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RÔMULO FARIAS CARNEIRO

PURIFICAÇÃO E CARACTERIZAÇÃO BIOQUÍMICA DE LECTINAS LIGANTES DE GALACTOSE ISOLADAS DE INVERTEBRADOS MARINHOS

FORTALEZA

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Tese apresentada ao curso de doutorado em Biotecnologia de Recursos Naturais do Departamento de Engenharia de Pesca da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Doutor em Biotecnologia de Recursos Naturais. Área de concentração: Purificação e caracterização de biomoléculas.

Orientador: Prof. Alexandre Holanda Sampaio

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“Nenhuma história tem começo e nenhuma história tem fim. Começos e fins são, em sua natureza, arbitrários e existem apenas como uma ideia conveniente na mente humana” (Caitlín R. Kiernan).

RESUMO

Lectinas animais podem ser definidas como proteínas ou glicoproteínas que reconhecem carboidratos de maneira específica, mas não participam do metabolismo dos mesmos e não pertencem a qualquer uma das principais classes de imunoglobulinas. As lectinas têm sido estudadas por mais de um século devido sua capacidade de decifrar o glicocódigo. Com exceção dos grupos Crustacea (Arthropoda) e Bivalvia (Mollusca), os invertebrados marinhos têm sido pouco estudados quanto à distribuição e presença de lectinas. Este trabalho objetivou isolar lectinas de grupos de invertebrados pouco estudados, caracterizar bioquimicamente as lectinas e avaliar seu efeito sobre a formação de biofilmes bacterianos. Lectinas foram isoladas da esponja *Aplysina lactuca* (ALL), de ovos do gastrópode *Aplysia dactylomela* (ADEL) e do ouriço do mar *Echinometra lucunter* (ELEL). ALL é um dímero formado por duas subunidades idênticas de 16 kDa unidas por pontes dissulfeto com afinidade por lactulose, lactose e mucinas. Sua estrutura primária é semelhante à da lectina da esponja *Axinella polypoides* e sua estrutura secundária é formada principalmente de folhas β. ADEL é uma glicoproteína do tipo *high mannose* composta por duas subunidades idênticas de 27 kDa unidas por pontes dissulfeto. A estrutura primária apresentou similaridade com outras lectinas de ovos de aplisídeos. A estrutura secundária de ADEL é rica em folhas β e sensível a presença de carboidratos. ADEL apresentou afinidade para diversos galactosídeos, em especial para ácido galacturônico (Ka = $1,5 \times 10^7$ M^{-1}). O modelo tridimensional de ADEL revela um novo domínio de reconhecimento a carboidratos. ELEL é uma lectina ligante de ramnose formada por duas subunidades de 11 kDa unidas por pontes dissulfeto, exibindo afinidade por carboidratos contendo a mesma orientação nas hidroxilas de C-2 e C-4 que a galactose. Sua estrutura primária foi semelhante a outras lectinas encontradas em ovos de ouriço e peixes. O modelo de estrutura tridimensional de ELEL sugere a predominância de folhas β em um domínio do tipo sanduíche β. Cálculos *ab initio* sugerem a interação de ELEL com um receptor comum em células tumorais, o Gb3. Ademais, as três lectinas apresentaram capacidade de aglutinar bactérias e/ou efeito inibitório do crescimento de biofilmes bacterianos. Em conclusão, este trabalho revela que invertebrados marinhos representam excelente fonte de novas lectinas ligantes de galactose e com efeito antibiofilme, sendo assim estas novas lectinas podem ser ferramentas biotecnológicas úteis em um futuro próximo.

Palavras-chave: lectina, biofilme, invertebrados marinhos, galactose.

ABSTRACT

Lectins, which are found in all living organisms, are sugar-binding proteins that differ from immunoglobulins and glycoenzymes because they are not produced as an adaptive immune response, and they contain no site for the enzymatic modification of their ligands. Lectins have been studied for more than a century because of their ability to decipher glycocode. Excepting Crustacea (Arthropoda) and Bivalvia (Mollusca), marine invertebrates have been poorly studied compared to the distribution and presence of lectins. This work aimed to isolate lectins from groups of invertebrates with poorly studied, biochemically characterize such lectins and to evaluate its effect on the formation of bacterial biofilms. Lectins were isolated from the sponge *Aplysina lactuca* (ALL), the eggs gastropod *Aplysia dactylomela* (ADEL) and the sea urchin *Echinometra lucunter* (ELEL). ALL is a dimer formed by two identical 16 kDa subunits linked by disulfide bonds with affinity for lactulose, lactose and mucins. Its primary structure is similar to the *Axinella polypoides* lectin and its secondary structure is formed mainly by β-sheet. ADEL is a high mannose glycoprotein formed by two identical 27 kDa subunits linked by disulfide bonds. Its primary structure showed similarity with other lectins obtained from aplysid eggs. The secondary structure of ADEL is rich in β-sheet and sensitive to the presence of carbohydrates. ADEL showed affinity for several galactosides, especially for galacturonic acid (Ka = 1,5 x 10^7 M^{-1}). The three-dimensional ADEL model reveals a new carbohydrate recognition domain. ELEL is a rhamnose binding lectin formed by two 11 kDa subunits linked by disulfide bonds. ELEL showed affinity for carbohydrates containing the same orientation in the hydroxyls of C-2 and C-4 as galactose. Its primary structure was similar to lectins found in eggs of sea urchins and fish. The three-dimensional ELEL structure model suggests the predominance of β-sheets in a β-sandwich domain. Ab initio calculations suggest the interaction of ELEL with a common receptor in tumor cells, Gb3. In addition, the three lectins showed ability to agglutinate bacteria and / or inhibitory effect of the growth of bacterial biofilms. In conclusion, this work reveals that invertebrates represent an excellent source of new galactose binding lectins and presenting inhibitory effect in the bacterial growth and/or antibiofilm, so these new lectins may be useful as biotechnological tools in the near future.

Keywords: lectin, biofilm, marine invertebrates, galactose.

SUMÁRIO

1 FUNDAMENTAÇÃO TEÓRICA

1.1 Lectinas

Os carboidratos são as biomoléculas orgânicas mais abundantes da natureza. Todos os organismos sintetizam e metabolizam algum tipo de carboidrato. Oligossacarídeos ligados a proteínas ou lipídios são componentes característicos em todas as superfícies celulares (glicocálice celular). Combinações de hexoses naturais com suas possíveis ligações e pontos de ramificação produzem uma diversidade de complexos arranjos oligoméricos, os quais incrementam amplamente a concisa informação genômica dos organismos (GHAZARIAN;IDONI; OPPENHEIMER, 2011).

Além das funções estruturais e energéticas as quais, classicamente, são atribuídas a essas moléculas, os carboidratos detêm importantes informações biológicas. Os glicoconjugados agem como portadores de informações: a porção sacarídica funciona como rótulo de endereçamento em algumas proteínas, agindo como mediador de processos multicelulares, nas interações específicas entre célula-célula e célula-matriz extracelular, na resposta imunológica, na coagulação sanguínea e na cicatrização de lesões (GHAZARIAN;IDONI; OPPENHEIMER, 2011; SHARON, 2007).

Recentemente, os carboidratos têm recebido bastante atenção, e pesquisas têm sido realizadas a fim de elucidar as complexas estruturas e papéis dessas moléculas. Sabe-se, por exemplo, que anomalias no padrão de glicosilação frequentemente acompanham condições fisiológicas anormais ou patológicas, como o câncer. Dessa forma, moléculas que reconheçam essas mudanças poderão fornecer uma nova estratégia em terapias de combate ao câncer (GHAZARIAN; IDONI; OPPENHEIMER, 2011).

A porção oligossacarídica dos glicoconjugados é muito heterogênea e rica em informações, formando locais extremamente específicos para o reconhecimento e a ligação de alta afinidade por proteínas ligantes de carboidratos como as lectinas. A existência dessas moléculas capazes de reconhecer carboidratos sem modificá-los, sugere que a interação proteína-carboidrato seja a forma de decodificar a informação contida nos glicoconjugados (SHARON, 2005; SHARON, 2007).

