ELBA VERÔNICA MATOSO MACIEL DE CARVALHO

ABORDAGENS BIOTECNOLÓGICAS DO TAMBAQUI (*Colossoma macropomum*)

Recife, Agosto, 2007

Universidade Federal de Pernambuco
Centro de Ciências Biológicas

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Tese apresentada pela aluna a banca Examinadora como um dos Pré-requisitos para a obtenção do Grau de Doutor do Programa de Pós Graduação em Ciência Biológicas da Universidade Federal de Pernambuco.

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SUMÁRIO

"Há duas formas para viver a sua vida:

Uma é acreditar que não existe milagre.

A outra é acreditar que todas as coisas são um milagre"

Albert Einstein

A meu marido, Darlan Karlo e a minha Mãe, Ione por toda paciência, amor e dedicação.

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RESUMO

O tambaqui (*Colossoma macropomum*) é um peixe com ampla distribuição nos rios da Região Norte e uma das espécies de maior importância na alimentação da população desta Região. Este peixe vem, nas últimas décadas, se tornando uma das principais espécies nativas para a piscicultura brasileira, apresentando um ótimo padrão de crescimento e alta produtividade, fato que torna abundante a sua oferta no mercado consumidor, contando, assim, com um alto interesse junto aos piscicultores de outros países da América do sul. A sua rusticidade, qualidade da carne e o fato de poder chegar a 1 m de comprimento e 30 kg de peso corporal no seu ambiente natural o torna um candidato promissor ao desenvolvimento da piscicultura na América Latina. Lectinas são proteínas ou glicoproteínas que reconhecem carboidratos com alto grau de especificidade através de sítio de ligação. O papel fisiológico das lectinas não está claramente definido, mas estudos recentes sugerem que lectinas são proteínas de defesa que podem proteger contra ataques de vírus, fungos e bactérias. O estudo com o soro do tambaqui revelou, através de PAGE, proteínas básicas. Uma destas proteínas foi parcialmente purificada e caracterizada obtendo-se uma lectina que reconhece galactose e fucose, da família das fucolectinas. A análise por espectrometria de massa destas proteínas básicas, retiradas do gel de eletroforese apresentou uma proteína similaridade com o componente do complemento C3-4 dos peixes *Oncorhynchus mykiss e Salmo gairdneri* de 95% (Q9DDV9_ONCMY). Posteriormente, durante os estudos dos genes que codificam as proteínas do soro detectou-se que o tambaqui não apenas expressa galectinas (lectina que reconhece galactose), mas também a MBL (Mannose Binding Lectin) e a Lectina tipo-C. A variedade de lectinas detectadas no tambaqui pode ser em parte responsável pela robustez da espécie, i.e. sua resistência a doenças.

Palavras Chave: Lectinas, Tambaqui (*Colossoma macropomum*), Peixe Amazônico, Classificação de ectinas animal, Sistema imunológico de peixes.

ABSTRACT

Tambaqui, *Colossoma macropomum* is one of the most important species of fish in feeding the population of the North region of Brazil, showing a broad distribution along the rivers of this region. In the last decades this fish has become one of major native species in the Brazilian aquiculture, presenting an excellent pattern of growing and high productivity, turning abundant its offer in the consumer market. This species has also aroused a high interest in the fish breeders of other Latin American countries, due to its rusticity, meat quality, being able to achieve 1 m length and 30 Kg of body mass within its natural environment. Its natural features turn the *C. macropomum* a promising candidate for the fish breeding development in Latin America. Lectins are proteins or glicoproteins that recognize carbohydrates with high degree of specificity through bond sites. The physiological role of lectins is not clearly defined, but recent studies suggest that lectins are defense proteins which can protect against attacks from viruses, fungi and bacteria. Throughout the study of the purification and partial characterization of the tambaqui serum lectin, we have discovered the it is a basic protein which recognize the monosaccharides galactoses and fucoses. Subsequently, during the study of the genes that codify these proteins, we have detected that the tambaqui not only express galectins (lectins that recognize galactoses), but also the MBL (Mannose Binding Lectin) and C-type lectin. The diversity of lectins found in tambaqui may be in part responsible for its robustness, i.e. its resistance to deseases.

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

LECTINAS E SUA IMPORTÂNCIA NO SISTEMA IMUNOLÓGICO DE PEIXES

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RESUMO

Lectinas são proteínas ou glicoproteínas que possuem a habilidade de se ligar específica e reversivelmente a carboidratos. Estas proteínas apresentam funções biológicas variadas e, atualmente vem crescendo a investigação do papel das mesmas no sistema imunológico de peixes. Operações na aqüicultura esforçam-se para produzir um aumento no número de peixes saudáveis através de meios biológica e economicamente eficientes. Por esta razão, a prevenção de doenças é muito importante para a indústria. Nos anos recentes a nutrição de peixes tem melhorado; igualmente, vacinas eficientes contra algumas doenças e esquemas para o manejo de peixes saudáveis têm sido desenvolvidos, com o objetivo de reduzir o impacto de doenças na aqüicultura. Contudo, um grande assunto permanece desconhecido no que diz respeito à imunidade de peixes. A resposta imune inata é independente de anticorpos e constitui a primeira linha de defesa contra infecção. Ao invés de contar com anticorpos para o reconhecimento de patógenos a imunidade inata de peixes consiste em um modelo baseado no reconhecimento de células não próprias, muitas vezes através do arranjo de carboidratos presentes na superfície. Portanto, as lectinas podem ter um papel igual ou mais importante em peixes, no qual a imunidade adquirida não é tão desenvolvida como em mamíferos. Recentemente, vários laboratórios têm iniciado trabalhos direcionados a identificação e caracterização estrutural e funcional de lectinas de peixe. Desenvolvimento nesta área pode prover novas abordagens para prevenção, monitoramento e tratamento de doenças em peixes.

LECTINAS

Breve histórico

Bruylants e Venneman em 1884 demonstraram que a toxicidade da semente *Abrus precatorius* devia-se a uma fração protéica, Abrina, que podia ser precipitada com álcool a partir de um de um extrato aquoso da semente (MOREIRA et al., 1991). O estudo de lectinas iniciou-se em 1888 quando um jovem doutor, Peter Hermann Stillmark (Figura 1A), da Universidade Dorpat (agora Universidade de Tartu na Estônia), apresentou sua tese de Doutorado intitulada "*Ueber Ricin, ein giftiges Ferment aus den Samen Von Ricinus comm. L. und einigen anderen Euphorbiaceen*" a qual se referia a descoberta de uma proteína tóxica, denominada Ricina, presente no feijão castor (Figura 1B) que tinha a propriedade de aglutinar células do sangue ampliando desta forma o estudo de aplicações destas proteínas. Este evento é internacionalmente conhecido como o nascimento de um novo ramo na ciência chamado de lectinologia (KENNEDY et al., 1995; GABOR et al., 2001; BIES et al., 2004).

A Abrina e a Ricina, proteínas com as mesmas propriedades, foram usadas por Paul Ehrlich, um médico cientista, em seus estudos imunológicos na última década de 1800 (LIS e SHARON, 1991). William Boyd e Elizabeth Shapleigh em 1954 nomearam este novo grupo de proteínas de Lectinas, do latim, *Lectus* (escolhido, selecionado), refletindo etimologicamente sua propriedade de aglutinar seletivamente grupos sanguíneos (BOYD e SHAPEIGH, 1954; KENNEDY et al., 1995; MATSUI et al., 2001).

Lectinas são definidas como proteínas que ligam, especificamente e reversivelmente, a mono, oligo ou polissacarídeo sem alterar sua estrutura (GOLDSTEIN e PORETZ, 1986; VAN DAMME et al., 1996; MACEDO et al., 2007; SITOHY et al., 2007). Estas proteínas têm a habilidade também de se ligar a resíduos de açúcares, através de sítio(s) de ligação a carboidrato, a qual estão conjugados com lipídeos ou proteínas (VORNHOLT et al., 2007). Inicialmente o estudo de lectinas era focado apenas em plantas, e apenas nas duas últimas três décadas foi quando se tornou evidente que lectinas também são distribuídas no reino animal (PROBSTMEIER e PESHEVA, 1999)

Uma Análise detalhada de mais de centenas de lectinas tem demonstrado que elas são um grupo heterogêneo de proteínas na qual se diferem fortemente uma da outras com respeito à especificidade do açúcar, estrutura molecular e atividade biológica. Devido as lectinas terem a habilidade de se ligar a carboidratos, cada ligação pode resultar em uma variedade de efeitos biológicos (MACHUKA et al., 1999) e estes efeitos atraem cada vez mais o interesse da comunidade científica (SATO et al., 2000; KONOZY et al., 2002; MONZO et al., 2007). Centenas de lectinas são, atualmente, bem caracterizadas, e o número de isolamento das mesmas vem crescendo muito rápido; elas têm sido isoladas de plantas, animais e microorganismos (LIS e SHARON, 1991, VORNHOLT et al., 2007). O contraste é impressionante com a situação de aproximadamente 30 anos atrás, quando justamente um pequeno número destas proteínas, quase todas de plantas, foram purificadas sendo duas lectinas de legumes, Concanavalina A (ConA) e aglutinina do feijão de soja (Soybean agglutinin – SBA) e uma de cereal, aglutinina de germe de trigo (Wheat germ agglutinin – WGA). Mesmo que os dados disponíveis naquela época fossem limitados, conclui-se que enquanto lectinas tem numerosas propriedades em comum, eles representam um grupo diversificado de proteínas com respeito a tamanho, composição e estrutura (SHARON, 1993; ELGAVISH e SHAANAN, 1997).

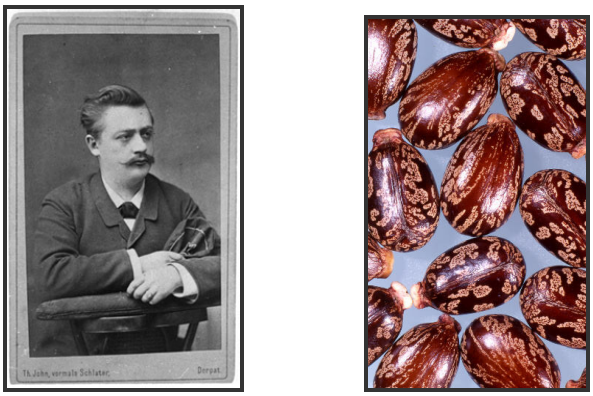

Figura 1: Peter Hermann Stillmark (A), sementes de *Ricinus communis* *da família* Euphorbiaceae (B).

Site: http://www.dadamo.com/wiki/wiki.pl

Detecção

As lectinas podem se ligar a açúcares livres ou à resíduos de carboidratos de polissacarídeos, glicoproteínas ou glicolipídeos, onde estes podem estar livres ou

ligados à membrana da célula (MONZO et al., 2007). A presença destas proteínas pode ser detectada principalmente através de um ensaio de hemaglutinação (Figura 2). Neste ensaio, uma diluição serial de uma solução contendo lectinas é realizada antes da incubação com eritrócitos (COELHO e SILVA, 2000; COUTIÑO-RODRÍGUEZ et al., 2001; OKAMOTO et al., 2005).

Lectinas também induzem a precipitação de polissacarídeos ou glicoproteínas em solução, sendo as reações de aglutinação por lectinas inibidas por seus carboidratos específicos.

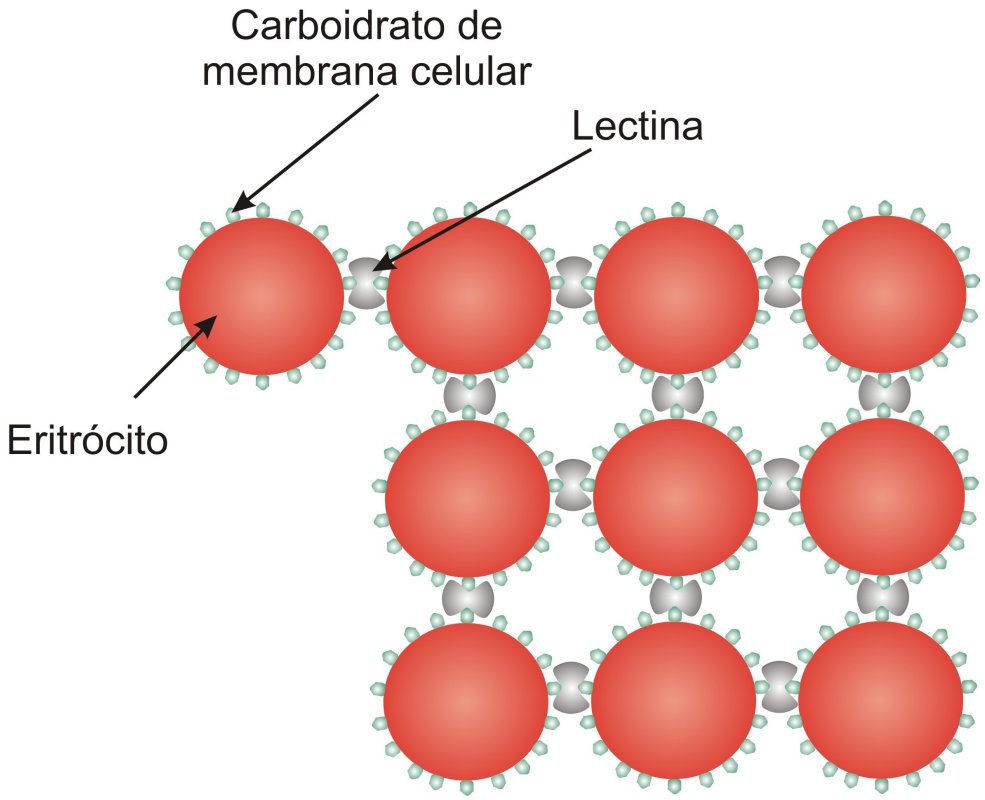

Figura 2: Atividade hemaglutinante mostrando a formação da malha de aglutinação.

Especificidade

Existem lectinas que possuem especificidade para mais de um carboidrato, aglutinando células de diferentes espécies. Também existem lectinas que só aglutinam as células em que houver a presença de um determinado carboidrato (KABIR, 1998; SATO et al., 2000; GABOR et al., 2001; COUTIÑO-RODRIGUÉZ et

al., 2001). Peumans e Van Damme (1998) observaram que lectinas de plantas exibem uma ampla especificidade para carboidrato, sendo que muitas apresentam maior afinidade para oligossacarídeos do que para açúcares simples, ou têm especificidade direcionada contra glicanos que não são próprios, além disso, lectinas estruturalmente diferentes podem reconhecer o mesmo carboidrato.

Os carboidratos específicos podem se ligar as lectinas através de pontes de hidrogênio, coordenações metálicas, interações de Van der Walls e interações hidrofóbicas (SCHWARTZ et al., 1993; DRICKAMER, 1998). Lectinas também induzem a precipitação de polissacarídeos ou glicoproteínas em solução, sendo as reações de aglutinação por lectinas inibidas por seus carboidratos específicos. Para detectar a especificidade de uma lectina, são necessários ensaios subseqüentes de inibição da atividade hemaglutinante, IAH (Figura 3) utilizando uma solução do carboidrato ligante (RÜDIGER, 1998; CAVADA et al., 2000; KAWAGISHI et al., 2001). Os eritrócitos utilizados para este ensaio podem ser de 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).

