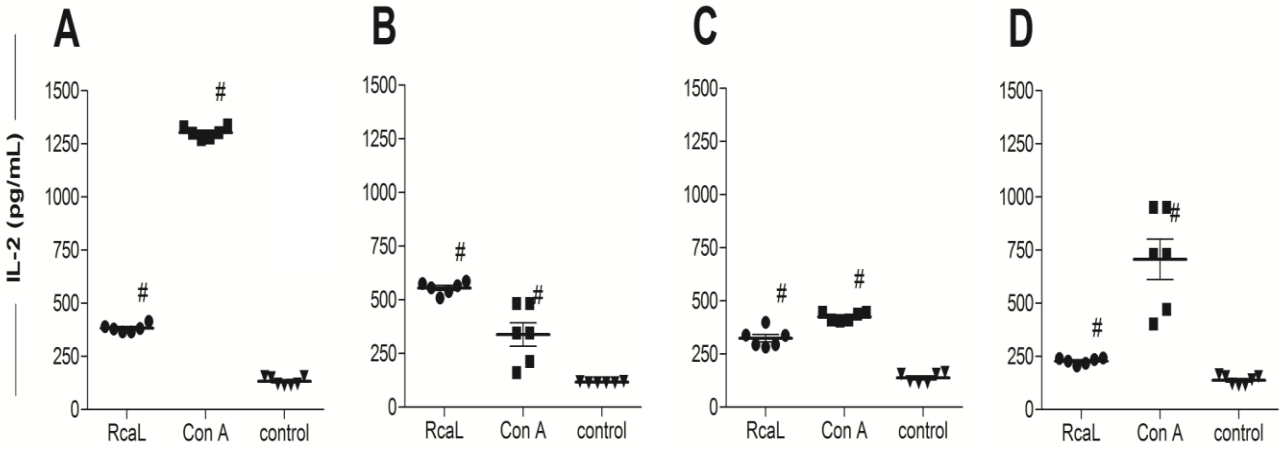

Figure 2 IL-2 production induced by RcaL in mice splenocytes cultures. **A, B, C and D** – 24, 48, 72 hours and 6 days of assay, respectively. RcaL and Con A showed similar behavior and induced higher IL-2 production in relation to control in all experimental times and Con A was superior to RcaL only at 24 hours (**A**). ***** $p < 0.05$.