As lectinas têm sido estudadas por mais de um século devido sua capacidade de apresentar efeitos biológicos de alto interesse para campos de aplicação bastante distintos, desde a agricultura até a biomedicina (SHARON, 2007).

Lectinas podem ser definidas como proteínas ou glicoproteínas que reconhecem carboidratos de maneira específica, mas não modificam estruturalmente os mesmos e não pertencem a qualquer uma das principais classes de imunoglobulinas (KILPATRICK, 2000).

Como carboidratos são abundantes na superfície de praticamente todas as células, as lectinas podem mediar interações célula-célula e célula-matriz extracelular. Hoje sabe-se que as interações proteína-carboidrato exercem papel importantíssimo em vários processos biológicos, tais como ativação celular, regulação do crescimento, metástase e apoptose (VASTA; AHMED, 2008).

Assim, a identificação de lectinas e seus ligantes, bem como o entendimento dos mecanismos moleculares e efeitos dessas interações proteína-carboidrato são objetos de interesse científico.

As lectinas são proteínas ubíquas na natureza, já sendo relatado seu isolamento, suas respectivas atividades biológicas e sua inclusão em rotas metabólicas em organismos diversos, incluindo fungos, bactérias, algas, plantas e animais (SHARON, 2007).

1.2 Lectinas Animais

Desde as descobertas das primeiras lectinas animais, na glândula albumínica do caracol *Helix pomatia* (CAMUS, 1899), nos cristais proteicos de Charcot-Leyden, atualmente galectina-10 (CHARCOT; ROBIN, 1853; LEYDEN, 1892; SWAMINATHAN, 1999), no veneno de cobras (FLEXNER; NOGUCHI, 1902; NOGUCHI, 1903), todos identificados no fim do século XIX, e das aglutininas do caranguejo ferradura (CATACUZENE, 1912) identificadas no início do século XX, notadamente o marco mais importante da história das lectinas animais foi a descoberta da natureza glicídica do grupo sanguíneo H por Walter J.T. Morgan e Winifred M. Watkins utilizando lectinas de enguia (WATIKINS; MORGAN, 1952).

Esta descoberta iniciou a transição das abordagens sorológicas para um período de intensa pesquisa bioquímica envolvendo lectinas nas décadas de 1960 e 1970. Durante este período, foram identificadas e bioquimicamente caracterizadas várias lectinas animais isoladas de invertebrados e vertebrados, incluindo mamíferos (KILPATRICK; GREEN, 1992).

Contudo, os estudos a respeito da presença e distribuição de lectinas em animais se concentram em poucos filos. Em particular, o número de lectinas isoladas de invertebrados marinhos é pequeno frente à diversidade de lectinas purificadas de plantas. Além disso, a fisiologia das lectinas animais não está completamente clara, embora haja evidências de que elas estejam envolvidas em processos diversos, tais como: interação célula-célula e célula-

matriz extracelular, simbiose e defesa contra microrganismos, sinalização celular e transporte de glicoproteínas e carboidratos (VASTA; AHMED, 2008).

As lectinas animais têm sido encontradas no citoplasma, no núcleo e associadas a membranas das mais diversas organelas e nos mais variados tipos celulares. Na superfície celular as lectinas podem atuar como receptores agindo na seletividade, adesão e migração celular (LEHMAN *et al*., 1990; ROSEN, 1993), bem como no reconhecimento de glicoproteínas circulantes (ASHWELL; HAVORD, 1982). As lectinas animais parecem também atuar como receptores para as glicoproteínas da matriz extracelular elastina e laminina (COOPER *et al*., 1994; HINEK *et al*., 1998), como receptores para glicosaminoglicanos que medeiam a ligação de proteoglicanos a carboidratos de outras glicoproteínas (DOEDGE *et al*., 1987), e, como primeiro passo na adesão de células imunes (ROSEN, 1993). Proteínas com afinidade por açúcares também funcionam como primeira linha de defesa contra infecções patogênicas (CASH *et al*., 2006).

Atualmente as lectinas animais podem ser classificadas em famílias distintas com base em suas características físicoquímicas, função e especialmente em sua identidade de sequência (KILPATRICK, 2000; VASTA; AHMED, 2008). A classificação recente indica que a maioria das lectinas animais pode ser reunida em um dos cinco principais grupos: (1) lectinas contendo o domínio de reconhecimento a carboidratos (DRC) do tipo C (tipo C), (2) galectinas (tipo S), (3) receptores de manose-6-fosfato (tipo P), (4) siglecs (sialic acid-binding-type lectins) e outras lectinas do tipo imunoglobulina ligantes de ácido siálico (tipo I) e (5) lectinas animais relacionadas as lectinas de leguminosas vegetais (tipo L) (VASTA; AHMED, 2008).

1.3 Lectinas de invertebrados marinhos

O termo invertebrado é usado para designar animais multicelulares que não possuem ou não desenvolvem uma coluna vertebral derivada do notocórdio. Os invertebrados representam 97% de todas as espécies animais conhecidas, e apenas o subfilo Vertebrata não possui representantes invertebrados. O grupo dos invertebrados não tem validade taxonômica, pois se trata na verdade de uma divisão polifilética, isto é, que não possui um ancestral comum. Interessantemente, esta condição polifilética faz com que alguns táxons de invertebrados estejam filogeneticamente mais relacionados aos vertebrados que aos demais invertebrados, é o caso dos grupos Chaetognatha, Hemicordata e Tunicata, por exemplo (HICKMAN; ROBERTSON; LARSON, 2004).

Os invertebrados estão distribuídos em todos os ambientes do planeta e são mais abundantes (em número de espécies) nos oceanos. Os filos de invertebrados marinhos mais conhecidos são: Porifera (esponjas), Cnidaria (corais e medusas), Mollusca (lulas, polvos, caracóis, ostras, mexilhões etc.), Arthropoda (insetos, crustáceos e aracnídeos), Equinodermata (ouriços, pepinos-do-mar e estrelas-do-mar) e Tunicata (ascídias) (HICKMAN; ROBERTSON; LARSON, 2004).

Em se tratando de lectinas, os invertebrados marinhos têm sido pouco estudados, com exceção dos grupos Crustacea (Arthropoda) e Bivalvia (Mollusca) que, devido ao elevado interesse econômico (estes animais são cultivados e consumidos em todo o mundo) são alvos de inúmeras pesquisas.

De uma maneira geral, as lectinas em invertebrados estão envolvidas na defesa contra patógenos, sendo parte importante do sistema imune inato destes animais. Desde que invertebrados não dispõem de um sistema imune adaptativo, isto é, eles não são capazes de produzir anticorpos, as lectinas representam a primeira linha de defesa contra infecção microbiana nos fluidos, células e tecidos de invertebrados (VASTA; AHMED; ODOM, 2004).

Não obstante, algumas lectinas de invertebrados marinhos parecem estar envolvidas em outros papéis fisiológicos, tais como captura de alimento, adesão celular e reprodução (OZEKI *et al*., 1991; PALES-ESPINOSA *et al*., 2009; WAGNER-HÜLSMANN *et al*., 2006).

1.4 Lectinas de esponjas

O filo Porifera é representado pelas esponjas, animais que possuem o corpo repleto de poros e canais que constituem um sistema de filtragem/alimentação adequado para o seu hábito de vida. As esponjas são sésseis e dependem de correntes de água passando pelo seu sistema de canais para trazer alimento e oxigênio (HICKMAN *et al*., 2004).

Estes organismos são considerados os animais metazoários mais antigos que existem e constituem um grupo de grande sucesso evolutivo que inclui vários milhares de espécies e uma grande variedade de formas e marinhas e de água doce (Figura 1).

A presença de lectinas em esponjas foi inicialmente descrita por Dodd *et al*., (1966) nas espécies *Cliona celata* e *Axinella* sp. As lectinas de ambas as espécies, embora capazes de aglutinar hemácias de maneira diferenciada, foram ambas inibidas por galactose.