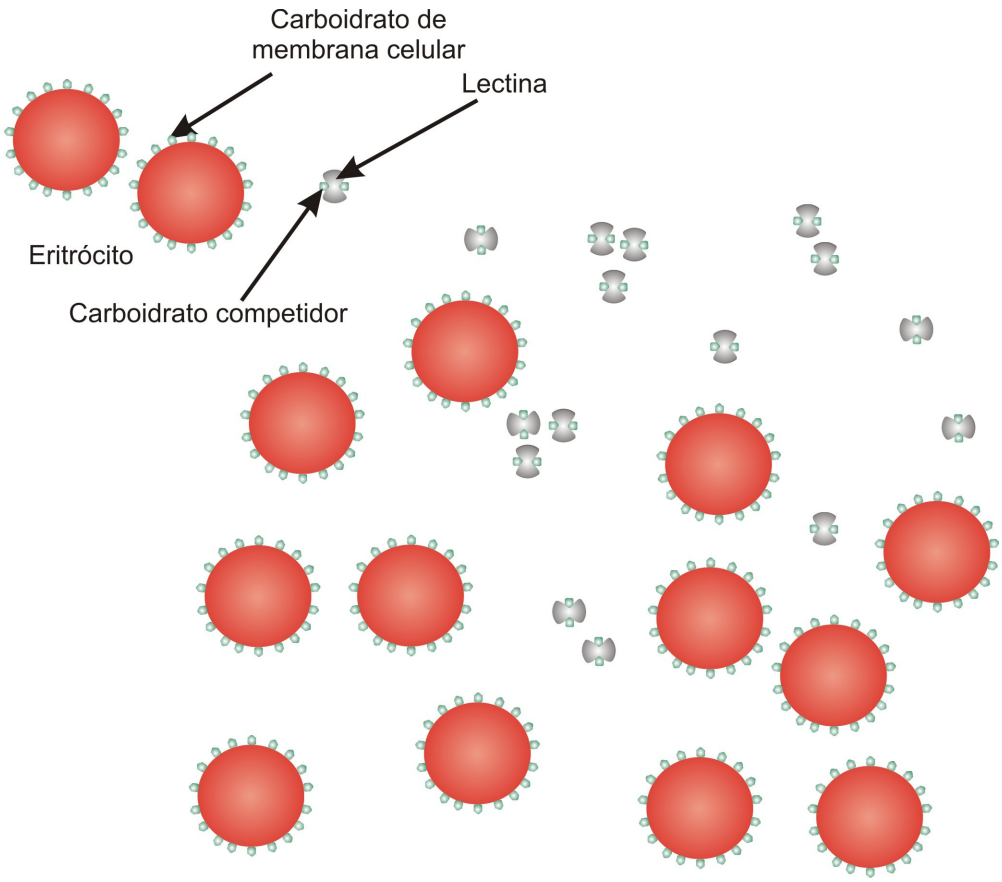

Figura 3: Inibição da atividade hemaglutinante.

Classificação

Devido a algumas novas discussões dentro da estrutura e genes de lectinas, a definição destas proteínas em plantas tem sido atualizada. Segundo Peumans e Van Damme (1998) lectinas de plantas foram classificadas de acordo com sua estrutura geral em: merolectinas, hololectinas, quimerolectinas e superlectinas. Merolectinas (Figura 4) consistem de apenas um domínio de ligação a carboidrato e devido a seu caráter monovalente são incapazes de precipitar glicoconjugados ou aglutinar células. Hololectinas também consistem exclusivamente de domínios de reconhecimento a carboidrato, mas possuem pelo menos dois domínios de ligação e estes podem ser idênticos ou muito similares. As hololectinas podem ser di ou multivalentes e assim podem aglutinar células e precipitar glicoconjugados. As superlectinas são compostas de no mínimo dois domínios de ligação a carboidrato e diferente das hololectinas os domínios de ligação a carboidrato não são idênticos ou similares reconhecendo açúcares estruturalmente diferentes. Ao contrário das

merolectinas, das holo e das superlectinas, as quimerolectinas não consistem exclusivamente de domínios de reconhecimento a carboidrato (*Carbohydrate Recognition Domain* – CRD), mas são formadas por domínio de ligação a carboidratos e outro domínio que possui uma atividade catalítica que age independentemente do CRD. As quimerolectinas comportam-se como mero ou hololectinas dependendo do número de sítios de ligação a carboidrato.

As lectinas ainda podem ser classificadas, de acordo com o carboidrato (Tabela 1) a que preferencialmente se ligam (NOMURA et al., 1998; PEUMANS e VAN DAMME, 1998; RABINOVICH et al., 1999).

Tabela 1: Classificação das lectinas de plantas nos grupos de reconhecimento a carboidrato.

GRUPO	ESPECIFICIDADE	LECTINA (FONTE)
Fucose	Fucose	*Ulex europaeus* aglutinina I (Tojo)
N-acetilglicosamina	GlcNAc	*Triticum aestivum* (Germe de trigo)
Galactose/N-acetilgalactosamina	Galactose>>GalNAc Gal= GalNAc Gal<< GalNAc	*Artocarpus integrifolia* (Jacalina) *Clerodendron trichotomum* (*Harlequin glorybower* ou *Clerodendro japonês*) *Glycine max* (Feijão de soja)
Manose	Manose/Glicose Manose/ Maltose Monose	*Canavalia ensiformis* (Concanavalina A) *Calystegia sepium* (Bons dias) *Galanthus nivalis* (Campanhia branca)
Complexo Glicano	Glicoproteína	*Phaseolus vulgaris* Aglutinina (PHA)

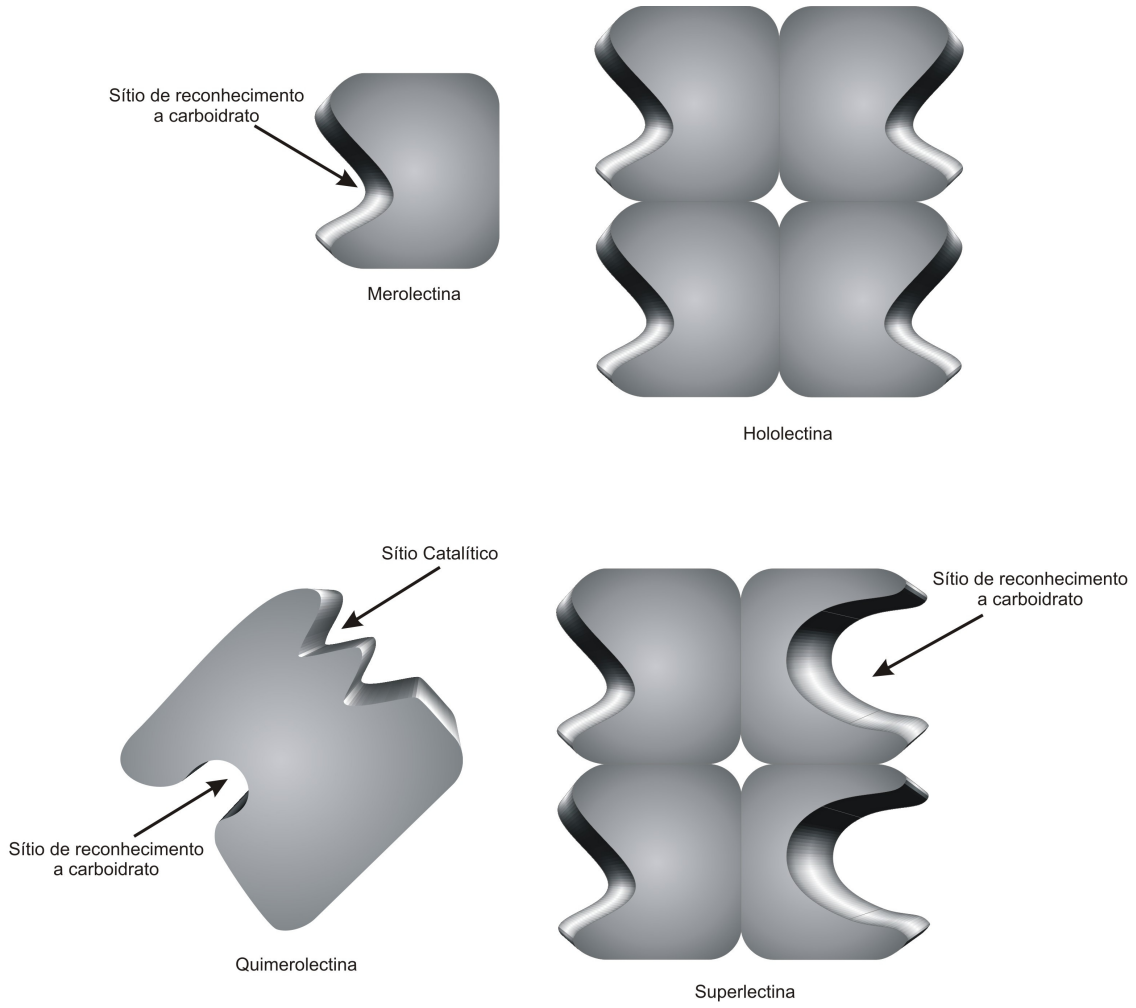

Figura 4: Representação esquemática da estrutura de lectinas de planta.

O reconhecimento de carboidratos por lectinas é mediado por CRD. Em relação à comparação das seqüências desses CRD de lectina de origem animal a classificação foi dividida em três tipos principais designadas como lectina tipo-C, tipo-P e tipo-S. Os CRD encontrados em cada tipo particular de lectina compartilham um padrão de resíduos de aminoácidos altamente conservados e invaráveis (KISHORE et al., 1997; RINI e LOBSANOV, 1999; EWART et al., 2001; LORIS, 2002; SUZUKI et al., 2003).

Lectinas tipo-C são proteínas de membrana e extracelulares que ligam uma variedade de carboidratos e que contêm um ou dois sítios de ligação ao cálcio e este(s) sítio(s) compartilhado(s) por todas as lectinas deste tipo, também é o mesmo sítio de ligação a carboidrato (EWART et al., 1999; RICHARDS et al, 2003). Os diferentes tipos de lectinas tipo-C reconhecem uma variedade de carboidratos. Entre

estas existem sete subgrupos de proteínas onde cada subgrupo tem um CRD em diferente arranjo estrutural (DRICKAMER e TYLOR, 1993, EWART et al, 2001). O papel no sistema de defesa imune inato tem sido descrito apenas para as lectinas do subtipo III e IV. As lectinas do subgrupo III são denominadas collectinas. Estas proteínas formam multímeros com subunidades compostas de colágeno tipo I próximo à extremidade amino-terminal e o CRD tipo-C está localizado na extremidade carboxi-terminal. Lectina que liga manose (*Mannose Binding Lectin*-MBL) é um tipo clássico deste subgrupo de lectinas do tipo-C. As lectinas do subgrupo VII possuem o CRD tipo-C com nenhum domínio associado distinguível. Proteínas deste subtipo incluem monômeros, dímeros e agregados multiméricos de CRD (EWART et al., 2001).

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

Lectinas 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. Quatorze membros da família de galectinas têm sido identificados e classificados em três subgrupos:

a) Protótipo (galectinas 1, 2, 5, 7, 10, 11, 13 e 14)
b) Quimera (galectina 3)
c) *Tandem repeat* (galectina 4, 6, 8, 9 e 12)

Todos estes subgrupos (Figura 5) contêm um CRD altamente conservado (DUMIC et al., 2006). A galectina do tipo protótipo contém um CRD por subunidade e são, usualmente, homodímeros de subunidades ligadas não-covalentemente (RABINOVICH et al., 1999; AHMED et la, 2004). Em contraste, galectina do tipo quimera são monômeros com um CRD C-terminal similar ao protótipo, e este está conectado a um domínio N-terminal rico em glicina e prolina (KISHORE et al., 1997; AHMED et al., 2004). A galectina *tandem repeat* é formada por uma única cadeia polipeptídica que forma dois distintos CRD, conectados por repetições de 7-10 aminoácidos com uma seqüência consenso (KISHORE et al., 1997; RABINOVICH et al., 1999; DUMIC et al., 2006).

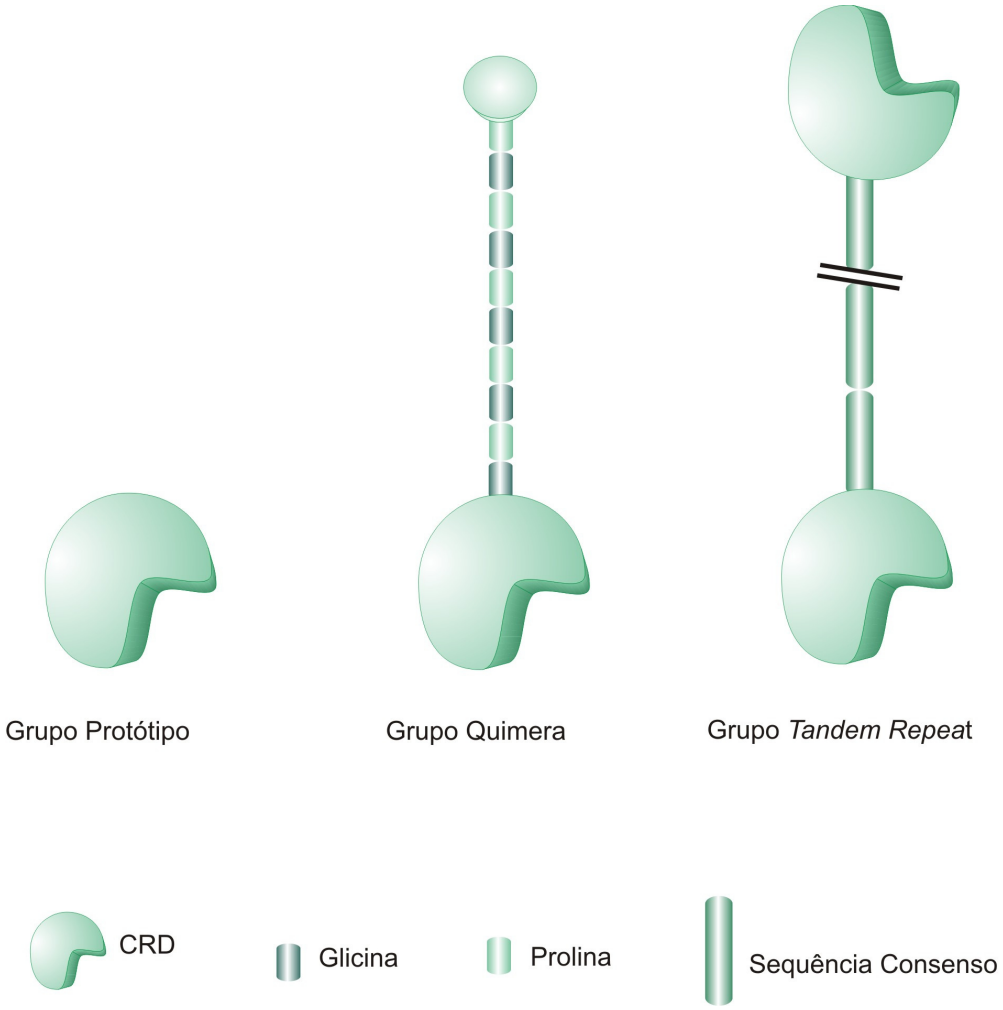

Figura 5: Representação esquemática da organização dos domínios de reconhecimento a carboidrato das galectinas.

Papel biológico

Lectinas são ubíquas na natureza e, devido à sua única propriedade de ligar carboidratos presentes na superfície celular, desempenham um importante papel em eventos celulares como aglutinação, reconhecimento celular, simbiose, estimulação da proliferação, opsonização, metástase e apoptose (VAN DAMME et al., 1997; WITITSUWANNAKUL et al, 1997; DUTTA et al., 2005).

O crescente estudo sugere que lectinas de plantas em geral podem ter um papel na proteção contra predadores animais, fungos e bactérias e por outro lado

elas podem estar envolvidas no processo simbiótico entre plantas e bactérias (CAVADA et al., 1998; RATANAPO et al., 2001; SACCHETTINI et al., 2001; VORNHOLT et al., 2007).

Lectinas de legume têm sido o grupo mais bem estudado de lectinas de plantas e o crescimento de evidências experimentais sugerem que estas lectinas são proteínas de defesa que possivelmente protegem sementes maduras contra o ataque de predadores, como insetos (MONZO et al., 2007; VORNHOLT et al., 2007).