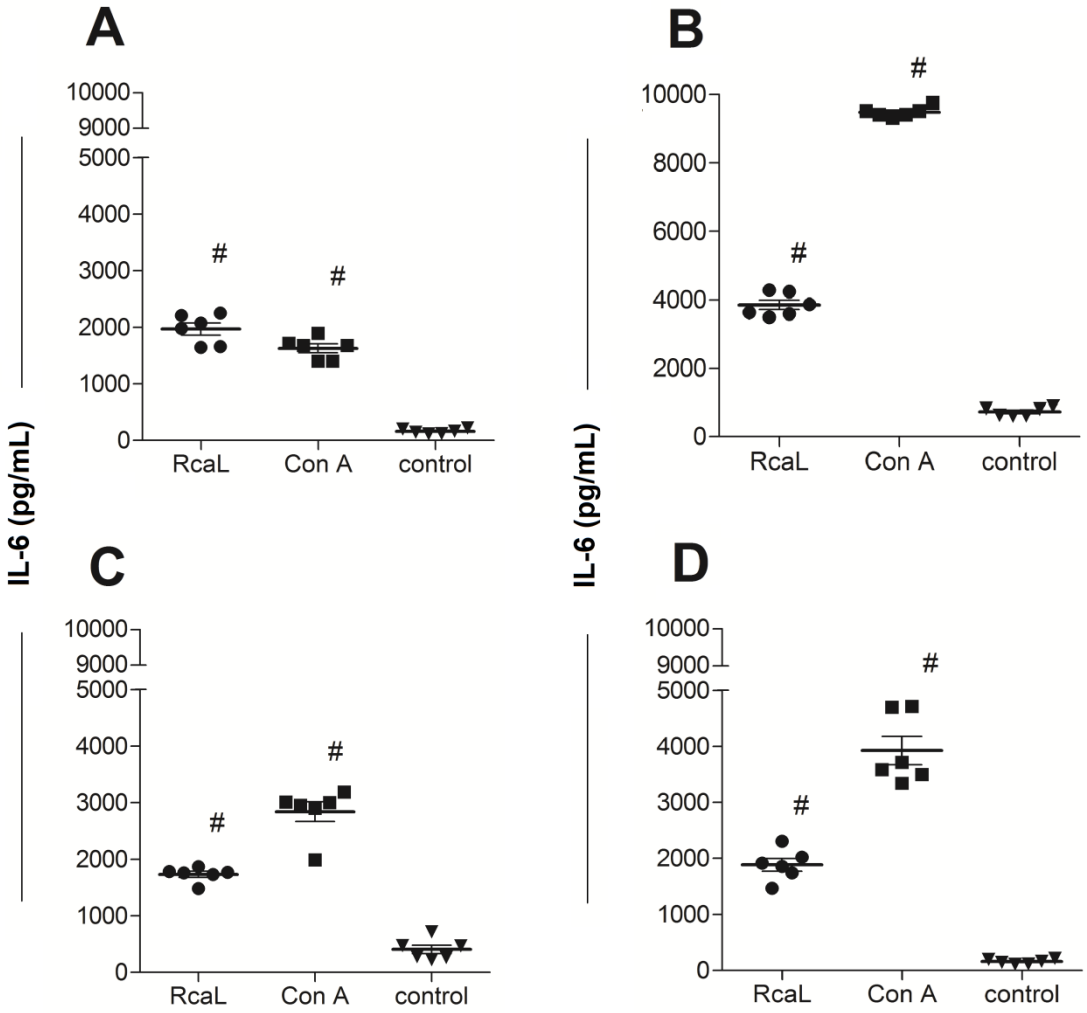

Figure 3 IL-6 production induced, in vitro, for RcaL lectin. **A**, **B**, **C** and **D** – 24, 48, 72 hours and 6 days of assay, respectively. RcaL and Con A showed similar behavior and induced higher IL-6 production in relation to control. Con A only was superior to RcaL at 48 hours (**B**) and 6 days (**D**) of assay. Points represent the average of five independent experiments per group. ***** $p < 0.05$.

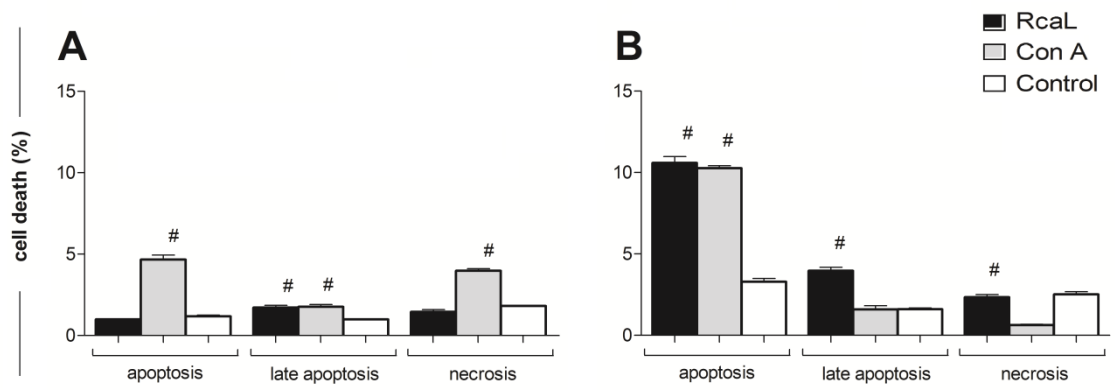

Figure 4 Cell viability of mice splenocytes treated with RcaL lectin. **A** – 24 hours of assay. RcaL induced only higher late apoptosis in relation to control. However, Con A induced higher apoptosis, late apoptosis and necrosis in cells treated *in vitro* with this lectin. **B** – 48 hours of assay. RcaL and Con A induced higher apoptosis in relation to control. RcaL was superior to Con A and control in relation to late apoptosis and necrosis. However, RcaL was similar to control in relation to necrosis cell death. Points represent the average of five independent experiments per group. ***** $p < 0.05$.

8 CONCLUSÕES

- Em protocolo eficiente, uma lectina foi purificada do soro do peixe *Rachycentron canadum* (RcaL) através de cromatografia de afinidade com uma coluna Concanavalina A-Sepharose 4B, e caracterizada.

- RcaL é dependente de cálcio e mostrou maior especificidade pelos carboidratos metil-α-D-manopiranosídeo e D-manose. Estruturalmente, RcaL é uma proteína ácida revelada em uma única banda com massa molecular aparente de 19.2 kDa.

- RcaL não apresentou atividade citotóxica em esplenócitos murinos, induziu preferencialmente resposta imune Th1 através da alta produção de IFN-γ e IL-2, e demonstrou atividade pró-inflamatória pela estimulação da produção de NO.

- RcaL modula a resposta imunológica por induzir a produção das citocinas IFN-γ, IL-2, IL-6 e IL-10.

- RcaL é também considerada uma lectina imunoestimulatória por demonstrar alta atividade proliferativa, sem promover aparente dano celular em esplenócitos murinos.

9 ANEXOS

Chapter AB Article (Advances in Environment Research)

Aimee Himelfarb 25 jan

Dear Dr. Coelho:

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Kind regards,

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N-5021 Bergen, Norway

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