Figura 1. Diversidade de formas de poríferos. A. *Haliclona caerulea* na praia do Pacheco, Caucaia, CE; B. *Aplysina fulva* na praia de Iracema, Fortaleza, CE; *Cinachyrella* sp. na praia do Paracuru, CE.

Fonte: Autor

Em detrimento ao enorme número de espécies de esponjas encontradas na natureza, apenas cerca de quarenta lectinas de esponjas estão descritas (Tabela 1), dez delas apresentam estrutura primária determinada e apenas uma possui estrutura tridimensional resolvida.

As lectinas isoladas de esponjas apresentam divergências em suas características bioquímicas. Suas massas moleculares variam desde 13 kDa até 175 kDa e a associação dos monômeros para formar estruturas oligoméricas também é variada, havendo interações fracas ou pontes dissulfeto para a formação de dímeros, trímeros, tetrâmeros, hexâmetos ou octâmeros (GARDÈRES *et al*., 2015).

No que diz respeito às atividades biológicas destas lectinas há também uma grande variedade de efeitos. As lectinas isoladas de *Geodia cydonium*, *Cinachyrella alloclada*, *Desmapsana anchorata*, *Pellina semitubulosa*, *Crambe crambe* e *Craniella australiensis* apresentam atividade mitogênica sobre diferentes linhagens celulares (ATTA *et al*., 1989; ATTA *et al*., 1990; ENGEL *et al*., 1992; DOGOVIC *et al*., 1996; WAGNER-HÜLSMANN *et al*., 1996; XIONG *et al*., 2005).

As lectinas isoladas de *Cliona varians* (CvL-1), *Haliclona cratera* e *Cinachyrella appion* exercem citotoxidade sobre linhagens malignas de leucemia, HeLa e câncer cervical, respectivamente (PAJIC *et al*., 2002; QUEIROZ *et al*., 2009; RABELO *et al*., 2012). Além disso, CvL-1 tem atividade pró-inflamatória, toxicidade contra bactérias patogênicas e aglutina formas promastigotas de Leishmania (MOURA *et al*., 2006; QUEIROZ *et al*., 2008).

A lectina de *Axinella corrugata* possui efeito quimiotático sobre neutrófilos, e a lectina de *Cinachyrella* sp. é capaz de modular receptores de glutamato, exercendo forte atividade sobre o sistema nervoso central de ratos (DRESCH *et al*., 2012; UEDA *et al*., 2013).

Tabela 1. Lectinas isoladas de esponjas.

Lectina	Espécie	Massa molecular	Ligantes	Cátions	Pontes dissulfeto
Galectinas					
CchGs	*Cinachyrella* sp.	4 x 13 kDa	Galactosídeos	Não	Não
GcG	*Geodia cydonium*	4 x 15 kDa	Galactosídeos	Não	Não
HoL-30	*Halichondria okadai*	2 x 30 kDa	Galactosídeos	Não	n/d
Sd galectin	*Suberites domuncula*	22 kDa	Galactosídeos	Não	n/d
Sd galectin-2	*Suberites domuncula*	31 kDa	Galactosídeos	Não	n/d
Lectinas tipo C					
APHRLEC	*Aphrocallistes vastus*	4x 20 kDa	Galactosídeos	Sim	Sim
Lb MBL	*Lubormirska baicalensis*	13 kDa	Manose	Sim	Sim
Lectinas tipo L					
Clathrilectin	*Clatrina chlatrus*	208/180 kDa	n/d	n/d	n/d
Tachylectinas					
Ef lectin	*Ephydatia fluviatis*	27 kDa	Lipopolissacarídeos	Sim	Sim
Sd lectin	*Subertites domuncula*	27 kDa	Lipopolissacarídeos	Sim	Sim
Lectinas órfãs					
ApL-1	*Axinella polypoides*	15 kDa	Galactosídeos	Não	Sim
ApL-2	*Axinella polypoides*	15 kDa	Galactosídeos	Não	Sim
Halilectina 2	*Haliclona caerulea*	2 x 14 kDa	Mucinas	Não	n/d
Halilectina 3	*Haliclona caerulea*	2 x 11 kDa + 18 kDa	GalNAc	Não	Sim
Lectinas sem classificação					
ApaL-I	*Aaptos papilata*	2 x 21 kDa	GlcNAc	n/d	n/d
ApaL-II e III	*Aaptos papilata*	16 kDa	GlcNAc/GalNAc	n/d	n/d
AaL	*Aplysina archeri*	4 x 16 kDa	Galactosídeos	Não	Sim
AlL	*Aplysina lacunosa*	4 x 16 kDa	Galactosídeos	Não	Sim
AcL-1	*Axinella corrugata*	78 kDa	*N*-acetil	Não	n/d
AcL-2	*Axinella corrugata*	80 kDa	Galactose	Não	n/d
CnL	*Chondrilla nucula*	15 kDa	Galactose	Não	n/d
CaL	*Cinachyrella apion*	17 kDa	Lactose	Não	n/d
CalL	*Cinachyrella alloclada*	17 kDa	Galactosídeos	Não	n/d
CvL	*Cliona varians*	4 x 27 kDa	Galactosídeos	Sim	Sim
CvL-2	*Cliona varians*	3 x 175 kDa	Galactosídeos	Sim	n/d
CcL	*Crambe crambe*	14 kDa	Fucose	Não	Não
CauL	*Craniella australiensis*	3 x 18 kDa	Mucina	Não	Sim
DaL	*Desmapsana anchorata*	2 x 18 kDa	Galactosídeos	n/d	n/d
Halilectina 1	*Haliclona caerulea*	40 kDa	Nd	Não	n/d

HcL	*Haliclona cratera*	2 x 15 kDa	Galactosídeos	Não	n/d
HL	*Haliclona* sp.	24 kDa	Lactose	n/d	n/d
Hol-1	*Halichondria okadai*	4 x 21 kDa	N-acetil	Não	n/d
Hol-2	*Halichondria okadai*	42 kDa	N-acetillactosamina	Não	n/d
Hol-18	*Halichondria okadai*	18 kDa	Quitotriose	Não	n/d
HpL	*Halichondria panicea*	4 x 21 kDa	Ácidos urônicos	n/d	n/d
HMA	*Haliclona manglaris*	2 x 15 kDa + 25 kDa	Tiroglobulina	Não	Sim
PsL	*Pelina semitubulosa*	6 x 34 kDa	Galactosídeos	Não	Sim

Adaptado de GARDERES *et al*., 2015. GalNAc – *N*-acetilgalactosamina; GlcNAc – *N*-acetilglicosamina; n/d – Não determinado

O papel fisiológico das lectinas de esponjas também é muito diversificado. Enquanto a lectina de *G. cidonium* parece estar envolvida juntamente com fatores de agregação celular na adesão das células, a lectina de *L. baikalensis* atua na regulação do crescimento da esponja agindo em conjunto com uma proteína *mago nashi* (WAGNER-HÜLSMANN *et al*., 1996; WIENS *et al*., 2006). Já as lectinas de *H.caerulea* e *H. manglaris* apresentaram atividade antioxidante *in vitro* e seu papel fisiológico pode estar relacionado à proteção foto-oxidativa destas esponjas (CARNEIRO *et al*., 2015).

Com relação à estrutura de lectinas de poríferos, apenas um pequeno número de trabalhos foi realizado e o que se observa é uma certa variedade estrutural no que diz respeito a classificação das lectinas estudadas (GARDERES et al., 2015).

As isolectinas da esponja *G. cidonium*, GcG-1 e GcG-2, apresentaram aminoácidos conservados e característicos do DRC das galectinas de vertebrados (STALZ *et al*., 2006). A estrutura primária de GcG-1 indica uma relação direta com a galectina-1. Entretanto, GcG-1 compartilha características inerentes a galectinas do tipo quimera, tais como a preferência por terminais relacionados aos carboidratos do epítopo sanguíneo A, em detrimento do reconhecimento a polilactosaminas (STALZ *et al*., 2006).