O papel das lectinas nos fungos continua desconhecido (KAWAGISHI et al, 2001), para bactérias e protozoários forai sugerido que estas lectinas têm uma função importante facilitando sua adesão ao epitélio intestinal. Para as lectinas de vírus, foi sugerido que, em humanos, elas se ligam à eritrócitos e outras células pelo reconhecimento do ácido *N*-acetil neuramínico presente na superfície celular e que esta ligação é um pré-requisito para o início da infecção (SINGH et al., 1999).

Lectinas têm um significante papel na resposta imune do hospedeiro. Elas ligam especificamente a moléculas de carboidratos expressas sobre patógenos, agindo como opsoninas e estimulando sua destruição por macrófagos (FOCK et al., 2001; RICHARDS et al., 2003; DUTTA et al., 2005).

A descoberta de lectinas de peixe tem adicionado uma nova dimensão em biologia de lectinas e na imunologia de peixe (DUTTA et al., 2005). Lectinas de peixe têm sido isoladas do soro, plasma, muco da pele e ovos. Há evidências de que estas lectinas têm também um importante papel na fertilização, morfogênese, embriogênese além do papel na defesa contra microorganismos (EWART et al., 1999; AHMED et al., 2004; DUTTA et al., 2005).

Aplicações de lectinas

Lectinas, devido à sua característica de reconhecer carboidratos, têm sido exploradas em vários ramos de pesquisa, podendo ser utilizadas para diferentes aplicações. As lectinas podem ser usadas como ferramentas para estimular a proliferação de linfócitos (MACIEL et al., 2004), para tipagem de grupos sanguíneos (MATSUI et al., 2001), para detectar componentes de carboidratos presentes em superfícies de células normais e cancerígenas (BELTRÃO et al., 2003; KOMATH et al., 2006). Podem ser imobilizadas em suporte insolúvel para purificação de outras glicoproteínas e glicolipídeos de interesse médico como, por exemplo, a purificação

de glicoproteínas do vírus HIV (do inglês - *human immunodeficiency virus*) o qual está sendo bastante utilizado em projetos de desenvolvimento de vacinas (GILLJAM, 1993; BERTRAND et al., 1998; AHMED et al., 2001).

Estas proteínas podem ser utilizadas como ferramenta para a produção dos chamados medicamentos-inteligentes, onde, estes medicamentos diferem dos tradicionais, por atuarem em células específicas do organismo evitando efeitos colaterais, do tipo provocado pela quimioterapia (CLARK et al., 2000; TORCHILIN et al., 2001; WOODLEY, 2001). A afinidade de lectinas por glicoproteínas de superfície celular tem sido usada para a identificação de microorganismos como, por exemplo, a *Neisseria gonorhoeae* que pode ser diferenciada de outras espécies de *Neisseria* (WU et al., 2000) ou, para identificação de grupos sanguíneos ABO (MATSUI et al., 2001).

Sistema imune de peixes

O sistema imunológico de peixes envolve a resposta imune adquirida e a inata. As vacinas se baseiam na resposta imune adquirida (mediada por anticorpo), a qual requer um tempo para se desenvolver após a exposição ao patógeno. A resposta imune adquirida ocorre exclusivamente em vertebrados enquanto que a resposta imune inata é encontrada em vertebrados e invertebrados (TSUTSUI et al., 2006). As imunoglobulinas ou anticorpos são sintetizados a partir da diferenciação dos linfócitos B, no baço. Os peixes parecem sintetizar apenas um tipo de imunoglobulina, IgM, de todas as imunoglobulinas conhecidas, IgG, IgM, IgA, IgD e IgE (MAGNADÓTTIR, et al., 1999; TSUTSUI et al., 2006). A resposta imune inata é independente de anticorpos e constitui a primeira linha de defesa contra infecção e, diferentemente da adquirida, é uma resposta rápida e não requer uma exposição anterior ao patógeno (EWART et al, 2001; KUBITZA e KUBTIZA, 2004; TSOI et al, 2004).

Várias proteínas agem na resposta imune inata de peixe (EWART et al, 2001). Algumas têm sido bem caracterizadas, incluindo as proteínas antimicrobianas bem como a lisozima (HANCOCK e LEHRER, 1998). Recentemente, pesquisas veterinárias e médicas descobriram que as lectinas estão envolvidas na imunidade inata (EWART et al., 1999; MAGNADÓTTIR, et al., 1999; FOCK et al., 2000; FOCK et al, 2001; DUTTA et al., 2005; RUSSELL e LUMSDEN, 2005). O reconhecimento

de vírus, fungos e bactérias é propriedade reportada de várias lectinas de peixe. Lectinas solúveis presentes no sangue podem agir na primeira linha de defesa do hospedeiro pela identificação dos patógenos como não próprios. Elas ligam especificamente a esses patógenos, "gerando uma maior atração" de macrófagos sobre os patógenos, e aumentando a lise celular mediada pelo complemento (EWART et al., 1999). Uma lectina que liga manose tem uma propriedade similar descrita acima. Contudo, uma lectina que liga galactose com a atividade de reconhecimento bacteriano foi identificada em ovos de salmão (*Oncorhynchus kisutch*), mas não parece agir como opsonina.

Peixes teleósteos apresentam características únicas, bem como dependência de temperatura para imunidade adquirida e algumas limitações em diversidade de anticorpo. Por esta razão se pensou que as funções imunes inatas podem ter um papel mais importante nesses animais que em outros vertebrados homeotérmicos bem como aves e mamíferos (TSOI et al., 2004).

Um número cada vez maior de lectinas de peixe tem sido reportado. Existem algumas lectinas identificadas no muco da pele de várias espécies de peixes, outras no soro e outras em ovos.

Entre alguns tipos de lectina animal, galectina compreende uma das maiores famílias e são bastante encontradas em peixes como *Conger myriaster, Arius thalassinus e Oncorhynchus mykiss* (INAGAWA et al., 2001; NAKAMURA et al., 2001; SUZUKI et al., 2003). Tem sido encotradas também lectinas tipo-C (BAYNE et al., 2001, NIKOLAKOPOULOU e ZARKADIS, 2006), MBL (NIKOLAKOPOULOU e ZARCADIS, 2006), fucolectinas, lectinas que reconhecem fucose e galactose (HONDA, 2000, ODOM e VASTA, 2006), lectina que reconhece L-raminose (OKAMOTO et al., 2005).

TAMBAQUI

Peixe típico da região Norte do Brasil

Historicamente os peixes amazônicos são mais conhecidos pela sua reputação do que por seu valor comercial. O mais mal afamado grupo é o das piranhas, devido aos contos dos viajantes sobre sua agressividade. O peixe comestível mais famoso da Amazônia desde meados do século XIX é o gigante

pirarucu (*Arapaima gigas*). Este peixe, salgado e seco, era servido como um substituto do bacalhau, tradicionalmente utilizado na cozinha portuguesa (LIMA e GOULDING, 1998). Com o crescimento dos centros urbanos nas décadas de 60 e 70, especialmente a cidade Manaus, o tambaqui substituiu o pirarucu na cozinha local. A tecnologia da rede de emalhar levou a uma rápida expansão da pesca de tambaqui, e nos meados de 1970 esta espécie sozinha era responsável por mais de 40% de todo o pescado consumido em Manaus (PETRERE JR., 1978).

O tambaqui é uma das espécies aquáticas de maior expressão na alimentação da Região Norte, apresentando ampla distribuição nos rios desta região (VAL e ALMEIDA-VAL, 1995). Este peixe nas últimas décadas vem se tornando uma das principais espécies nativas para a piscicultura brasileira, com um ótimo padrão de crescimento e produtividade, fato que torna abundante a sua oferta no mercado consumidor. Esta espécie conta também com grande interesse de piscicultores de outros países da América do Sul, devido à sua rusticidade, qualidade da carne e o fato de poder chegar a 1 m de comprimento total e 30 kg de peso corporal no seu ambiente natural (GOUDING e CARVALHO, 1982).

Apesar de seu cultivo ser possível em todo o Brasil, o risco de alta mortalidade durante os meses de inverno tem desencorajado o cultivo nos estado das regiões Sul e Sudeste, particularmente, em locais onde as águas podem atingir temperaturas abaixo de 17 ºC (KUBITZA e KUBITZA, 2004); a temperatura ideal é em torno dos 25 ºC a 30 ºC (SILVA e GURGEL, 1999). Desse modo, o cultivo do tambaqui tem se concentrado nas regiões Norte, Nordeste e Centro Oeste do país, onde além do clima favorável, o tambaqui desfruta de grande aceitação no mercado (KUBITZA e KUBITZA, 2004).

O tambaqui (Figura 6), da palavra tupi *tãba'ki*, que significa resíduo de ostra (*tãba* – concha, ki – amontoado), foi a primeira espécie de peixe amazônico que atraiu um número relativamente grande de pesquisadores, como engenheiros de pesca, biólogos, fisiologistas e aqüicultores. Isto é devido principalmente à sua importância na pesca e pelo reconhecimento de que ele teria um grande potencial para a aqüicultura. Segundo Lima e Goulding (1998), o tambaqui tornou-se o peixe símbolo da Amazônia porque ele incorpora, em uma única espécie, a maioria dos problemas que precisam ser resolvidos para se manejar a pesca e ao mesmo tempo desenvolver a aqüicultura.

Figura 6: Tambaqui da Estação de Aqüicultura Continental Prof. Johei Koike, do Departamento de Pesca da Universidade Federal Rural de Pernambuco.

História da taxonomia

O exemplar no qual a descrição científica do tambaqui foi baseada, foi coletado pela expedição de Alexandre Rodrigues Ferreira, naturalista Português. Os exemplares zoológicos capturados pelo explorador no período de 1784-1785 foram levados para Barcelos, e nessa época Barcelos era a capital da Capitania de São José do Rio Negro. Em Agosto de 1785, uma grande coleção de exemplares (incluindo o tambaqui) zoológicos e antropológicos foi enviada de Barcelos para Lisboa e de lá para o Museu de História Natural de Ajuda, que na época era parte dos jardins do Palácio Real de Lisboa. Alexandre Ferreira, por motivo de doença, nunca pode completar o estudo sobre suas coleções amazônicas (LIMA e GOULDING, 1998).

Após a invasão napoleônica a Portugal, em 1811, a França pôde remover exemplares do Museu de Lisboa. O zoólogo Auguste Saint-Hilaire escolheu, dentre outros exemplares, o tambaqui para levar para o famoso *Jardin des Plantes* em Paris. Alguns deles, inclusive o tambaqui, foram parar no laboratório do paleontólogo e anatomista comparativo, Barão George Von Cuvier, do Museum d'Histoire Naturelle, em Paris. O tambaqui foi cientificamente batizado em 1818 por Cuvier como *Colossoma macropomum*, pertencendo à família das Characidae e subfamília Serrasalminae (LIMA E GOULDING, 1998).

Características gerais do tambaqui

O tambaqui, *C. macropomum*, é um peixe de água doce cujo tamanho pode atingir até 1 m de comprimento e um total de 30 kg de peso corporal no seu ambiente natural (GOUDING e CARVALHO, 1982). Este peixe é o segundo maior peixe de escamas nas águas sul-americanas, perdendo apenas para o gigante pirarucu (*Arapaima gigas*).

Comparando com a maioria das outras espécies de peixes amazônicos, o tambaqui possui lábios muito carnosos; possui dentes molares afiados e fortes (Figura 7). Esta característica permite que a alimentação do tambaqui seja onívora e as preferências dietéticas do tambaqui mudam do zooplâncton às sementes e frutas conforme o peixe cresce. Adultos e subadultos continuam comendo zooplâncton, mas este item alimentar progressivamente se torna secundário. O tambaqui possui muitos rastros branquiais e o número destes rastros aumenta à medida que o peixe cresce. As estruturas altamente desenvolvidas dos rastros branquiais são associadas com a filtragem de zooplâncton (LIMA e GOULDING, 1998).

A idade média na qual o tambaqui atinge a maturidade sexual é de 3 a 4 anos. O tambaqui é um peixe reofílico, portanto não desova naturalmente em cativeiro; peixes dessa natureza precisam vencer as correntezas dos cursos de água para maturar as suas gônadas (PROENÇA e BITTENCOURT, 1994). Na região Nordeste, a reprodução do tambaqui ocorre do período de outubro a março, sendo observada uma maior concentração das desovas no período de novembro a fevereiro (KUBITZA e KUBITZA, 2004).

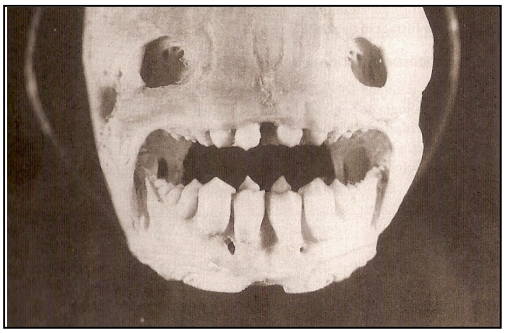

Figura 7: Dentição do tambaqui adulto.

Fonte: Lima e Goulding,1998

Doenças

A proteção contra doenças é primordial para a aqüicultura. Até o momento nenhuma doença endêmica foi registrada para o tambaqui cultivado ou silvestre, e isto tem contribuído para sua reputação de ser uma espécie robusta (LIMA e GOULDING, 1998). É de extrema importância investir no controle de doenças e isto pode ser alcançado através de medidas preventivas, tais como manutenção de estoques não infectados e dietas apropriadas, prescindindo, assim, do uso de remédios. O completo isolamento de adultos e jovens é provavelmente o maior passo que poderia ser dado para reduzir o parasitismo (THATCHER, 1991).

Condições ambientais extremas, a idade e o estresse são os principais fatores determinantes da intensividade de doenças. Em geral peixes jovens são os mais suscetíveis, uma vez que não tiveram tempo de criar resistência. O estresse comumente causado por mau manuseio, excesso da população, alimentação inadequada, ou uma combinação destes fatores também aumentam muito as chances de parasitismo intenso (LIMA e GOULDING, 1998).

O número de macroparasitas registrados para o tambaqui é relativamente pequeno, quando comparado com o total para as espécies de peixes das regiões temperadas (WOOTTON, 1992). A sardinha, o esturjão e algumas espécies de pescadas são atacados por mais do que o dobro da quantidade de macroparasitas registrados até agora para o tambaqui (CHOUDHURY e DICK, 1993; THONEY, 1993). As infecções do tambaqui por microparasitos, como fungos, bactérias e protozoários, foram menos estudados.

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2 JUSTIFICATIVA DO TRABALHO

Um grande número de lectinas de animais tem sido identificado só em termos de atividade hemaglutinante (AH) e especificidade do carboidrato. Em muitos casos, pouco se conhece a respeito de sua função biológica. Operações em aqüicultura esforçam-se para produzir um grande número de peixes saudáveis através de recursos que sejam economicamente e biologicamente eficientes. Por esta razão, a prevenção de doenças é muito importante tanto para os piscicultores da região, como também para a indústria. Muitas vacinas têm sido desenvolvidas, a nutrição dos peixes tem melhorado, com o objetivo reduzir significativamente o impacto de doenças na aqüicultura. Contudo, pouco se conhece com respeito à imunidade dos peixes.

Recentemente, pesquisas veterinárias e médicas descobriram que as lectinas estão envolvidas na imunidade inata. O papel fisiológico das lectinas não está claramente definido, mas o crescente estudo sugere que lectinas são proteínas de defesa que podem proteger contra ataques de predadores como vírus, fungos e bactérias.