As isolectinas de *Cinachyrella* sp. também são consideradas galectinas, CchG possui 147 aminoácidos, incluindo cinco resíduos conservados do DRC de galectinas: Arg^{47}, Asn^{60}, Trp^{68}, Glu^{71} e Arg^{73}. A estrutura primária de CchG apresentou 16% de identidade com a galectina da enguia europeia *Anguila anguila*, 19% com galectina-1 humana e 23% de identidade com as galectinas de *G. cydonium*. Os dados obtidos por Ueda et al. (2012) sugerem que CchG é uma galectina do tipo proto.

CchG é a única lectina de esponja com estrutura tridimensional resolvida, em que o monômero de CchG consiste em duas folhas β antiparalelas, compostas por cinco fitas β cada. Esta estrutura é característica de outras protogalectinas e a topologia das folhas β é similar àquelas encontradas em outras galectinas. Os monômeros de CchG associam-se formando dímeros canônicos. A estrutura tetramérica (dímero de dímeros) que ocorre em CchG é mediada por uma rara estrutura estabilizada por uma ponte dissulfeto entre cisteínas adjacentes (FREYMANN *et al*., 2012).

Em *Suberites domuncula* e *Ephydatia fluviatilis*, duas lectinas relacionadas às tachylectinas foram identificadas. As lectinas Sd e a Ef, assim como as lectinas do caranguejo ferradura, possuem um domínio transmembranar e seis domínios repetidos ao longo da estrutura (FUNAYAMA *et al*., 2005; SCHRODER *et al*., 2003). As duas lectinas apresentaram 62% de

identidade entre si, além de mais de 50% de identidade com as lectinas do caranguejo ferradura *Tachypleus tridentatus* (FUNAYAMA *et al*., 2005).

Duas isolectinas do tipo C foram identificadas em *Aphrocallistes vastus*. Ambas as lectinas possuem um sítio de *N*-glicosilação, três pontes dissulfeto intracadeia e um domínio transmembranar (GUNDACKER *et al*., 2001).

As lectinas de *Axinella polypoides* foram inicialmente agrupadas na família de lectinas animais do tipo R, mas a baixa similaridade das lectinas de *A. polypoides* com outros membros da família tipo R fez com que estas lectinas fossem consideradas órfãs (BUCK *et al*., 1998).

Outras lectinas órfãs foram isoladas de *Haliclona caerulea* e *H. manglaris* (H-2 e H-3 de *H. caerulea* e HMA de *H. manglaris*) não apresentaram similaridade com qualquer lectina conhecida. Interessantemente, as duas últimas compartilham entre si a interação com cromóforos, entretanto, não apresentam similaridade de sequência entre si (CARNEIRO *et al*., 2015; CARNEIRO *et al*.,2013a; CARNEIRO *et al*., 2013b).

Apesar de todas as divergências bioquímicas, biológicas e estruturais observadas nas lectinas de esponjas, estas proteínas convergem em um fator primordial: a especificidade. Majoritariamente as lectinas de poríferos reconhecem galactose e/ou derivados (GARDERES et al., 2015).

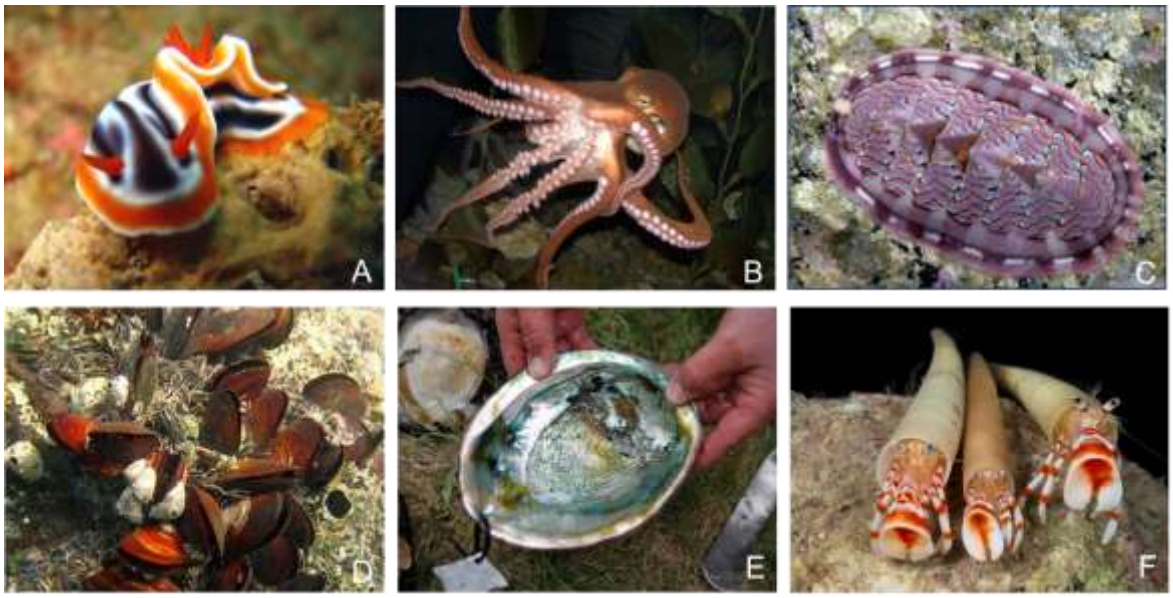
Lectinas ligantes de galactose foram isoladas de *A. polypoides*, *C. alloclada*, *C. nucula*, *H. okadai* e *C. varians*. *H. caerulea*, *C. appion*, *A. lacunosa* e *A. archeri* possuem lectinas que reconhecem formas derivadas da galactose, tais como galNAc e lactose (BRETTING *et al*., 1981; CARNEIRO *et al*., 2013; KAWSAR *et al*., 2008; MIARONS; FRESNO, 2000; MOURA *et al*., 2006; MOURA *et al*., 2015; SCHRÖDER *et al*., 1990; YAMAWAKI *et al*., 1994). Há ainda lectinas que reconhecem glicoconjugados ricos em resíduos de galactose, como é o caso das lectinas de *C. australiensis*, *H. caerulea* e *H. panicea* (CARNEIRO *et al*., 2013; MÜLLER *et al*., 1981; XIONG *et al*., 2006).

Não está claro o motivo desta convergência de especificidade entre as lectinas de poríferos, mas, de uma maneira geral, os estudos envolvendo tais lectinas ainda estão em sua infância e há ainda muito a ser feito para que seja possível compreender esta e outras questões relevantes sobre este grupo de moléculas de elevado potencial biotecnológico.

1.5 Lectinas de moluscos

O filo Mollusca é um dos mais numerosos e importantes grupos de animais; estima-se que existam pelo menos 50 mil espécies distribuídas no ambiente marinho, terrestre e de água doce. O termo Mollusca indica uma característica distintiva do grupo: o corpo mole (HICKMAN; ROBERTSON; LARSON, 2004). Este grupo muito diversificado inclui quítons, mexilhões, ostras, lulas, polvos, caracóis e lesmas (Figura 2).

Figura 2. Principais classes de moluscos. **A**. Nudibrânquio (Gastropoda); **B**. Polvo (Cephalopoda); **C**. Quíton (Poliplacophora); **D.** Mexilhão (Bivalva); **E**. Abalone (Monoplacophora) e **F**. Conchas de escafópodes (Scaphopoda).



Fonte: https://pt.wikipedia.org/wiki/Moluscos#Classes

As lectinas de moluscos estão entre as mais estudadas proteínas de origem animal. Provavelmente o interesse por estas moléculas deve-se ao fato de estes animais possuírem elevada importância econômica e as lectinas estarem envolvidas na defesa dos moluscos contra patógenos (CHERNIKOV *et al*., 2013).

Nestes animais, as lectinas são comumente encontradas nos hemócitos, ou como constituintes livres da hemolinfa (BELOGORTSEVA *et al*., 1998; CASTAGNA *et al*. 1996; CHIKALOVETS *et al*., 2013). Lectinas também têm sido detectadas nas glândulas digestivas, células reprodutivas e brânquias de ostras, mexilhões e gastrópodes (ESPINOSA *et al*., 2009; GILBOA-GARBER *et al*., 1985; SPRINGER *et al*., 2008).