O Brasil possui, talvez, a maior e mais variada ictiofauna do planeta. Só na Bacia Amazônica brasileira calcula-se que existam cerca de 2.000 espécies de peixes. Muitas destas espécies são de extrema importância para alimentação, e, absolutamente, nenhum estudo foi realizado com lectinas de peixe da região Norte e Nordeste do Brasil (região tropical). O tambaqui é uma das espécies aquáticas de maior expressão na alimentação da Região Norte, e vem nas últimas décadas se tornando uma das principais espécies nativas para a piscicultura brasileira, devido ao seu ótimo padrão de crescimento e produtivodade, fato que o torna abundante a sua oferta no mercado consumidor (VAL e ALMEIDA-VAL, 1995). Portanto o tambaqui foi o peixe de escolha para a nossa pesquisa.

A purificação de lectinas do tambaqui e, conseqüentemente, o estudo de suas características podem levar à sua utilização em aplicações biotecnológicas. Segundo Fassina et al. (2001) o uso de lectinas purificadas para imobilização em suportes cromatográficos pode abrir novos caminhos para o desenvolvimento de procedimentos mais eficientes, menos custosos e mais seguros de purificação.

Com a detecção do gene de lectina(s) do tambaqui, novas ferramentas serão oferecidas para uma melhor compreensão tanto em relação à expressão dos genes

para os diferentes tipos de lectinas nos diversos tecidos de tambaqui como também uma melhor compreensão do papel destas proteínas no sistema imunológico de peixes. Estas informações podem servir como base para programas de melhoramento genético visando aumentar a resistência dos peixes frente a uma determinada doença economicamente importante a fim de facilitar o seu manejo, melhorando, em última instância, a rentabilidade dos piscicultores da Região Norte e Nordeste.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Purificação e caracterização parcial da lectina do soro de tambaqui, bem como detecção do gene da(s) lectina(s) do tambaqui (*C. macropomum*).

3.1 OBJETIVOS ESPECÍFICOS

⇨ Pré-purificar por fracionamento salino o soro de tambaqui (*Colossoma macropomum*);
⇨ Determinar o perfil da atividade hemaglutinante (AH) das diferentes frações obtidas com diferentes eritrócitos;
⇨ Determinar o efeito da temperatura e íons sobre a AH das diferentes frações;
⇨ Caracterizar as diferentes frações através de eletroforese, inibição por carboidratos;
⇨ Purificar parcialmente a(s) lectina(s) do tambaqui *(C macropomum)* a partir da fração de atividade hemaglutinante;
⇨ Sintetizar e amplificar cDNAs da lectina para identificar a presença da família;
⇨ Analisar o perfil de expressão das lectinas nos tecidos do tambaqui.
⇨ Clonar os genes de lectina em plasmídio.

CAPÍTULO II

PURIFICATION AND PARTIAL CHARACTERIZATION OF THE LECTIN(S) PRESENT IN THE SERUM OF AMAZON FISH TAMBAQUI (*Colossoma macropomum*, CUVIER, 1888)

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PURIFICATION AND PARTIAL CHARACTERIZATION OF THE LECTIN(S) PRESENT IN THE SERUM OF AMAZON FISH TAMBAQUI (*Colossoma macropomum,* CUVIER, 1888)

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Abstract

Lectins are that recognize and bind specifically and reversibly to carbohydrates. They are broadly distributed in nature, within microorganisms, plants and animals. Tambaqui, *Colossoma macropomum* is one of the most important species of fish used as food by the population of the Northern region of Brazil, showing a broad distribution along the rivers of this region. In the last decades, this fish has become one of major native species in the Brazilian aquiculture, presenting an excellent pattern of growing and high productivity, turning abundant its offer in the consumer market. In the present work the tambaqui serum lectin was partially purified through the saline precipitation of ammonium sulfate. The obtained fractions were analyzed and the one with the biggest activity was chosen to be used in the following steps. The fraction (F) 0-50% (F0-50) lost its hemaglutinant activity at 60 ^{0}C being partially inhibited by the fucoses and galactoses monosaccharides, and its activity was not changed neither in the presence of ions or in the presence of different buffers. Two peaks of lectinic activity were obtained when the F0-50 was subjected to a chromatography in a column of Mono S HR 5/5 (cationic exchanger). Through the use of SDS-PAGE under reducing and non-reducing conditions, both peaks presented three major bands with approximately 21, 19,3 e 15,6 kDa and, through PAGE for basic and native proteins it has been observed the same profile for both peaks. Therefore, it can be concluded that the lectins present in the serum of tambaqui have basic character and are capable to recognize fucoses and galactoses, being members of the group of the fucolectins.

Key Words: *Colossoma macropomum*, purification, *MALDI-TOFF MS, fucolectins*

INTRODUCTION

Lectins are proteins that are capable to bind specifically to carbohydrates expressed over cellular surface. Initially, these proteins have been identified in plants but nowadays it is well known that they are broadly distributed in nature, including eukaryotic and prokaryotic organisms (Sato *et al*., 2000; Sun *et al*., 2007). Due to their ability to specifically recognize certain sugars on the surface of bacteria, it has been suggested that animal lectins participate actively of the immune innate response through the induction of the bacterial agglutination or, like opsonins, by the increase of the phagocytosis of microorganisms by hemocyts (Bayne *et al*., 2001; Jimbo *et al*., 2007; Sun *et al*., 2007). Lectins of fish have been isolated from serum, plasma, mucus, skin and eggs. It seems that these lectins have an important role in fertilization, morphogenesis and embryogenesis, in addition to their role on the defense against microorganisms (Ewart *et al*., 1999, Ahmed *et al*., 2004; Dutta *et al*., 2005).

Tambaqui, *Colossoma macropomum* is one of the most important species of fish used as food by the population of the Northern region of Brazil, showing a broad distribution along the rivers of this region (Val & Almeida-Val, 1995). In the last decades, this fish has become one of major native species in the Brazilian aquiculture, presenting an excellent pattern of growing and high productivity, turning abundant its offer in the consumer market. This species has also aroused a high interest in the fish breeders of other Latin American countries, due to its rusticity, meat quality, being able to achieve 1 m length and 30 Kg of body mass within its natural environment. (Gouding & Carvalho, 1982) The main goal of the present work was to partially purify and characterize the lectins present in the tambaqui serum.

METHODS

BLOOD COLLECTING AND SERUM SEPARATION

The tambaqui blood collecting was performed in the Continental Aquaculture Station Prof. Johei Koike of the Fishing Department of the Federal Rural University of Pernambuco (UFRPE). The blood from an adult tambaqui was collected using a 5ml syringe through its caudal vein. Immediately after the collecting the blood was kept in glass tubes for approximately three hours at ambient temperature. Subsequently, after blood coagulation, the serum was removed with the aid of a Pasteur pipette being centrifuged at 1300 x g by 5 minutes at 4 ^{0}C. The serum was removed and centrifuged again at the same conditions. After the re-centrifugation the serum was distributed in 5 ml aliquots and kept at -20 ^{0}C until the moment to be used.

HEMAGGLUTINATING ACTIVITY (HA)

The AH was established in microtiter plates with 96 wells, consisting in eight horizontal rows, with eight wells each, using rabbit blood following the work of Correia e Coelho (1995). 50 μL of NaCl 0,15 M were added to each well; later, 50 μL of the sample were put in the second well of a horizontal row, and, from left to right, successive dilutions were performed, rejecting the last 50 μL from the last well of a horizontal row. After each dilution of the sample, 50 μL of the suspension of the rabbit's erythrocytes were added to all wells. After 30 minutes the analysis of the plates was performed. The AH corresponds to the inverse of the last dilution that presents hemagglutination relative to the control. The specific hemagglutinating activity (SHA) was obtained dividing the HA by mg of proteins by ml.

The evaluation of AH was performed in the presence of different buffers (citrate, phosphate, sodium phosphate, Tris-HCl) at different molarities (0,01 M; 0,1 M; 0,2 M; 0,4 M) and values of pH (4,5 a 8,5), as well as different concentrations of NaCl, ranging from 0,01 M to 0,1M. In order to establish the AH, a quantitative evaluation of proteins was performed for all samples according to Lowry *et al*. (1975).

FRACTIONING WITH AMMONIUM SULPHATE

The serum diluted (1:2) in a sodium phosphate buffer 50 mM, pH 7,5 was precipitated with ammonium sulphate with 0-50% e 50-80% saturation according to Green and Hughens (1995). The fractions (F) and final supernatant (FS) were dialyzed against distilled water, followed by abovementioned sodium phosphate buffer, obtaining F0-50, F50-80 and FS80. After dialyses the SHA were obtained from these fractions.

MASS SPECTROMETRY (MALDI-TOF MS) OF BASIC PROTEINS FROM THE TAMBAQUI SERUM

The fractions of the serum obtained from the saline precipitation with ammonium sulphate were subjected to an electrophoresis for basic, native proteins according to Reisfeld *et al*. (1962). The bands of interests were cut from the gel with aid of a sterile stiletto. In sequence, the bands were subjected to a gel digestion and, subsequently, to an enzymatic digestion with trypsin according to Gourdine *et al.* (2007). At the end of treatment, samples were dried in *speed vac* and sent to mass spectrometry analysis (MALDI TOFF).

CARBOHYDRATE SPECIFICITY, IONS AND THERMAL STABILITY

The evaluation of hemagglutinating activity inhibition (HAI) against carbohydrates (monosaccharide, disaccharadide and glycoprotein), ions and thermal stability was performed according to Correia and Coelho (1995) in microtiter plates with 96 wells.

HAI assay, the concentration of the utilized of carbohydrates varied from 200 to 1,56 mM. The HAI was established by the HA from the sample in the presence of carbohydrates which was compared to the HA of the sample in the absence of carbohydrate, and correspondent to a reduction of HA of the studied sample.

The influence of ions over the HA was established with the ions solutions: $CaCl_2$, $MgCl_2$ and $MnCl_2$ with the concentration ranging from 40 to 0.625 mM.

For the determination of the thermal stability, 100 µg/mL of each sample were heated in water bath in the following temperatures: 40, 50, 60, 70, 80 e 90 ^{0}C for 30 minutes. In sequence, the samples were immediately cooled and centrifuged at 3000 x g for 20 min. The HA was performed with the supernatant of each heated sample.

PURIFICATION OF TAMBAQUI SERUM LECTIN

In order to purify the lectin of the serum of tambaqui, different chromatographic matrices were evaluated using affinity chromatografy techniques, ionic exchange and molecular exclusion in conventional or fast resolution systems. Among the evaluated supports (Guar gel, Sepharose 4B, immobilized fetuins, Cm-cellulose, Sephadex-G100, G50 and G25, Mono Q HR 10/10, Hitrap SP XL), the Mono S HR 5/5 (GE Healthcare) was chosen for the purification which was performed, in accordance to the manufacturer norms, with the High Performance Liquid Chromatography ÄKTA-Purifier (GE Healthcare) system. After the equilibrium of the column with sodium phosphate buffer 50 mM pH 7,5 was achieved, it was applied 1 ml (15 mg) of F0-50. The non adsorbed material was removed with 5 column volumes (cv) with the phosphate buffer. The adsorbed proteins were eluted with gradient of NaCL of 0 - 1,0 M during 20 cv. The flux velocity used was 0,5 mL/minute.

POLYACRILAMIDE GEL ELECTROPHORESIS (PAGE)

PAGE were performed for native and basic proteins according to Reisfeld *et al*. (1962) and Davis (1964), respectively. SDS-PAGE at 10% was performed according to Laemmli (1970) in denaturing or reducing and denaturing conditions, in the absence and in the presence of β-mercaptoethanol, respectively.

RESULTS

BUFFER ASSAYS

There was no difference in HA for the serum diluted in different tested buffers with distinct molarities and pHs. Also, the HA was not changed with the different salt concentrations.

SALINE FRACTIONING WITH AMMONIUM SULPHATE

Only the F0-50 presented HA (Table 1). Through the SDS-PAGE en reducing conditions, differences were observed among the fractions which can be verified by the falling of the number of bands of F50-80 e FS80 (Figure 1). Therefore, the fraction F50-80 was chosen to continue with the purification process.

Table 1: Saline fractions of the tambaqui serum.

Samples	HA	Protein (mg/mL)	SHA
Serum*	512	24,67	20,75
F0-50	512	18,96	27,01
F50-80	0	7,28	0
FS80	0	1,32	0

*Serum diluted (1:2) in sodium phosphate buffer.

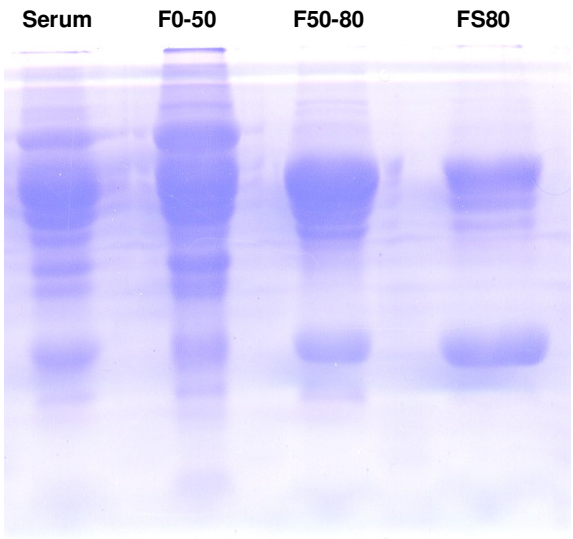

Figure 1: Reducing SDS-PAGE analysis of serum and fractions.

MASS SPECTROMETRY (MALDI-TOF MS) OF BASIC PROTEINS OF THE TAMBAQUI SERUM

The extracted bands from the polycrylamide gel for native basic proteins were analyzed by mass spectrometry (MS), and, with these data, a search was performed in the Mascot database. The comparison of the means of the peaks of the highest band of F0-50% (indicated in figure 2 by the arrow) presented a 95% similarity with the complement component C3-4 of the fishes *Oncorhynchus mykiss and Salmo gairdneri* (Q9DDV9_ONCMY).

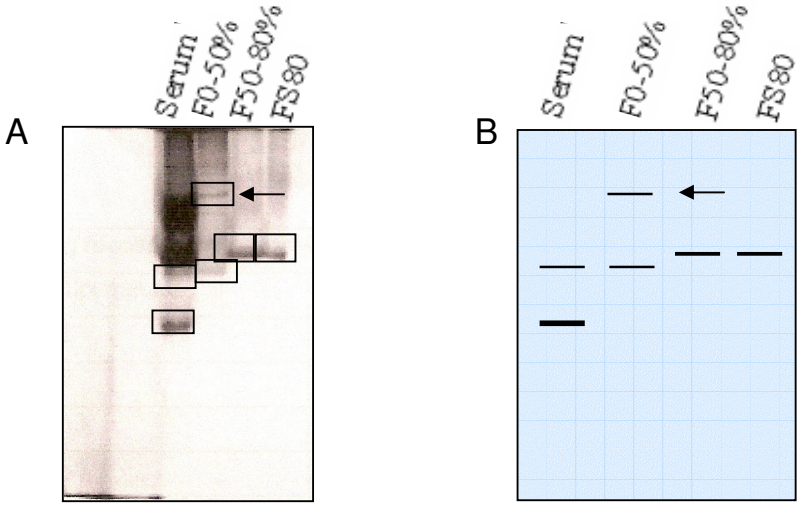

Figure 2: PAGE for native and basic protein (A) and scheme (B). Extracted band of the gel (inside the box), and complement component C3-4 (indicated by the arrow).

HEMAGGLUTINATING ACTIVITY INHIBITION (HAI)

The HAI of F0-50 was tested with the carbohydrates: D(+)Glucose, D(+)Fucose, D(+)Galactose, D(+)Mannose, D(-)Fructose, methyl-α-D-mannopiranoside, 1-O-Methyl-α-D-glucopyranoside, D(+)xylose, N-Acetyl-D-glucosamine, D(+)Lactose, D(+)Threalose, D(+)Saccharose, 1-O-Methyl-β-D-arabinopyranoside . The fraction was partially inhibited by fucose, galactose and lactose; rabbit serum and fetuin also inhibited F0-50 HA (tabela 2).