As lectinas encontradas no plasma e nos hemóctios estão fundamentalmente relacionadas a mecanismos de defesas destes animais. Estas proteínas atuam como proteínas de reconhecimento de padrões (*Pattern recognition proteins* - PRPs), ou seja, proteínas responsáveis por reconhecer padrões bioquímicos não próprios (*Pathogen-associated molecular pattern* - Padrões moleculares associados a patógenos - PAMPs). Em geral estas lectinas apresentam especificidade bastante variada, tendo sido relatadas lectinas ligantes de galNAc, fucose, manose e carboidratos complexos (ALPUCHE *et al*., 2010; BULGAKOV *et al*., 2004; LI *et al*., 2011; NAGANUMA *et al*., 2006).

As lectinas encontradas nos demais tecidos parecem estar envolvidas em funções bastante distintas, desde a seleção e captura de alimentos até a secreção e biomineralização de carbonato de cálcio (ESPINOSA *et al*., 2009; WEISS *et al*., 2000).

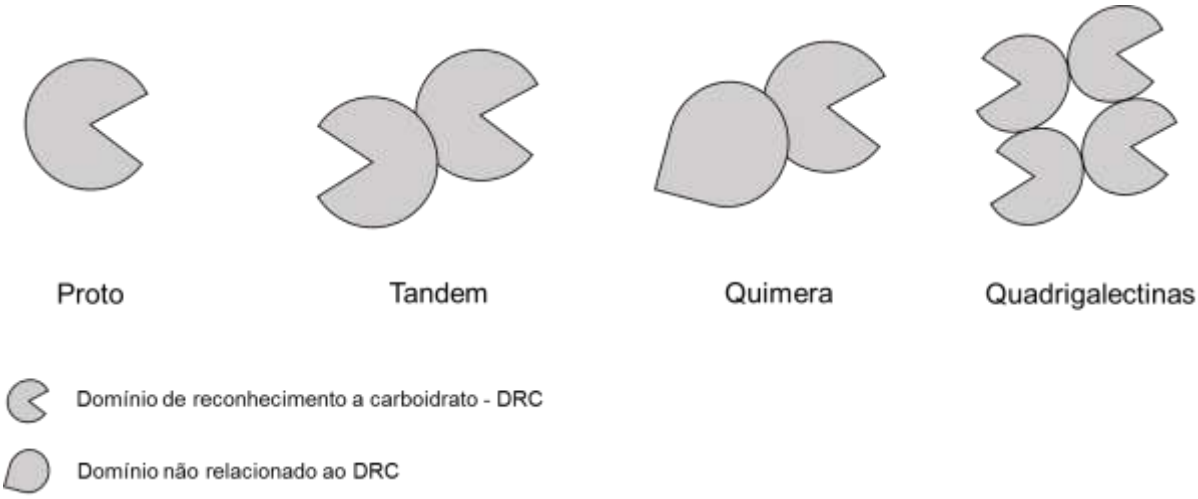
Dentre as mais de duzentas lectinas identificadas em moluscos destacam-se as lectinas do tipo C e as galectinas. As primeiras destacam-se por serem a imensa maioria, enquanto que as últimas destacam-se porque em moluscos está presente um subtipo de galectinas não identificadas em outros organismos: as quadrigalectinas (D. ZHANG *et al*., 2011; H. ZHANG *et al*., 2009; SONG *et al*., 2011;TASUMI;VASTA, 2007).

Galectinas constituem uma família de lectinas ligantes de β-galactosídeos, não dependentes de cálcio e que possuem similaridade de sequência em seus DRCs. Em geral, o DRC de uma galectina possui 135 aminoácidos, dos quais sete formam o sítio de ligação ao carboidrato (COOPER; BARONDES, 1999).

De acordo com a quantidade de DRCs por cadeia polipeptídica, as galectinas podem ser incluídas em diferentes subfamílias: *proto* - um DRC por subunidade; *tandem* - dois DRCs similares em uma mesma cadeia polipeptídica e; *quimera* - um DRC e mais um domínio não relacionado à atividade lectínica, em uma mesma cadeia polipeptídica (Figura 3) (HIRABAYASHI; KASAI, 1993).

Entretanto, algumas das galectinas descritas em moluscos apresentam quatro DRCs por subunidade, as quadrigalectinas (Figura 3), um fato único que faz deste grupo de invertebrados um ótimo objeto de pesquisa para o melhor entendimento a respeito da evolução das galectinas (SONG *et al*., 2011).

Figura 3. Subfamílias de galectinas



Adaptado de HIRABAYASHI; KASAI, 1993.

Além de lectinas do tipo C e galectinas, lectinas de outras famílias e mesmo lectinas que não se encaixam em qualquer das famílias estruturais foram encontradas em moluscos.

A lectina do *escargot Helix pomatia*, por exemplo, apresenta um arranjo quaternário do tipo hexamérico que consiste em um dímero de trímeros, em que três monômeros estão unidos por pontes dissulfeto e os trímeros estão associados por interações fracas, enquanto que sua estrutura tridimensional consiste em um tipo de sanduíche β singular (SANCHEZ *et al*., 2006). A estrutura primária de HPA é semelhante apenas a SLL2, uma lectina do coral *Sinularia lochmodes* (JIMBO *et al*., 2000). HPA e SLL são membros de uma pequena família de lectinas, denominadas lectinas do tipo H, em alusão à *Helix pomatia*.

A lectina de *Pteria penguim* é considerada uma RBL (*rhamnose binding lectin* – lectina ligante de ramnose). Interessantemente, esta é a única RBL encontrada em moluscos e uma das poucas presentes em tecidos não reprodutivos (NAGANUMA *et al*., 2006).

Uma lectina do tipo R foi isolada de *Mytillus galloprovincialis* (Mytilec-1). Inicialmente Mytilec-1 foi considerada como uma lectina sem classificação definida (FUJII *et al*., 2012) e posteriormente, foi agrupada na família de lectinas do tipo R, os membros desta família se destacam por apresentarem pouca similaridade entre si, mas apresentam a repetição de uma sequência típica (QxW), onde "x" é qualquer aminoácido (HIRABAYASHI; DUTTA; KASAI, 1998).

A família do tipo R é a única família de lectinas com representantes dos reinos Animalia, Plantae e Monera. A denominação R é em alusão à cadeia lectínica da ricina, uma proteína inativadora de ribossomos (RIP – *ribossome inactivating protein*) do tipo II isolada da mamona (*Ricinus comunis*) (HIRABAYASHI; DUTTA; KASAI, 1998).

Assim como a cadeia b da ricina, as demais lectinas do tipo R apresentam DRCs repetidos ao longo de sua cadeia, em que três domínios semelhantes são arranjados dentro de uma mesma cadeia polipeptídica. A dependência de íons divalentes pode ou não ser observada (HIRABAYASHI; DUTTA; KASAI, 1998).

Os ovos do gastrópode *Aplysia kurodai* também apresentam uma lectina com domínios repetidos ao longo de sua cadeia polipeptídica. No entanto, AKEL (*Aplysia kurodai* eggs lectin) não pode ser incluída na família de lectinas do tipo R, pois não apresenta o motivo (QxW) e tampouco apresenta similaridade com a cadeia b da ricina (KAWSAR *et al*., 2009; OZEKI, 1998).

1.6 Lectinas de equinodermos

O filo Echinodermata compreende um grupo de invertebrados deuterostômios exclusivamente marinhos. Estes animais possuem espinhos por todo o corpo além de um sistema hidráulico interno responsável pela locomoção (HICKMAN; ROBERTS; LARSON, 2004). Dentre os equinodermos destacam-se as estrelas do mar, os ouriços do mar e os pepinos do mar (Figura 4).

Figura 4. Classes de equinodermos. **A**. Estrela do mar (Asteroidea); **B**. Ofiuróide (Ophiuroidea); **C**. Ouriço do mar, *Echinometra lucunter* (Echinoidea); **D**. Bolacha da praia (Echinoidea); **E.** pepino do mar (Holothuroidea); **F.** Lírio do mar (Crinoidea).

Fonte: http://www.poppe-images.com

Atualmente, cerca de trinta lectinas de equinodermos foram purificadas, doze possuem estrutura primária determinada e somente três possuem estrutura tridimensional resolvida (Tabela 2).

Tabela 2. Lectinas isoladas de equinodermos.

Lectina	Espécie (n°de lectinas)	**Família de lectinas**	**Ligantes**
Equinoidina	*Anthocidaris crassispina*	Tipo C	Glicoproteínas do tipo O
SUEL	*Anthocidaris crassispina*	RBL	Galactosídeos
SJL-1	*Apostichopus (Sticopus) japonicus*	Tipo C	Galactosídeos
SJL-2	*Apostichopus (Sticopus) japonicus*	-	Galactosídeos
SPL-1	*Apostichopus (Sticopus) japonicus*	-	Galactosídeos
SPL-2	*Apostichopus (Sticopus) japonicus*	-	Galactosídeos
MBL-AJ	*Apostichopus (Sticopus) japonicus*	Tipo C	Manana
AJCTL	*Apostichopus (Sticopus) japonicus*	Tipo C	Galactosídeos
Starfish lectin	*Asterina pectinifera*	Tipo C	Antígeno Tn
AMUL	*Asteria amurensis*	Tipo C	Ácidos neuramínicos
CEL-I	*Cucumaria echinata*	Tipo C	Galactosídeos
CEL-II	*Cucumaria echinata*	Tipo C	Galactosídeos
CEL-III	*Cucumaria echinata*	Tipo R	Galactosídeos
CEL-IV	*Cucumaria echinata*	Tipo C	Galactosídeos
MBL-CJ	*Cucumaria japônica*	Tipo C	Manana
Sperm lectin	*Hemicentrotus pucherrinus*	-	GalNAc
HGA	*Holothuria grisea*	-	Mucina
HGA-2	*Holothuria grisea*	Tipo C	Galactosídeos
HGL	*Holothuria grisea*	-	Mucina
HsL	*Holothuria scabra*	Sem família definida	Antígeno T
LvL	*Lytechinus variegatus*	-	Galactosídeos
OXYL	*Oxycomanthus japonicas*	-	Fetuína
SN-MBL	*Strongylocentrotus nudus*	Tipo C	Manana
SUL-I	*Toxopneustes pileolus*	RBL	Galactosídeos
SUL-II	*Toxopneustes pileolus*	-	Galactosídeos
SUL-III	*Toxopneustes pileolus*	RBL	Galactosídeos

Fonte: Revisado pelo autor

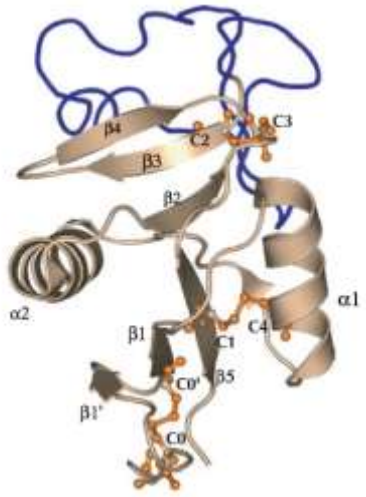
Dentre as cinco classes de equinodermos, Asteroidea (estrelas do mar), Holothureoidea (pepinos do mar), Echinoidea (ouriços do mar e bolachas da praia), Crinoidea (lírios do mar) e Ouphiuroidea (ofiuroides), a classe dos pepinos do mar é a mais explorada em se tratando de lectinas.

O fluido celomático é a principal fonte de lectinas em equinodermos. As lectinas presentes na cavidade celomática destes animais supostamente desempenham papéis de defesa, estando envolvidas no reconhecimento específico de substâncias ou organismos estranhos (BULGAKOV *et al*., 2013).

As lectinas em equinodermos representam a primeira linha de defesa destes organismos contra infecções; elas atuam como opsoninas reconhecendo e ligando-se a bactérias, fungos e superfícies virais e, posteriormente, desencadeando a fagocitose de patógenos por células especializadas (BULGAKOV *et al*., 2007; BULGAKOV *et al*., 2013; PETROVA *et al*., 2009).

Dentre as lectinas isoladas de equinodermos, destacam-se as lectinas do tipo C. A superfamília de proteínas contendo um domínio lectínico do tipo C (CTLD - C-*type lectin like*) compreende um vasto grupo de proteínas extracelulares de metazoários com funções diversificadas. A estrutura do CTLD (Figura 5) consiste em uma alça dupla estabilizado por duas pontes dissulfeto altamente conservadas localizadas na base da segunda alça (ZELENSKY; GREADY, 2005).

Figura 5 - Estrutura de um CTLD típico. Os *loops* responsáveis pela ligação a carboidratos estão destacados em azul. As pontes disulfeto apresentam-se como bastões laranja, incluindo as duas pontes invariáveis: C2 e C3.



Fonte: Adaptado de ZELENSKY; GREADY (2005).

O termo lectina do tipo C foi introduzido inicialmente para designar um grupo de lectinas dependentes de Ca^{2+}. Com o acúmulo de informações estruturais a respeito de tais lectinas ficou claro que a atividade relativa à ligação a carboidratos era mediada por uma compacta região da proteína, o DRC. A comparação entre os DRCs de diferentes lectinas do tipo C revelou resíduos conservados e motivos característicos do domínio (DRICKAMER, 1993). Estudos cristalográficos confirmaram que o DRC das lectinas tipo C tem uma estrutura globular compacta, diferente de todos os dobramentos lectínicos até então conhecidos. Este domínio ficou conhecido como DRC tipo C ou domínio lectínico do tipo C (WEIS *et al.*, 1991). Com o crescimento do número de sequências protéicas determinadas ficou claro que nem todas as proteínas que contêm os resíduos chave e o padrão estrutural conservado de lectinas do tipo C são verdadeiramente capazes de ligar-se a Ca^{2+} ou mesmo a carboidratos. Para resolver esta contradição, o termo CTLD é usado para designar toda proteína que apresente o DRC do tipo C independentemente da habilidade de ligar-se a Ca^{2+} ou mesmo a carboidratos (ZELENSKY; GREADY, 2005).

Lectinas do tipo C foram isoladas dos pepinos do mar *Cucumaria echinata* (CEL-I, II, e IV), *Apostichopus japonicus* (CTLAJ, MBL-AJ e SJL-1), *Holothuria grisea* (HGA-2) e *C. japonica* (MBL-C), dos ouriços do mar *Anthocidaris crassispina* (equinoidina) e *Strongylocentrotus nudus* e das estrelas do mar *Asterina pectinifera* e *A. amurensis* (BULGAKOV *et al*., 2007; BULGAKOV *et al*., 2013; GIGA *et al*., 1987; HAN *et al*., 2012; HATAKEYAMA *et al*., 1994; HIMESHIMA *et al*., 1994; IMAMICHI; YOKOYAMA, 2013; KAKIUCHI *et al*., 2002; MELO *et al*., 2014;). Interessantemente, com exceção das lectinas ligantes de manana (MBL – *mannan binding lectin*) isoladas de *A. japonicus* e *S. nudus*, todas as demais possuem especificidade por galactosídeos.

Além de três lectinas do tipo C, *C. echinata* possui uma lectina do tipo R, CEL-III (NAKANO *et al*., 1999). Todas as quatro lectinas reconhecem galactosídeos e três delas exibem efeito citotóxico sobre células malignas. Ademais, CEL-III é capaz de hemolisar eritrócitos humanos. A atividade hemolítica pode ser inibida por galNAc, indicando que a habilidade de hemolisar células é dependente do reconhecimento celular específico (UNNO; GONNA; HATAKEYAMA, 2014).