Table 2: Inibição da AH de F0-50

Saccharides	**Agglutination titer (HA)**
D-Fucose	16
D-Galactose	16
Lactose	128
Fetuin	128
Rabbit serum	64

HA de F0-50 = 512^{-1}

DETERMINATION OF THE THERMAL STABILITY AND EVALUATION OF THE HA IN THE PRESENCE OF IONS

The HA of the serum and of the F0-50 decreased from 40 ^{0}C being totally abolished after heating at 70 and 60 ^{0}C (table 3), respectively. The tested ions (Ca^{2+}, Mg^{2+} e Mn^{2+}) did not affect the HA from the serum and fractinos.

Table 3: Thermal stability of serum and F0-50.

TEMPERATURE ^{0}C	SERUM (HA)	F0-50
30	512	512
40	256	128
50	128	16
60	8	0
70	0	0
80	0	0
90	0	0
100	0	0

PURIFICATION OF THE TAMBAQUI SERUM LECTIN

From all trial column chromatographies, the column of Mono S HR 5/5 (GE Healthcare) presented the best results with two peaks of the adsorbed material. Both peaks were eluted in the saline gradient of 0 – 1,0 M (figure 3). Peak 1 was eluted with saline concentration 0,69 M, while peak 2 was eluted with saline concentration 0,75 M; the HA were 64^{-1} e 32^{-1}, respectively.

For peak one, under non reducing conditions, 4 bands were observed through SDS-PAGE: the first one was weaker with 32 kDa and the other three were stronger, with 21,5, 19,9 and 15,9 kDa. For peak 2, two bands were observed: a weaker with 21,5 kDa and a stronger with 15,6 kDa (figure 4A). Under reducing conditions it was observed that peak one presented three main bands with molecular weights of approximately 21,0, 19,3 and 15,6 kDa, while peak 2 presented two very tenuous bands of 20,9 and 18,8 kDa and a stronger band with 16,3 kDa (figure 4B). The gel for native and basic proteins presented the same electrophoretic profile for both peaks with two bands only (figure 5).

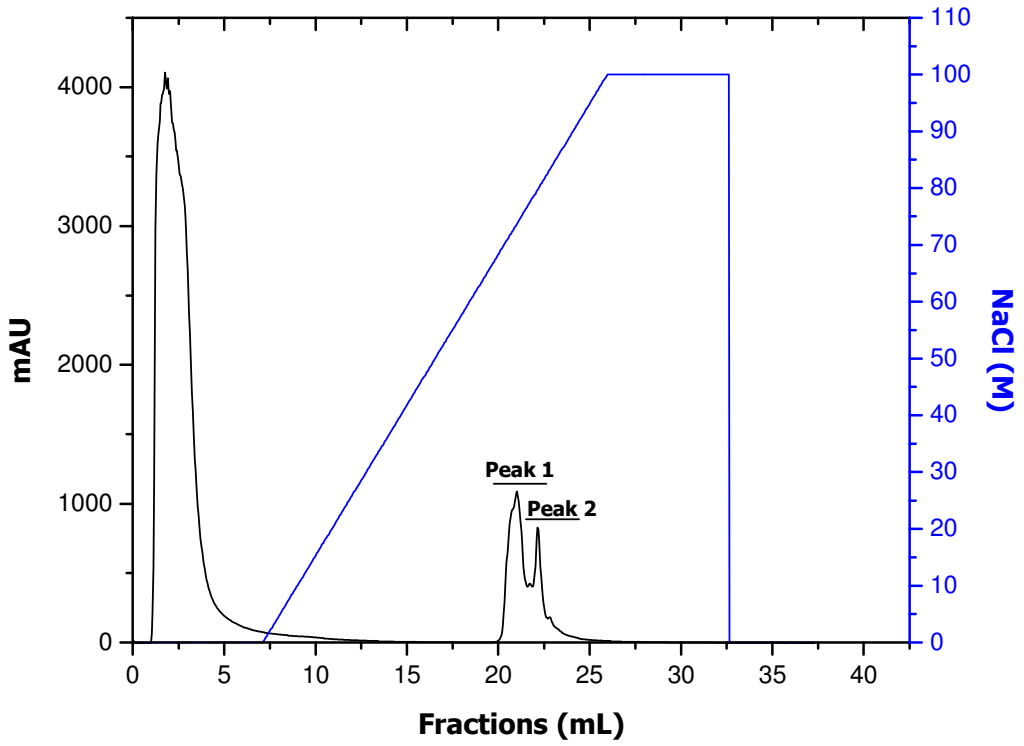

Figure 3: Cationic exchange chromatography in MONO S HR 5/5 column.

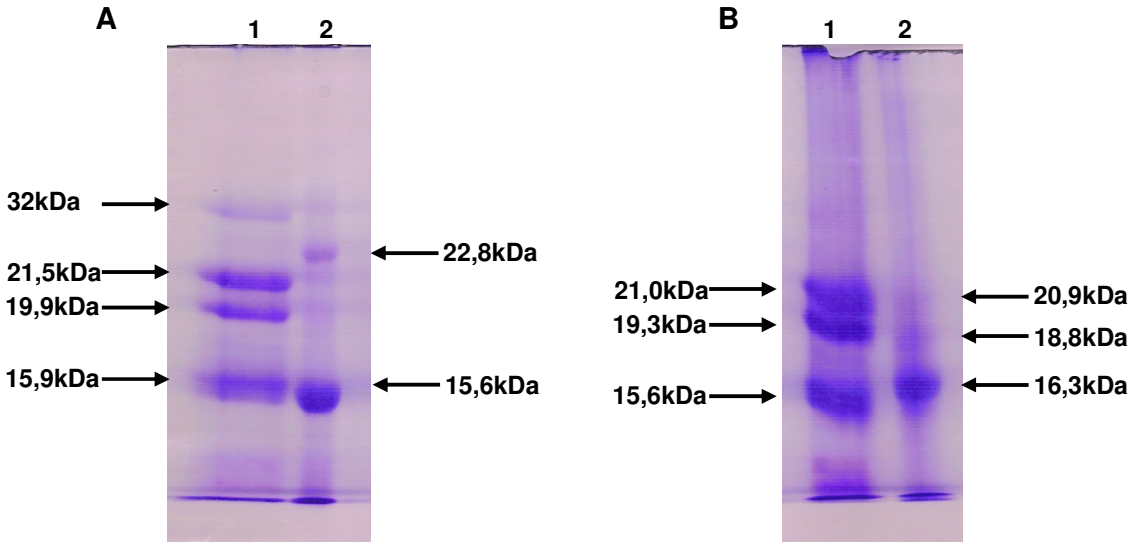

Figure 4: SDS-PAGE 10% (p/v) of the obtained peaks of the Mono S. column. Peak 1 (1) and peak 2 (2) were subject to both, unreduced (A) and reduced (B) SDS-PAGE.

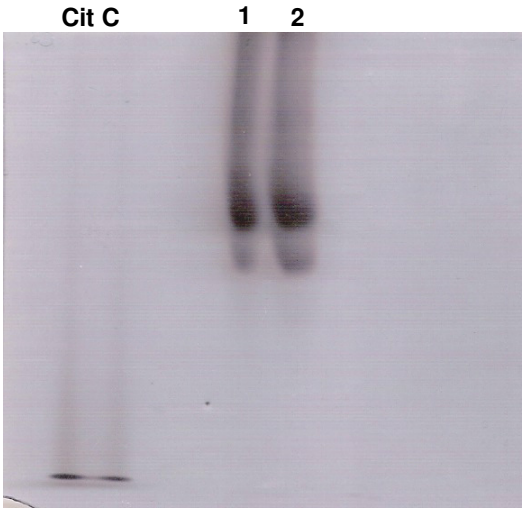

Figure 5: PAGE for native and basic protein. Peak 1 (1), Peak 2 (2).

DISCUSSION

Although tambaqui is a freshwater fish, the HA of its serum was not affected by high concentrations of NaCl. Similar results were obtained with Anguilla japonica, a catadrome fish that adapts well in both, saltwater and freshwater (Suzuki *et al*., 2003; Martinho *et al*., 2007). This is an important feature to this particular fish because it spends part of its lifecycle in freshwater and another part in saltwater, and, consequently, its proteins are very tolerant to the salinity change. The hemaggutinating activity of the lectin present in the tambaqui serum have shown to be stable for the pHs tested (4,5 to 8,5).

The serum of some vertebrates contain over 100 distinct proteins (Bayne *et al*., 2001) and the saline fractioning can be used as a first step to discard some non important proteins (Kennedy *et al*., 1995).

The saline fractioning of tambaqui serum has shown to be effective to select the protein of interest, since F0-50 was the only one that presented HA.

Due to the fact that teleostean fishes present unique features, such as the dependence of the acquired immunity from the temperature and some limitations in antibody diversity, some researchers suggested that the innate immune functions are much more important in these fishes than in other homeothermal animals (Kubitza & Kubitza, 2004; Tsoi *et al*., 2004). As a matter of fact, the lectinic activity of tambaqui lingered active until 70 ^{0}C showing a certain degree of resistance of these proteins to high temperatures, despite the fact that tambaqui lives in relative warm waters.

The loss of ions during the purification process may cause a disturbance in the protein structure, leading to a loss of hemagglutinanting activity (Kennedy *et al*., 1995). It was not detected any alteration neither in the lectinic activity of the serum nor in the tabaqui serum fractions despite of their incubations with ions. Maybe, the tambaqui serum lectins CRD recognizes the carbohydrate in an ion independent manner (Ewrat *et al*., 2001; Suzuki *et al*., 2003).

The fact that occured lectinic activity of tambaqui serum by fucose and galactose independent of Ca^{++} suggests that this lectin is included in recently identified family of fucolectins (Cammarata *et al*., 2001; Cammaratta *et al*., 2007).

From all tested matrices the only one in which the lectin did not lose its activity was the Mono S HR 5/5. This result may be inferred to the features of the matrix. This particular matrix has particles that are extremely homogeneous and it is not

strongly cationic and it works at low pressure. The last two factors destroy the structure of the protein or make it difficult the desorption from column. Therefore, this matrix provided an adequate environment for the partial purification of the tambaqui serum lectin. The observed differences for the two peaks obtained at the chromatography in Mono S HR 5/5 column, mainly by SDS-PAGE in non reducing conditions, suggests the presence of isoforms and isolectins in the tambaqui serum. It is common to find more than one type of lectin in serum of animals (Suzuki *et al.,* 2003; Ahmed *et al*., 2004).

In fishes and mammals the complement may be activated by three different paths: the classical path, the alternative path and the path of the lectin that activates the complement system by the MBL (Mannose Binding Lectin) linking to carbohydrates present on the surface of microorganisms Magnadottir *et al*., 2005; Ourth *et al*., 2005). In tambaqui serum was detected a basic protein showing high similarity with one component of the complement system C3-4 of the fishes *O. mykiss e S. gairdneri*. Recent studies point that the complement may have an important role in different biological processes, such as the muscular and skeletal development, the reproduction and the regeneration of members and organs (Magnadottir *et al*., 2005).

The purification of this protein may lead to a better comprehension of the function of this complement in fishes.

It can be concluded that the tambaqui serum presents thermo-stable lectins from the fucolectin family that are independent of ions for their activity, and with basic pI. This serum also presents a protein that is similar to a component from the complement system C3-4 of some fishes.

In the near future the authors intend to totally purify and characterize the lectin(s) from the tambaqui serum. The physiologic role of these lectins will be better understood, in the hope of improving the general comprehension of the immune system of the fishes.

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CAPÍTULO III

MOLECULAR DIVERSITY OF THE EXPRESSED LECTINS IN TAMBAQUI (*Colossoma macropomum*) TISSUES

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MOLECULAR DIVERSITY OF THE EXPRESSED LECTINS IN **TAMBAQUI (*Colossoma macropomum*) TISSUES**

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ABSTRACT

Lectins represent a diverse group of proteins that recognize and bind specifically to carbohydrates. These proteins play a significant role in the immune responses of fishes. Tambaqui (*Colossoma macropomum*) is a native fish from Amazon basin, and it is one of the most important species in Brazilian aquaculture. The main objective of the present work is to analyze the profile of the expressed genes of different types of lectins in the tissues of tambaqui. We have analyzed the expression of these lectins due to the use of reverse transcriptase coupled to polymerase chain reaction (PCR). The gene of galectin (*Gal*) is constitutively expressed in all tested tissues of tambaqui. For the other lectins (MBL. FucolectinAA and Fucolectin) the expression occurred mainly in the skin of the fish. The sequencing of the PCR product has shown that *Gal* belongs to the galectins subfamily tandem-repeat.

Keywords: *Colossoma macropomum,* tambaqui lectin; RT-PCR; galectin.

Introduction

Lectins are proteins or glycoproteins which have the ability to bind specifically to mono or oligosaccharides reversibly (Elgavish and Shaanan 1997; Vijayan and Chandra 1999; Sato et al. 2000; Souza et al. 2001).

The physiological role of lectins in plants is not clearly elucidated, but increasing studies suggest that lectins are defense proteins which protect against predator attacks, such as viruses, fungi and bacteria (Cavada et al. 1998; Ratanapo et al. 2001; Sacchettini et al. 2001). The presence of lectins have been identified in eggs of some fish families, and some researchers suggest that these lectins are involved in fertilization and in defense against pathogens (Shiomi et al. 1989). Lectins can also be found in fish serum (Fock et al. 2000; Cammarata et al. 2001; Buchmann and Lindenstrom, 2002). According to Ewart et al. (2001) lectins of the fish serum can bind to a set of carbohydrates structures on the cell surface of microorganisms (bacteria, viruses, yeast and parasitic protozoary) mediating an antibacterial effect, or acting on the direct destruction by complement systems through an attack of the lytic membrane, or even promoting fagocitosis.

Fishes differ from mammals in relation to the immuno-acquired response

In fishes, which differ from mammals in relation to the immuno-acquired response, because they synthetize only one type of immunoglobulin (IgM), lectins have na important role in the inate imunity (Magnadóttir et al. 1999; Dutta et al. 2005).

Probably, Brazil has the most varied fish fauna of the planet. Only in Brazilian Amazon basin, it is calculated that there are approximately 2.000 species of fishes. Many species are extremely important for human feeding, and, as far as the authors know, no study was carried out with lectins of fishes from the north and northeast regions of Brazil (tropical regions). In the last decades, tambaqui (*Colossoma macropomum*) is becoming one of the major native species for Brazilian inland fishculture, showing a great pattern of growth and productivity, becoming abundant its offers in the consumer markets. The main objective of the present work is to analyze the profile of the expressed genes of different types of lectins in the tissues of tambaqui.

Materials and Methods

Tambaqui tissue

Tambaqui (3), gently given by Mar Doce Company (Recife, Pernambuco-Brazil company) were anaesthetic in ice and the spleen, liver, heart, skin, gill, brain, kidney and pyloric caecae were surgically removed following the anatomic localization (Kubitza and Kubitza 2004). The tissues were immediately conditioned in liquid nitrogen until the moment of the isolation of the total RNA.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from the tissues of three individuals using the Trizol reagent (Invitrogen, Carlsbad, EUA) according to the manufacturer´s instructions. The concentration and the quality of the total isolated RNA were determined by measuring their absorbance at 260-280 nm. The integrity of the RNA samples was visually examined by electrophoresis in 1% agaroses gel.

The cDNA was synthesized from total RNA using the 3'Oligo$(dT)_{18}$-adapter, named QT (5'-CAGTGAGCAGAGTGACGAGGACTGGAGCTCAAGC(T_{18})-3') and the Moloney Murine Leukemia Virus Reverse Transcriptase, M-MLV RT (Promega). The reaction was carried out at 37 ^{0}C for 60 min and inactivated at 70 ^{0}C for 15 min. Finally, the cDNa was diluted 10 times in ultrapure water to a final volume of 50 µl.