CEL-III também exibe forte citotoxicidade sobre algumas linhagens de células, especialmente sobre MDCK (*madin-darby canine kidney epithelial cells*), contudo não afeta outras linhagens como CHO (*chinese hamster ovary*), sugerindo mais uma vez que a atividade biológica de CEL-III é mediada pelo reconhecimento celular específico (UCHIDA *et al*., 2004).

Além disso, CEL-III afeta o desenvolvimento de *Plasmodium falciparum* e *P. berghei*, os protozoários causadores da malária (YOSHIDA *et al*., 2007).

Apostichopus (Stichopus) japonicus possui pelo menos seis lectinas dependentes de Ca^{2+}, das quais duas (CTLAJ e MBL-AJ) são MBLs, três reconhecem gal/galNAc (SJL-1, SJL-2 e SPL-II) e uma é ligante de ácidos urônicos, tais como ácido galacturônico e glucurônico (SPL-2) (HAN *et al*., 2012; MATSUI *et al*., 1994; TAYLOR *et al*., 1993).

MBL-AJ é capaz de interagir com carboidratos presentes em antígenos carcinogênicos, mas não interage com células saudáveis, o que faz de MBL-AJ a ferramenta principal de um método para o diagnóstico de câncer cervical, através de um teste simples e extremamente confiável (BULGAKOV *et al*., 2011).

Em *Holothuria grisea*, três lectinas foram isoladas e uma delas, HGA, apresenta efeito anti-inflamatório em ratos. HGA é um dímero de 114 kDa por subunidade com especificidade por mucina de estômago de porco (PSM – *porcine stomach mucin*) e seu efeito anti-inflamatório é único em se tratando de lectinas de invertebrados marinhos (MOURA *et al*., 2013). A segunda lectina de *H. grisea*, HGA-2, é do tipo C com especificidades por galactosídeos. HGA-2 foi capaz de aglutinar bactérias Gram-positivas, o que sugere seu envolvimento na defesa do animal (MELO *et al*., 2014).

Em estrelas do mar, lectinas foram isoladas da hemolinfa e dos órgãos de *Asterina pectinifera* e do fluido celomático de *A. amurensis* (IMAMICHI; YOKOYAMA, 2013; KAKIUCHI *et al*., 2002).

Não há até o momento lectinas isoladas a partir de ofiuroides, enquanto que as lectinas de lírios do mar possuem somente um representante conhecido: OXYL, lectina isolada de *Oxycomanthus japonicus*. OXYL é um dímero formado por duas subunidades idênticas de 14 kDa. OXYL reconhece oligossacarídeos contendo *N*-acetilactosamina quando estes contêm ácido siálico ligado ($\alpha 2 \rightarrow 3$) na extremidade não redutora (MATSUMOTO *et al*., 2011).

Na classe Echinoidea lectinas foram isoladas somente de ouriços do mar. *Strongylocentrotus nudus* possui uma MBL, MBL-SN. Esta lectina foi isolada a partir do fluido celomático do ouriço do mar e consiste em um dímero de 34 kDa, formado por subunidades idênticas de 17 kDa unidas por uma ponte dissulfeto. MBL-SN foi inibida por mananas presentes em bactérias patogênicas, tais como *Vibrio alginolyticus*, *Marimonas communis* e *Pseudoateromonas atlantica* (BULGAKOV *et al*., 2013).

No ouriço do mar *Anthocidaris crassispina*, uma lectina do tipo C foi isolada a partir do fluido celomático. Equinoidina foi uma das primeiras lectinas isoladas a partir de invertebrados deuterostômicos. Equinoidina é um dímero unido por ponte dissulfeto e cada

subunidade possui 13 kDa. Cada dímero pode interagir com outros para formar um oligômero de 400 kDa. O melhor inibidor para a lectina parece ser AcNeuα2→3Galβ1→3(AcNeuα2→6)GalNAc→Ser/Thr (GIGA; SUTOH; IKAI, 1985).

A determinação da estrutura primária de Equinoidina em 1987 foi um marco no estudo da origem das lectinas do tipo C. Este trabalho continua sendo até hoje um dos mais influentes sobre lectinas (GIGA *et al*., 1987).

A segunda lectina de *A.crassispina* (*Sea Urchin Eggs Lectn* - SUEL) foi isolada a partir dos ovos não fecundados do ouriço. SUEL apresenta similaridade com lectinas ligantes de ramnose isoladas de ovos e gônodas de peixes. SUEL é um homodímero formada por duas subunidades idênticas de 11 kDa cada, ligadas por pontes dissulfeto. Cada subunidade é composta de um único DRC de 105 aminoácidos (OZEKI *et al*., 1991).

SUEL é expressa em todo o tecido embrionário, principalmente nos ovos, onde é encontrada na periferia da célula depositada na forma de grânulos. Após a fertilização, SUEL migra para o córtex celular, onde permanece até a primeira divisão. Durante o desenvolvimento embrionário, SUEL está presente na superfície do embrião, e na blástula, ela pode ser encontrada na invaginação característica deste estágio. A migração de SUEL ao longo do desenvolvimento embrionário parece ser mediada por filamentos de actina (OZEKI *et al*., 1995).

Considerando todas as informações destacadas até aqui, fica claro que os invertebrados marinhos são ótimos alvos para a prospecção biotecnológica de lectinas, especialmente aqueles que são pouco explorados. Sendo assim, esta tese se destina aos estudos estruturais e avaliação do efeito antibiofilme e antibacteriano de lectinas isoladas dos invertebrados *Aplysina lactuca* (esponja), *Aplysia dactylomela* (gastrópode) e *Echinometra lucunter* (ouriço do mar). O presente estudo será apresentado na forma de três artigos descritos a seguir: artigo I - Isolation, biochemical characterization and antibiofilm effect of alectin from the marine sponge *Aplysina lactuca*, artigo 2 - Purification, biochemical characterization, and amino acid sequence of a novel type of lectin from *Aplysia dactylomela* eggs with antibacterial/antibiofilm potential e artigo 3 - L-rhamnose-binding lectin from eggs of the *Echinometra lucunter*: amino acid sequence and molecular modeling.

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Artigo I

Isolation, biochemical characterization and antibiofilm effect of a lectin from the marine sponge *Aplysina lactuca*

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Isolation, biochemical characterization and antibiofilm effect of a lectin from the marine sponge *Aplysina lactuca*

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ABSTRACT

A new lectin was isolated from the marine sponge *Aplysina lactuca* (ALL) by combining ammonium sulfate precipitation and affinity chromatography on guar gum matrix. ALL showed affinity for the disaccharides α-lactose, β-lactose and lactulose (K_a = 12.5, 31.9 and 145.5 M^{-1}, respectively), as well as the glycoprotein porcine stomach mucin. Its hemagglutinating activity was stable in neutral acid pH values and temperatures below 60 °C. ALL is a dimeric protein formed by two covalently linked polypeptide chains. The average molecular mass, as determined by Electrospray Ionization Mass Spectrometry (ESI–MS), was 31,810 ±2 Da. ESI–MS data also indicated the presence of three cysteines involved in one intrachain and one interchain disulfide bond. The partial amino acid sequence of ALL was determined by tandem mass spectrometry. Eight tryptic peptides presented similarity with lectin I isolated from *Axinella polypoides*. Its secondary structure is predominantly β-sheet, as indicated by circular dichroism (CD) spectroscopy. ALL agglutinated gram-positive and gram-negative bacterial cells, and it were able to significantly reduce the biomass of the bacterial biofilm tested at dose- dependent effect.

1. Introduction

Lectins, which are found in all living organisms, are sugar-binding proteins that differ from immunoglobulins and glycoenzymes because they are not produced as an adaptive immune response, and they contain no site for enzymatic modification of their ligands. However, lectins are important proteins of both vertebrate and invertebrate innate immune systems, and they interact with carbohydrates in a specific and reversible manner [1–3].

Several lectins from different sources have been isolated and characterized. Among the exploited invertebrates, lectins from the phylum Porifera has revealed promising pharmacological and biotechnological application potential [4]. Currently, about 150 sponges have been screened for hemagglutinating activity [4–8], and the isolation of about 40 resulted [4].