Oligonucleotides (primers) and PCR

Degenerated primers (table 1), forward and reverse for four kinds of lectins, were synthesized according to the homologous region of the conserved oligonucleotides sequences that were searched from the genes and genomas of other species of fishes (attach II).

cDNA (diluted 10 times) amplification was carried out at 94 ^{0}C for 3 min for denaturation following 30 cycles of 94 ^{0}C for 50 s, 48 ^{0}C at 52,0 ^{0}C (see table 1) for 50 s and 72 ^{0}C for 2 min.

Table 1: Primer list.

Lectins	Primers	annelling
Galectin (Gal)	5- TCACTTYAACCCACGCTATGA -3 (forward) 5- AGGCRATAGAKTTCACYTCCA C -3 (forward)	48 ^{0}C
Mannose Binding Lectin (MBL)	5- MKSCCTGGCAGAGATGGAAGA -3 (forward) 5- GTARTATTTCTGTCCCACTTTCCT -3 (reverse)	48 ^{0}C
Fucolectin *Anguilla anguilla* (FucoAA)	5- KSCARGCWATGCCATTATGG -3 (forward) 5- ACAGCGAAAGGTTYTAGTCTC -3 (reverse)	52 ^{0}C
Fucolectin (Fuco)	5- ATGGAAACMGTRASTCTGACT -3 (forward) 5- TGTAWAGAGTTGCCGATGT -3 (reverse)	52 ^{0}C

PCR and Amplicons purification

After analyzing each PCR product by electrophoresis in 1% agarose gel, it was performed a DNA amplification using the galectin primer forward (5-TCACTTYAACCCACGCTATGA -3) and the Q0 primer reverse (5'-CCAGTGAGCAGAGTGACGA-3') and all the tissues as templates. Denaturation was performed at the same conditions mentioned above. After the visualization through electrophoresis agarose gel, the amplicons of spleen (spleen – Sp highest e Sp smallest) and pyloric caecae (Pc highest e Pc smallest) were removed from the agaroses gel, purified with Wizard SV Gel and PCR Clean-Up System kit (Promega). It was removed one aliquot from the above mentioned PCR products in order to perform another PCR using galectin specific primers. All PCR products were sequenced by the dideoxy termination method using a DyeNAMIC ET Terminater Cycle sequencing kit (Amersham Bioscience K.K., Tokyo, Japan) and an ABI PRISM 377 DNA sequencer (Applied Biosystems, CA, USA).

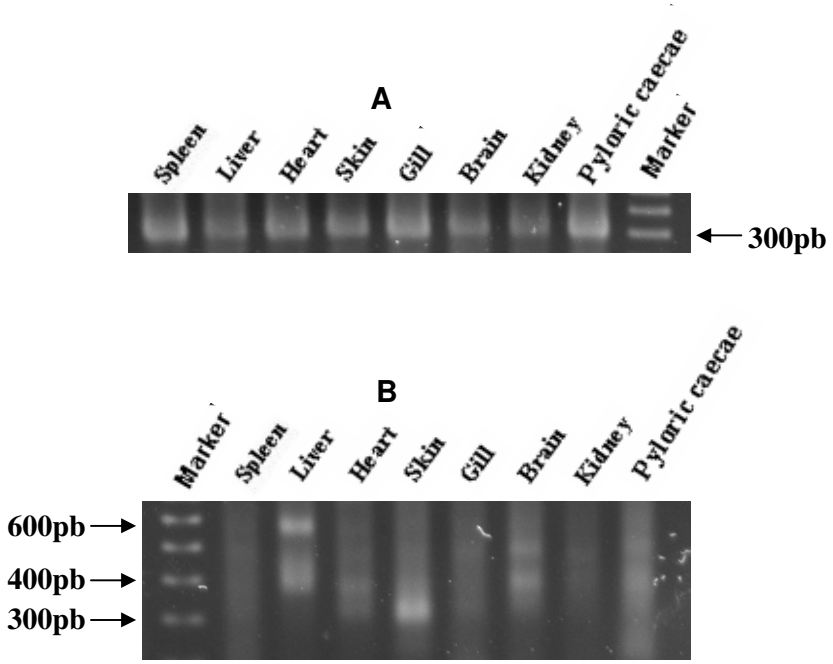
Homology search was performed using BLAST computer program (Altschul et al. 1990). The amino acid sequence of each domain of Sp and Pc were aligned and checked by the software Bioedit (Hall. 1997).

Results

Total RNA isolation and cDNA synthesis

The reverse transcription coupled to polimerases chain reaction (RT-PCR), using a protocol with QT adapter, allowed the synthesis of complete cDNA molecules (data not shown).

By using specific primers, PCR of cDNA resulted in amplicons with expected sizes: galectin with amplicon of approximately 300 bp in all tissues (figure 1A), MBL resulted in an amplicon of about 300 bp in skin (figure 1B), FucoAA resulted in an amplicon of about 500 bp in all tissues, except in brain and other of about 400 bp in liver and in pyloric caecae (figure 1C), and finally, Fuco resulted in an amplicon of 400 bp in skin (figure 1E).



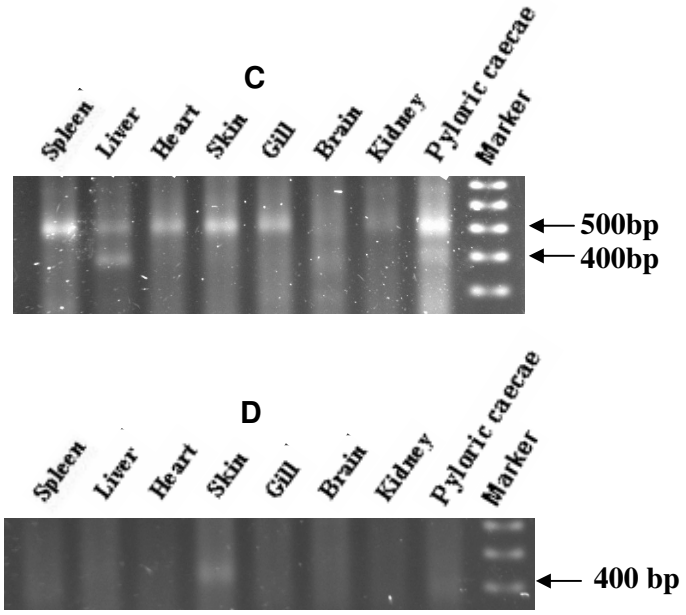


Figure 1: Gene profile analysis of the GAL, MBL, FucoAA and Fuco by RT-PCR. Identical amounts of total RNA from spleen, liver, heart, skin, gill, brain, kidney and pyloric caecae were isolated and reverse-transcribed to produce cDNA. PCR amplifications were then performed specific primers to amplify galectin (A), MBL (B), FucoAA (C) e Fuco (D).

PCR and sequencing

Galectin was constitutively expressed in all tissues, therefore we immediately decided to investigate this lectin. PCR was performed using the forward primer for galectin and reverse primer for Q0. The product of this reaction resulted in two strong amplicons (figure 2A) of approximately 600 and 950 bp in spleen (Sp1/600 and Sp1/950) and caecae (Pc1/600 e Pc 1/950). These amplicons were purified from gel (figure 2B), and another PCR was performed using specific primers for GAL. This reaction resulted in amplicons of expected sizes of 300 bp (figure 2C).

The sequencing of PCR products using the specific primers Sp1/600 (Sp1), Sp1/950 (Sp2), Pc1/600 (Pc1) and Pc1/950 (Pc2) shows that GAL transcripts belongs to the galectin subfamily Tandem Repeat (group 9 galectin). The search for homology to Sp1 (figure 3) showed that there was approximately 53% of homology to the galectin of group 9 from *Danio rerio* (AAR84192) and 41% of homology to the galectin from O*ncorhynchus mykiss* (BAA88670). The search for homology to the Pc1 (figure 4) demonstrated that there was approximately 57% of homology.

The sequencing reaction didn't work for the PCR products Sp1/600, Sp1/900, Pc1/600, Pc1/950, Sp2 and Pc2.

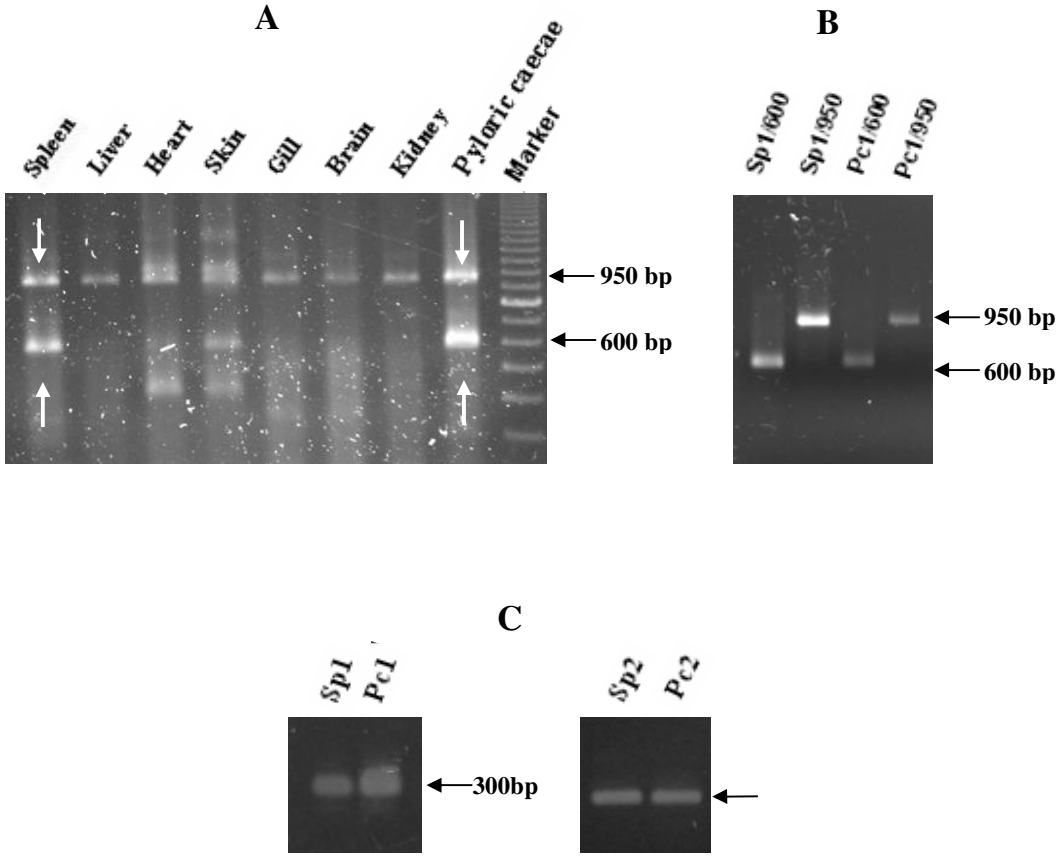


Figure 2: RT-PCR of the gal specific primer forward and Q0 primer reverse (A), purification of the spleen and pyloric caecae amplicons (B) and PCR using gal specific primers (C), Sp1 (Sp1/600), Pc1 (Pc1/600), Sp2 (1/950), Pc2 (1/950).

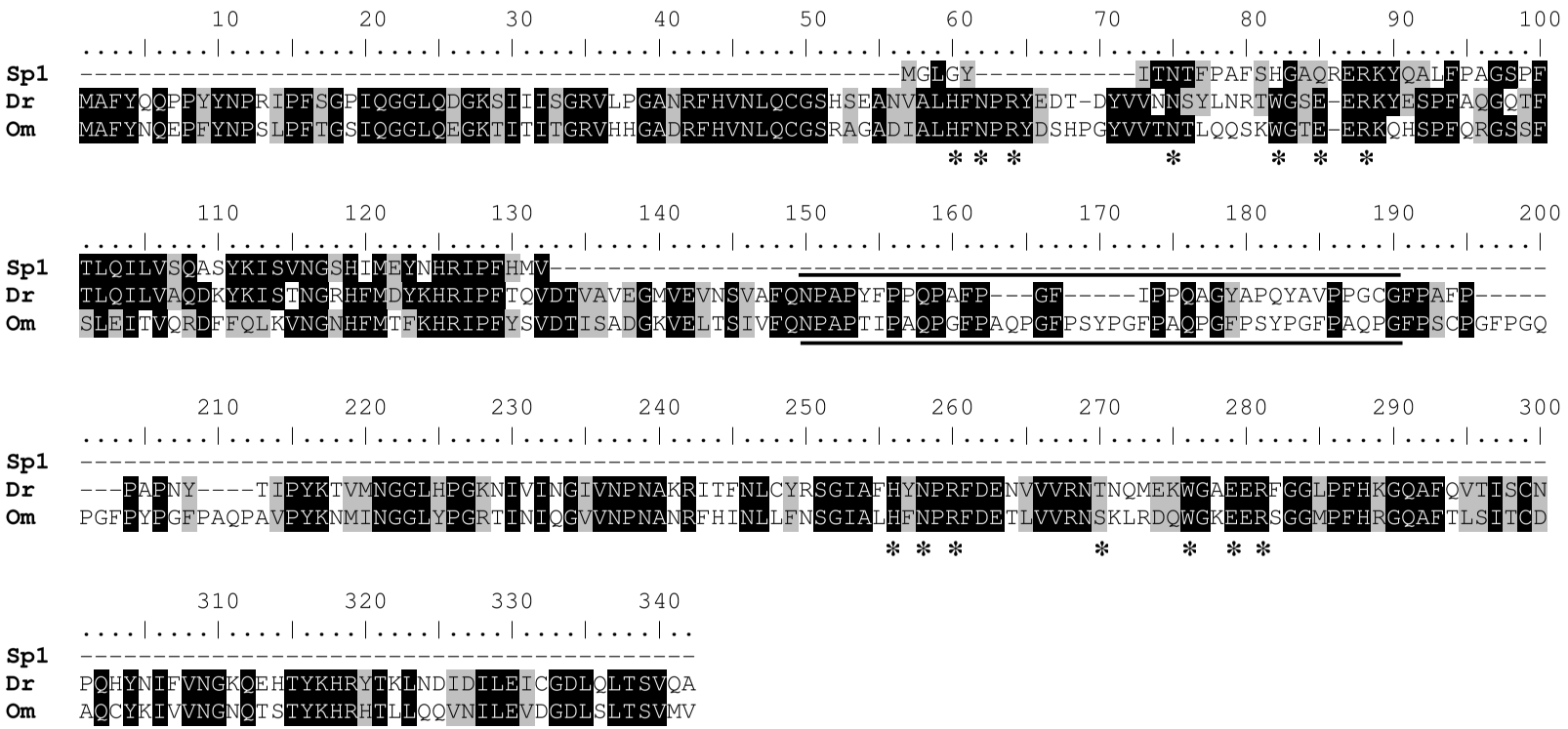


Figure 3: Amino acid sequence comparison of the Sp1 with other galectins from fishes. Conserved residues and identical residues are indicated with grey and black respectively. The critical amino acid residues that are known to interact with carbohydrate are marked by an asterisk. Link peptide (underscored). *Danio rerio* (Dr) and *Oncorhynchus mykiss* (Om).

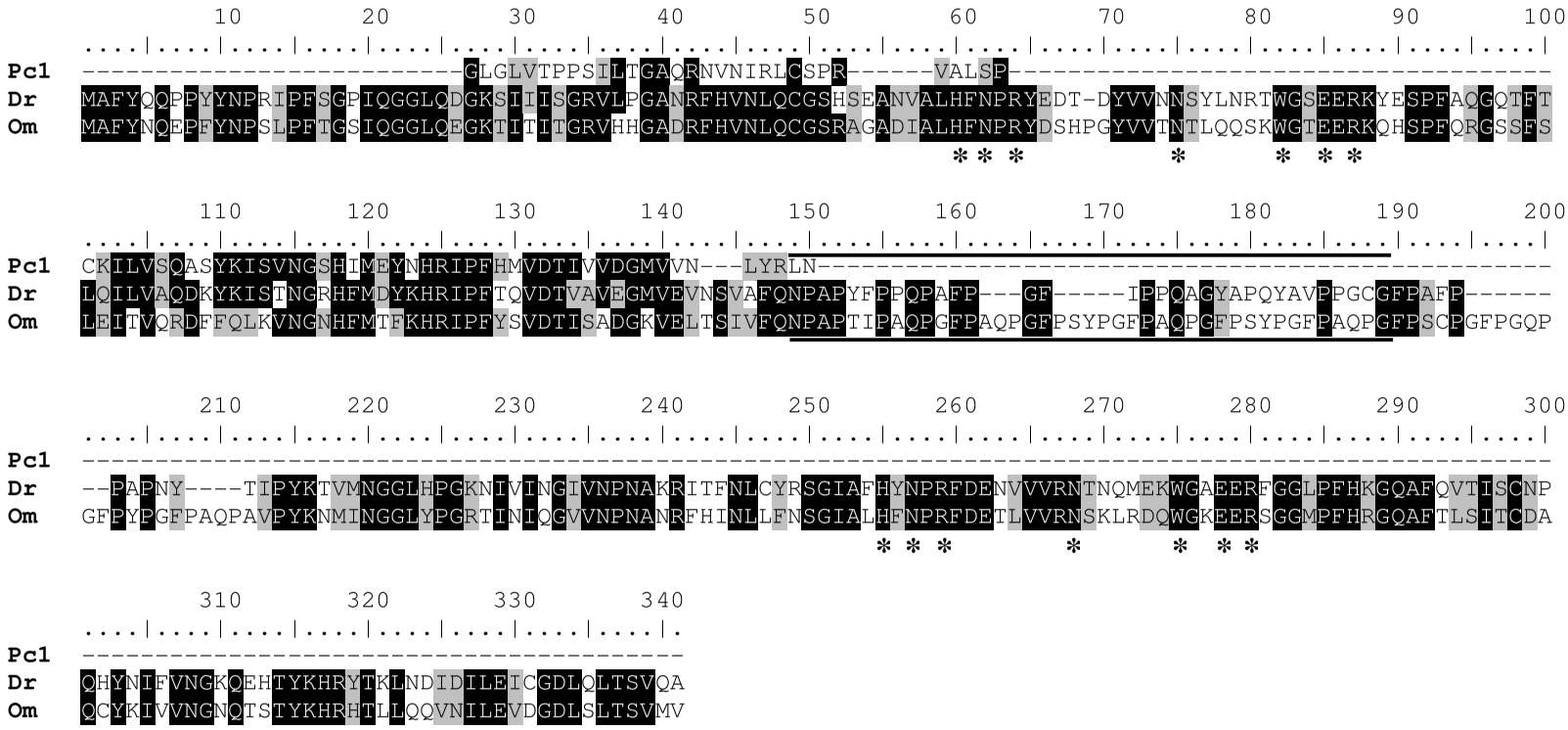


Figure 4: Amino acid sequence comparison of the PC1 with other galectins from fishes. Conserved residues and identical residues are indicated with grey and black respectively. The critical amino acid residues that are known to interact with carbohydrate are marked by an asterisk. Link peptide (underscored). *Danio rerio* (Dr) and *Oncorhynchus mykiss* (Om).

Discussion

In recent studies, lectins of fish have been isolated from serum, plasma, skin mucus and eggs. Some lectin functions were studied, showing an important role in the innate immunity of these animals (Rini and Lobsanov 1999; Ewart et al. 2001; Dutta et al. 2005; Okamoto et al. 2005; Russell and Lumsden 2005; Podolsky et al. 2006). These soluble and extracellular proteins recognize specifically carbohydrates which cover the pathogen surface and allow fishes to identify it as non-self cell. It has been reported in literature that some lectins, such as galectins, have an important role in recognition of fish pathogens, being also important in the embryonic development in these animals (Ahmed et al. 2004; Dutta et al. 2005). Many lectins were already purified and characterized, and there are an increasing number of studies related to the genes involved in innate immunity, but, as far as the authors know, there is no literature about fish lectins from North and Northeast region of Brazil.

In tambaqui, it was found the presence of 4 types of lectins (GAL, MBL, FucoAA and Fuco), and all of them presented a different expression profile in all tissues, except the galectin which was constitutively expressed in all tissues, From the analyzed tissues it was observed the expression of all lectins in tambaqui skin. Since the water is a perfect medium for proliferation of bacteria and parasite microbes, the skin is constantly exposed to pathogen attacks. Moreover, there are defense mechanisms against pathogen invasion on the surface of fish body and the skin works like a mechanical and biochemical barrier (Suzuki et al. 2003; Russell and Lumsden 2005; Tsutsui et al. 2006). Until the present moment, no endemic disease was registered for raised or wild tambaqui, which has contributed for its reputation of being a robust species (Lima and Goulding 1998). Possibly, such fact could be explained by the presence of a reasonable number of lectins in the skin (all tested lectins) and in all testes internal tissues of tambaqui (GAL, fucoAA and fuco).

The sequencing reaction for Sp1/600, Sp1/900, Pc1/600 and Pc1/900 did not work. Therefore, we have decided to perform a PCR with these samples using specific primers for galectin. In the sequencing for Sp1 and Pc1, it was demonstrated that the transcripts of GAL belongs to the galectin subgroup Tandem Repeat (galectin group 9). In the alignment with homologue proteins of the fishes *Danio rerio* and *Oncorhynchus mykiss,* it was observed the possibility to found in tambaqui the

first domain with presence of some amino acids involved with carbohydrate binding. The two aligned domains of respective fishes are connected by a linker peptide. This linker peptide is smaller in *Danio rerio* causing a greater approximation of the two domains and, consequently, this lectin binds more to monosaccharides (galactose, N-acetyl galactosamine) than polysaccharides (Ahmed et al. 2004). The presence of galectin was already confirmed in tambaqui serum and this protein has its lectinic activity strongly inhibited by the monosaccharides galactose and fucose, and its activity was weakly inhibited by glycoprotein.

The study of these lectins can provide a better comprehension of the immune system of the fish allowing the development of applications for disease prevention. The investigation of fish lectins can also serve as a base for genetic improvement aiming to increase fish resistance for economically important diseases (Kubitza and Kubitza 2004).

The tambaqui lectin is a new protein and additional experiments of quantitative PCR are necessary to exactly evaluate the expression of these lectins in tambaqui. The cloning of complete genes, containing the regions 3`-and 5`-UTR, in which regulatory sequences can be found, are currently in progress and a more complete panorama of the lectins of tambaqui will be obtained soon, improving the comprehension of the role of these proteins in its defense system.

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CAPÍTULO IV

PARTIAL CLONING AND ANALYSIS OF THE EXPRESSION PROFILE OF GENES OF THE LECTINS FROM TAMBAQUI (*colossoma macropomum*) DURING BACTERIAL INFECTION.

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PARTIAL CLONING AND ANALYSIS OF THE EXPRESSION PROFILE OF GENES OF THE LECTINS FROM TAMBAQUI (*COLOSSOMA MACROPOMUM*) DURING BACTERIAL INFECTION

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ABSTRACT

Lectins are proteins which recognize and bind specifically to carbohydrates. The discovery of lectins of fish added a new dimension in the study of the biology of lectins and in fish immunology. In the last decades, tambaqui has became one of the main native species for the Brazilian inland fishculture, with a good growth patern and productivity, fact that makes abundant its offers in the consumer markets. Tambaqui is a fish known by its strength. The objective of this paper is to investigate the role of the lectins in the fish defense against bacterial infections by *Pseudomonas spp*. and *Aeromonas spp*. We have analyzed some groups of lectins, in liver, kidney and spleen of tambaqui, by using reverse transcriptase coupled to the polymerase chain reaction (*reverse-transcription-polymerase chain reaction*, RT-PCR). The genes of lectins which recognize galactose (galectin) and C-type lectin are constitutively expressed in all of the three organs of tambaqui, while the gene of lectin which recognizes mannose (*Mannose-Binding Lectin*-MBL) was expressed in major quantity in kidney and more tenuously in spleen. The search for homology showed that the MBL has approximately 65% of homology with the MBL of the fish *Danio rerio.*

Keywords: *Colossoma macropomum, Tambaqui lectin; RT-PCR; fish galactolectin;* fish mannose-binding lectin; fish C-type-lectin

1 INTRODUCTION

Lectins are defined as proteins that bind, specifically and reversibly, to mono, oligo or polysaccharides without alterations in its structure (GOLDSTEIN and PORETZ, 1986; VAN DAMME et al., 1996; MACEDO et al., 2007; SITOHY et al., 2007). These proteins have the ability of binding themselves to residues of carbohydrates, through carbohydrates binding site(s) which can be conjugated to lipids or proteins (VORNHOLT et al., 2007). Initially, the study of lectins was focused just in plants, and only in the two last decades became evident that lectins are also distributed in the animal kingdom (PROBSTMEIER and PESHEVA, 1999).

Many proteins act in the innate immunoresponse of fish (EWART et al, 2001). Some have been well characterized, including the antimicrobial proteins such as lisozime (HANCOCK and LEHRER, 1998). Recently, medical and veterinary researches discovered that lectins are involved in the innate immunity of animals (EWART et al., 1999; FOCK et al, 2001; DUTTA et al., 2005; RUSSELL and LUMSDEN, 2005). The physiological role of lectins is not clearly defined, but recent studies suggest that lectins are defense proteins which can protect against viruses, fungi and bacteria (TSUTSUI et al. 2006).

Tambaqui (*Colossoma macropomum*) is a species of Amazon fish that have been attracting the attention of a relatively great number of researchers, such as fishing engineers, biologists, physiologists and aquaculturists (LIMA and GOULDING, 1998). This fact must occur mainly because of its importance in fishing and the recognition that it would have a big potential for aquaculture. According to Lima and Goulding (1998), tambaqui has become the symbol fish of Amazon because it incorporates, in one species, most of the problems that need to be solved for managing fishing and, at the same time, to develop aquaculture. The present work objectives to investigate the expression profile of lectins in tambaqui during bacterial infection.

2 MATERIALS AND METHODS

2.1 Fish Treatment

Suspensions of *Aeromonas spp*. (group A), *Pseudomonas spp*. (group B) and saline (Control group) were injected intraperitoneally in tambaqui fishes (approximately 300 g). Fishes were kept in current water in temperatures variating from 22 ^{0}C to 24 ^{0}C (mean 23 ^{0}C). After 15 days, fishes were anaesthetized in ice and the liver, kidney and spleen were excised surgically following its anatomic localization (KUBITZA and KUBITZA, 2004). Finally, organs were stored immediately in liquid nitrogen until the moment of total RNA isolation.

2.2 Total RNA isolation from tissues and Purification of messenger RNA

The total RNA of isolated tissues was extracted from 3 tambaqui fishes using trizol reagent (invitrogen, Carlsbad, USA) according to the manufacturer norms. The concentration and the quality of the total isolated RNA were determined by measuring the absorbances at 260-280nm. The integrity of the RNA samples was visually examined through electrophoresis in 1% agarose gel.

The poly(A)-rich RNA, was isolated from total RNA using a purification kit of mRNA (PolyATract® mRNA isolation system, Promega, Madison-WI, USA) by capturing mRNA hybrids and biotinylated oligo(dT) probe with Magnesphere® (Promega, Madison-WI, USA), a complex of streptavidine coupled to paramagnetic particles. The mRNA from fish tissues was subsequently used for the amplification of lectin genes of fish.

2.4 RT-PCR semi-quantitativo

The cDNA was synthesized from mRNA using the adapter 3'Oligo(dT)$_{18}$, called QT (5'-CAGTGAGCAGAGTGACGAGGACTGGAGCTC AAGC (T_{18})-3' and reverse transcriptase of Moloney Virus of Murin Leukemia, M-MLV-RT (Promega). The reaction was performed at 37 ^{0}C during 60 min and inactivated at 70 ^{0}C for 15 min. Finally, the cDNA was diluted 10 times in ultra pure water to be used in further assays.

2.5 Oligonucleotides (primers) and PCR

Degenerated primers (Table 1) for 5 types of lectins were synthesized according to homologue region of conserved nucleotide sequences of genes from another species of fish, (annex II). The amplification of cDNA was carried out at 94 ^{0}C during 3 min for denaturation following 30 cycles at 94 ^{0}C for 50 s, 48 ^{0}C for 50 s and 72 ^{0}C for 2 min.

Table 1: List of primers.

Lectins	*Primers*
Galectin (GAL)	5- TCA CTTYAA CCC ACG CTATGA -3 (forward) 5- AGGCRATAGAKTTCACYTCCA C -3 (reverse)
C-type lectin (CTL)	5- ACTGTTTGGCACTTG GTGGA -3 (forward) 5- GCTCACCAGTGTTCCAGTT -3 (reverse)
Mannose binding lectin (MBL)	5- MKSCCTGGCAGAGATGGAAGA -3 (forward) 5- GTARTATTTCTGTCCCACTTTCCT -3 (reverse)

2.6 cDNA Cloning

Each PCR product, purified from agarose gel using the Wizard SV Gel kit and PCR Clean-Up System (Promega, Madison, USA) was ligated into the pGEM-T Easy vector (Promega). The transformation was performed in *Escherichia coli* (JM109, Promega) using the calcium chloride method. Quickly, cells were concentrated by centrifugation and resuspended in a solution containing calcium chloride. Competent cells (*E. coli*) were mixed with plasmidial DNA and after that, submitted to a heat shock. The cells grew up in non-selective medium and plated in a medium containing ampiciline. The purification of plasmids was performed using a Wizard plasmid purification system kit (Promega, Madison-WI, USA) according to manufacturer norms.

2.7 cDNA sequentiation and analysis of sequences

The positive clones were sequentied using the flankers primers of the M13 insert forward and reverse, synthesized by IDT (Integrated DNA Technologies, Coralville, USA). The sequence of nucleotide was carried through the chain termination method (Dya terminator chemistry) using a kit (DYEnamic ET Dye

Terminator kit), sequentied on MegaBACE 750 DNA Analysis System (GE Healthcare, Piscataway, USA).

The database search for related nucleotides were conducted using the software NCBI (ALTSCHUL et al., 1997) and for sequences alignment, we used the Bioedit software (HALL, 1997 - version 5.0.6).

3 RESULTS

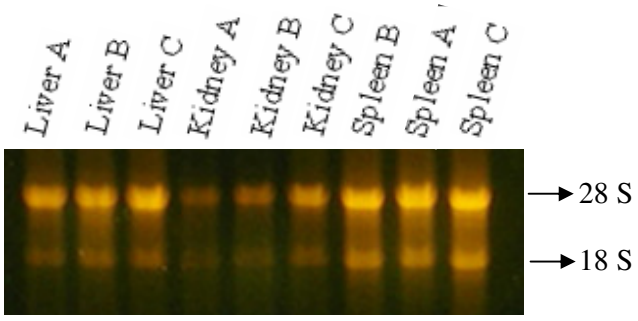
3.1 Fish treatment

In 4 days, there was no aparent signal of disease in fishes, except the excessive production of mucus. On the fifth day, we have applied a second dosage of bacteria in each fish. The control was also submitted for a second serum aplication. On the tenth day, tambaqui fishes started to present some slight signals of infection: blackout on the skin, excessive production of mucus, breath difficulty, loss of appetite and erratic swimming. On the fifteenth day, the skin of fishes were with furunculosis, for infected the fishes with *Pseudomonas spp.* and white dots spreading on the skin, for the fishes infected with *Aeromonas spp.* At the same day, all fishes were sacrificed. Internally, all the organs (liver, stomach, spleen, intestine, gallbladder, heart and gills) were apparently normal, except the kidneys of all the infected fishes, which were swollen.

3.2 RT-PCR

In the total RNA isoleted were found intact (figure 1A). The reverse transcription coupled to the polimerase chain reaction (RT-PCR), using a protocol with QT adapter, allowed the synthesis of intact cDNA molecules (figure 1B). The quantity of the kidney tissue of each group was corrected for the synthesis of cDNA.

A



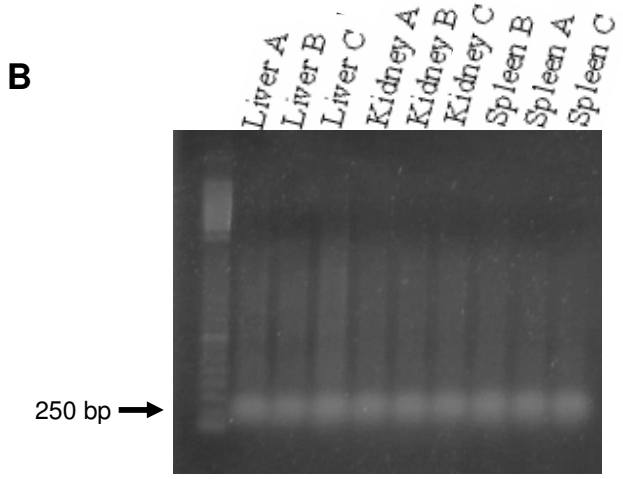
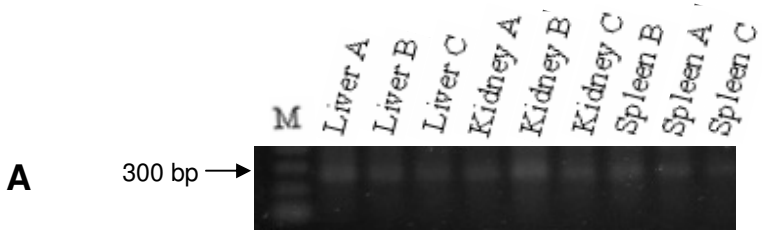


Figura 1: Isolation of total RNA (A) and cDNA (B) from the tambaqui tissues of liver, kidney and spleen. Marker (M); Groups: *Aeromonas spp.* (A), *Pseudomonas spp.* (B) and control group (C).

3.3 PCR

The amplification reactions of cDNA with specific primers showed different expressions in each tissue. For GAL (figure 2A), it was observed the presence of an amplicon of approximately 300 of base pairs (bp) in all of the treated tissues (groups A, B and C). For CTL (figure 2B), it was observed the presence of an amplicon of approximately 300 of bp in all the treated tissues (groups A, B and C) and for both lectins it was detected any expression difference between the different tissues and treatments. However, for MBL (figure 2C), it was detected the presence of an amplicon of about 300 bp in the kidney (A, B and C) and, tenuously, in spleen, and another amplicon of 400 bp in the kidneys.



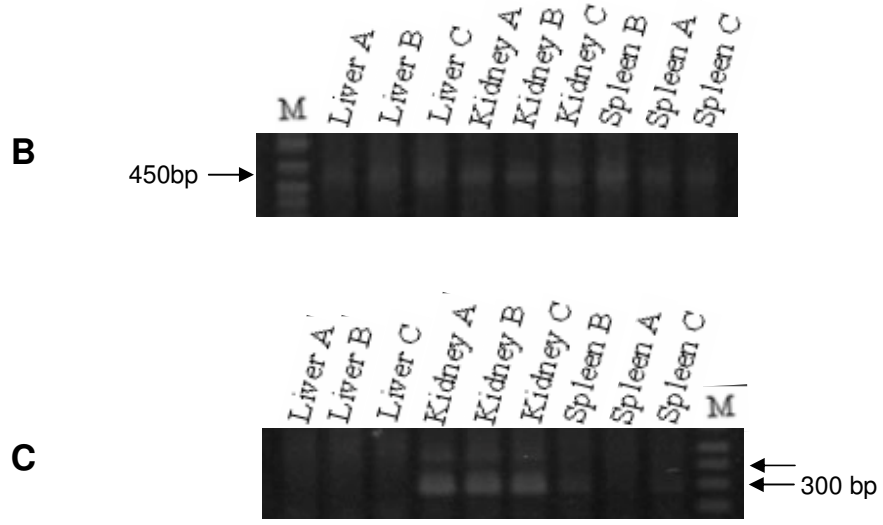


Figura 2: PCR referent to the expression of the lectins genes in different tissues of tambaqui. GAL (A), CTL (B) and MBL (C). Groups: *Aeromonas spp.* (A), *Pseudomonas spp.* (B) and control group (C). Marker (M)

3.4 Analysis of nucleotidic sequences

The cloning of PCR products (GAL, CTL and MBL) selected and purified from agarose gel produced bacterial recombinant clones. The plasmids of the clones selected by antibiotic and *PCR* colony using flanker primers of the insert forward and reverse were purified using a purification system of plasmid and submitted to sequencing.

The sequence of the found recombinant clones was analyzed on the NCBI database (GenBank). The result of the nucleotide sequences for tambaqui MBL (dates don't showed) showed low similarity (<66%) with *Danio rerio* (gi|68356567), (<63%) with *Danio rerio* (gi|125806397), (<56%) with *Carassius auratus* (gi|7542473), (<47%) with *Cyprinus carpio* (gi|38603529) and (<47%) with *Cyprinus carpio* (gi|38603527). When the search was made with aminoacid sequences deduced from lectins genes of tambaqui, the values of these similarities increased to: 76% with *Danio rerio*, 73% with *Carassius auratus* and 64% with *Cyprinus carpio* (figure 3). The sequencing reaction for GAL and CTL didn't work.

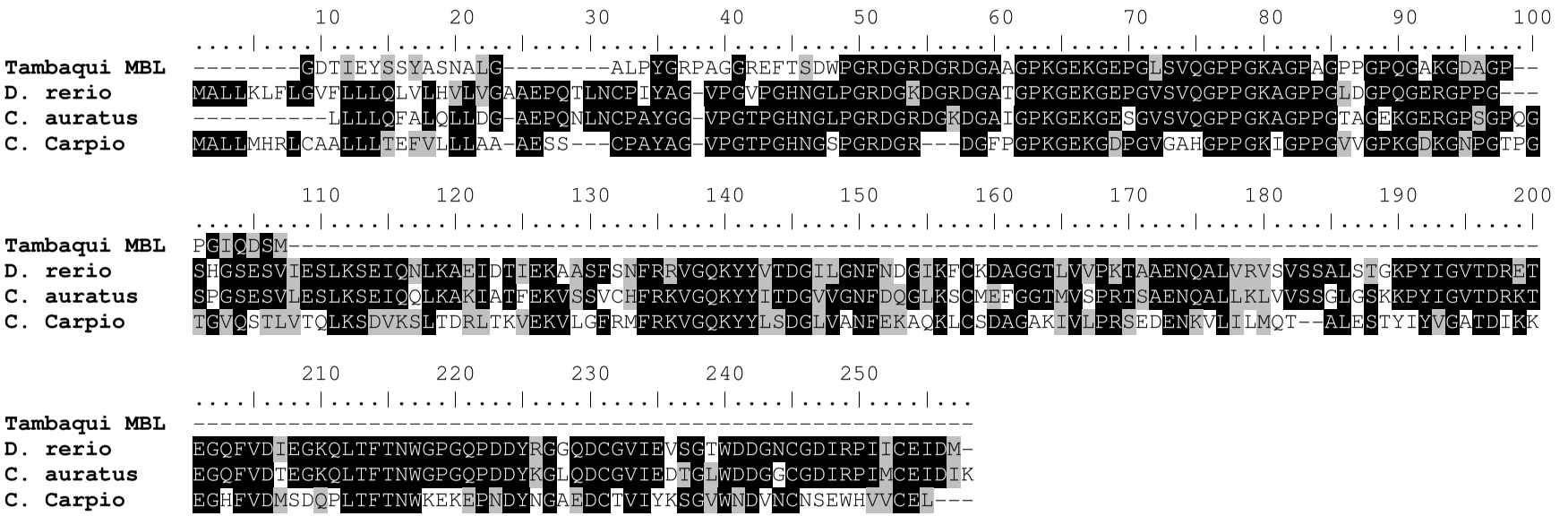


Figure 3 : Amino acid sequence comparison of the tambaqui MBL with other MBL from fishes. Conserved residues and identical residues are indicated with grey and black respectively.

4 DISCUSSION

Lectins have a significant role in the immune response of the host in most animals. They bind specifically to carbohydrate molecules present on the cell surface of the pathogens, acting as opsonins estimulating their destruction by macrophages (FOCK et al., 2001; RICHARDS et al., 2003; DUTTA et al., 2005). The recognition of bacteria is a property reported by for many fish lectins (TSUTSUI et al. 2006). A galectin activity which recognizes bacteria was identified in salmon eggs (OTTINGER, et al., 1999). In the work of EWART et al. (1999), it was detected a salmon serum lectin that binds to *Aeromonas salmonicida* and *Vibrio anguillarum*.

Tambaqui is a teleost fish extremely robust and, as far as authors know, it wasn't registered any endemic disease to raised or wild tambaqui. In experiments with infected tambaquis, at least two injections, with five days interval, were necessary to observe the typical signs of a*eromonas* and *pseudomonas spp* disease and only in the eleventh day fishes started to show the first slight signs of infection. It is worth mentioning that, in experiments with infected salmons to identify genes that are differentially regulated in liver, spleen and kidney, with *Aeromonas salmonicida,* the characteristic signs of the disease appeared in seven days of infection and fishes were sacrificed in the thirteenth day (TSOI et al., 2004).

All the experimental group of tambaqui was sacrificed and the RNA was obtained from different tissues (liver, kidney and spleen). The analysis of the profile expression for three main lectin genes of fish (CTL, MBL, GAL) was performed using the protocol of reverse transcription coupled to PCR. Surprisingly, there was no difference between CTL and GAL expression, in all tambaui tissues in both groups the control group and the treated group. On the other hand, was verified that only MBL is expressed exclusively in kidney and, tenuously, in spleen of infected and control tambaqui. The cDNA segment of the sequenced MBL shows that this lectin presents about 76% of similarity with MBL of *Danio rerio* (zebrafish), 73% with *Carassius auratus* (goldfish) and 64 % with *Cyprinus carpio* (carp). This similarity is very significant, because it refers to the CRD region. It is worth noting that, despite the CRD is much conserved among the MBL of these fishes, it refers to genes that evolved independently, since the sequences of nucleotides of MBL from *Danio rerio*, *Carassius auratus*, *Cyprinus carpio* are divergents. This fact indicates that the mutations were silent and synonyms, that is, even if the codons are different they

codify the same amino acids. Consequently, there is less similarity among the sequences of nucleotide than among the amino acids. Thus, in the present work, new lectin genes of fish are being described for tambaqui (GAL and MBL).

In case of the galectin, additional experiments need to be done in relation to its antimicrobial activity, since its sequence show high similarity with galectin tandem repeat group (galectin 9), which is known to participate in embryogenesis and, therefore, in the recognition among cells in tissue organization. Whether it is involved on the innate immune system, as thought for its constitutive expression, further confirmation is necessary. It is known that MBL has a crucial role on the innate defense of fishes, activating the complement system (KONSTANTINA et al., 2006). Due to its CRD, they bind to mannose residues on the pathogens surface (DRICKAMER, 1992). In teleosts, the MBL gene was identified in: common salmon (*Salmo salar*), carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), and in Japanese eel (*Anguilla japonica*).

As previously mentioned CTL was expressed in all tissues of both, treated and control fishes. This fact is not observed in salmon (TSOI et al., 2004). For this fish the expression occurred in the liver of both infected and control groups, on the other hand, the expression did not occur on the other tissues (kidney and spleen) for the control group. The GAL expression also occurred in all tambaqui tissues. In no other fish in found in literature, this lectin expression was observed in all three tissues.

The expression profile of these three important lectins can justify the fact of the tambaqui to be such a robust fish. The constitutive expression observed in all tissues for these families of lectins (GAL and CTL) may reflect the role of these proteins in the defense system of the tambaqui against high microbial loads present in tropical waters.

Teleost fishes present unique characteristics, such as temperature dependence of the acquired immunity, as well as certains limitations in antibodies diversity. Therefore, it is believed that the defense functions which depend on innate immune system could have a more important role in these animals than in other homoeothermic vertebrate species, such as birds and mammals (TSOI et al., 2004).

The study of galectin, MBL, C-type lectin and other lectins present in tambaqui, can be useful for a better comprehension about the functioning of the tambaqui immune system and other tropical fishes, possibly facilitating the

development of techniques for the prevention of diseases, in a way that could help its managing by pisciculturists.

As far as the authors know, this is the first report of molecular cloning of the tambaqui lectin genes. Additional experiments of quantitative PCR are in progress to evaluate exactly the expression of these and other lectins in tambaqui. In addition, the complete genes cloning, containing the regions 3`-and 5`-UTR (untranslated region), in which regulatory sequences can be found, is being done. Therefore, it is believed that a more complete panorama about lectins of the tambaqui will amplify the comprehension about the role of these proteins in the defense system of the tambaqui and other tropical fishes as well.

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CONCLUSÕES

⇨ A lectina do soro de tambaqui (*Colossoma macropomum*) é uma proteína com características básicas que reconhece galactose e fucose;

⇨ Nos tecidos avaliados do tambaqui (coração, fígado, rim, baço, pele, cérebro, ceco, brânquias) ocorreu diferente expressão do gene das lectinas (GAL, MBL, FUCO AA, FUCO);

⇨ A pele foi o único tecido em que foi detectada a expressão de todas as lectinas testadas (GAL, MBL, FUCO AA, FUCO);

⇨ O tambaqui infectado com bactérias foi resistente por muitos dias;

⇨ Galectina e lectina tipo-C foram expressadas constitutivamente nos diferentes tecidos, o que justifica a robustez do tambaqui;

⇨ O seguimento de cDNA da galectina do tambaqui pertence ao grupo de galectinas do subtipo *Tandem Repeat;*

⇨ O seguimento sequenciado de cDNA da MBL mostra que a lectina foi similar à MBL de *Danio rerio* (76%, zebrafish), de *Carassius auratus* (73%, goldfish) e de *Cyprinus carpio* (64%, carpa);

⇨ Em peixes infectados a MBL foi expressa com intensidade nos rins mostrando sua importância na ativação do sistema complemento.

ANEXO I

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ANEXO II

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LECTINA	PEIXE	REFERÊNCIA
Galectina	*Danio rerio*	gi|68393907
	Danio rerio	gi|49227306
	Oncorhynchus mykiss	gi|6681409
	Oncorhynchus mykiss	gi|20270910
	Oreochromis mossambicus	gi|115280042
	Zebrafish/*Danio rerio*	gi|28316273
C-type	*Oncorhynchus mykiss*	gi|20385162
	Oncorhynchus mykiss	gi|11095790
	Oncorhynchus mykiss	gi|11095794
	Oncorhynchus mykiss	gi|68697200
MBL	*Carassius auratus*	gi|7542473
	Cyprinus carpio	gi|38603527
	Cyprinus carpio	gi|38603529
	Cyprinus carpio	gi|7542469
	Cyprinus carpio	gi|38603531
	Danio rerio	gi|7542471
	Danio rerio	gi|18858996
FucolectinaAA	*Anguilla japonica*	gi|9651020
	Anguilla japonica	gi|9651022
	Anguilla japonica	gi|9651024
	Anguilla japonica	gi|9651026
	Anguilla japonica	gi|9651028
Fucolectina	*Danio rerio*	gi|78191595
	Danio rerio	gi|78191597
	Danio rerio	gi|78191599
	Danio rerio	gi|38488748
	Fundulus heteroclitus	gi|52001210
	Morone saxatilis	gi|78191587
	Morone chrysops	gi|78191593
	Oncorhynchus mykiss	gi|78191585

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