Sponge lectins form a heterogeneous group of proteins, which present a wide variety of biochemical characteristics (*i.e.*, molecular size, glycosylation, Ca^{2+} binding site and isoelectric point) and biological activities, including antimicrobial and cytotoxic activities, modulation of inflammatory response, and neuromodulatory activity [4,9,10,11–13].

Despite their biotechnological potential, only a few lectins from sponges have thus far been structurally characterized. Among those that have had their primary structure determined are representatives of some structural lectin families, including C-type lectins, tachylectins, galectins [14–16], and "orphan" lectins that do not belong to any specific family [17,18].

Irrespective of their structural features, most sponge lectins converge in one primordial characteristic: specificity. Several galactophilic lectins were isolated from sponges [4,7,10,19,20]. The genus *Aplysina*, for instance, presents galactose-binding lectins isolated from *A. archeri* and *A. lacunosa* [8].

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Aplysina lactuca is a recently described endemic species from the northeastern Brazilian coast. Specimens can be found on the upper part of rocks or coral heads, in depths varying from 5 to 22 m *A. lactuca* is a yellowish-brown sponge of very soft and flexible consistency that presents a lamellar form, which is reminiscent of the root buttresses of large tropical canopy trees [21].

In this work, we report the purification and biochemical characterization of a new lectin isolated from the marine sponge *A. lactuca* (ALL). We then describe the effect of the lectin against bacterial biofilm formation.

2. Methods

2.1. Materials

Guar gum, protein mix, α-cyano-4-hydroxycinnamic acid (CHCA), sugars and glycoprotein were purchased from Sigma (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile (ACN), formic acid (FA), trifluoracetic acid (TFA), vinyl-pyridine (VP), iodoacetamide (IAA), β-mercaptoethanol (β-ME) and dithiothreitol (DTT), acrylamide, bis-acrylamide, ammonium persulfate and sodium dodecyl sulfate were HPLC/Spectro grade. Sodium phosphate, ammonium sulfate, Tris hydrochloride, EDTA, glycine, sodium chloride, sodium acetate violet crystal were the purest grade commercially available (for analysis grade).

2.2. Animal collection

Sponge specimens were collected on the intertidal zone of Pacheco Beach (3°41′14″S, 38°37′58″W), Caucaia, Brazil. The sponges were transported in seawater to the laboratory and stored at −20 °C until use. The species was identified, and a voucher was deposited (ID: UFPEPOR 1929) at the Zoology Department of the Universidade Federal de Pernambuco, PE, Brazil. Collections were authorized and certified by responsible environmental institutions (SISBIO ID: 33913-8).

2.3. Lectin purification

The frozen sponges were cut into small pieces, ground in a mortar and homogenized in Tris-HCl 50 mM, pH 8.0, containing NaCl 150 mM (TBS) at 1:10 (w/v). Smashed sponges were filtered through nylon tissue, and the extract was centrifuged at 7000*g* for 30 min at 4 °C. The supernatant (crude extract) was stored at −20 °C until use.

Crude extract was submitted to precipitation with 70% ammonium sulfate saturation. After 4 h at 4 °C, precipitated proteins were pelleted by centrifugation and solubilized in a small volume of TBS (F 0–70). Approximately 45 mL of F 0–70 were applied on cross-linked guar gum prepared according to Appukuttan et al. [22]. The column (1.0 × 8.0 cm) was previously equilibrated with TBS, unbound proteins were washed with equilibrium buffer and retained proteins were eluted with glycine buffer (50 mM, pH 2.6, containing NaCl 150 mM). Fractions of 2 mL were manually collected at a flow rate of 1 mL min^{-1} and monitored by absorbance measurement using an Ultrospec 2100 spectrophotometer (GE Healthcare). Retained fractions (ALL) were pooled, dialyzed against distilled water, and freeze-dried.

2.4. Hemagglutinating activity and inhibition assays

Hemagglutinating activity (HA) and inhibition assays were performed following pre-established methods [23] using human (A, B and O) and rabbit erythrocytes, both treated with proteases and untreated.

The following sugars and glycoproteins were used in the inhibition assay: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, α-D-lactose, β-D-lactose, D-lactulose, D-maltose, D-raffinose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-thiogalactose, phenyl-β-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside, *O*-nitrophenyl-β-D-galactopyranoside and type 2 porcine stomach mucin (PSM).

The effects of pH, temperature, EDTA and divalent cations on hemagglutinating activity were evaluated as described by Sampaio et al. [23].

2.5. Molecular mass and sugar content

Molecular mass of ALL under denaturing condition was estimated by SDS–PAGE [24] in the presence and absence of β-ME. LMW-SDS Marker kit (GE Healthcare, UK) was used as standard.

ALL relative molecular mass was estimated by size exclusion chromatography in the BioSuite column coupled to an Acquity UPLC system (Waters Corp, MA, USA). Chromatography was conducted in TBS at a flow rate 0.5 mL min^{-1}. The column was previously calibrated with protein mix containing BSA (66 kDa), ovalbumin (45 kDa), *carbonic anhydrase* (29 kDa), ribonuclease (14 kDa) and aprotinin (6.5 kDa).

Average molecular mass was determined by Electrospray Ionization-Mass Spectrometry (ESI–MS). ALL (10 pmol μl^{-1}) was dissolved in a solution of ACN 50% containing 0.2% FA. An aliquot of 100 μL of this solution was centrifuged at 8000 × *g* for 5 min and then infused into a nanoelectrospray source coupled to a Synapt HDMS ESI-Q-ToF mass spectrometer (Waters Corp. MA, USA) using a Hamilton syringe. The instrument was calibrated with [Glu1]-Fibrinopeptide B fragments. Mass spectra were acquired by scanning at m/z ranging from 1500 to 3500, at 2 scans s^{-1}. The mass spectrometer was operated in positive mode, using a source temperature of 363 K and capillary voltage at 3.2 kV. Data collection and processing were controlled by Mass Lynx 4.1 software (Waters).

Additionally, the average molecular mass of ALL was determined by MALDI-ToF on an Autoflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), using matrix solution (10 mg mL^{-1} of CHCA on acetonitrile, water, TFA, 50, 47, 3% v/v). The spectra were acquired in linear positive mode and processed with Flex Analysis 3.4 software (Bruker Daltonics, Germany).

Neutral carbohydrate content in ALL was evaluated in accordance with Dubois et al. [25], using lactose as the standard.

2.6. Quantification of sulfhydryl groups

To quantify free cysteines, ALL (1 mg mL^{-1}) was incubated with 55 mM IAA in the dark for 45 min at room temperature and subjected to desalting by gel filtration chromatography on a Zeba™ Spin Desalting column, 7 K MWCO (Thermo Scientific, MA, USA).

To quantify total cysteines, ALL was reduced with DTT 10 mM at 56 °C for 1 h and alkylated with 55 mM IAA in the dark for 45 min at room temperature. Then, carboxamidomethylated (CAM)-ALL was desalted in Zeba™ column. Alternatively, ALL was treated with β-ME 5 mM for 45 min at 56 °C and then with *VP* 20 mM for 1 h in dark at room temperature. After that, pyridylethylated (PE)-ALL was desalted.

All samples were freeze-dried, solubilized in ACN 50% containing FA 0.2%, and subjected to ESI–MS analyses as described above.

2.7. MS/MS

SDS-PAGE was performed as described above. Protein bands were excised and treated with DTT and IAA according to pre-established methods [26]. Digestion with trypsin was carried out as described by Carneiro et al. [18]. Peptides were extracted from gel according Shevchenko et al. [26].

Tryptic peptides were separated on a reverse phase C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system. The eluates were analyzed in a hybrid mass spectrometer (ESI-Q-ToF) (Synapt HDMS, Waters Corp. MA, USA). The instrument parameters were adjusted as described by Carneiro et al. [18].

Collision induced dissociation (CID) spectra were manually interpreted, and sequenced peptides were searched online against NCBI and Uniprot databanks.

2.8. Circular dichroism measurement

Circular Dichroism (CD) spectroscopic measurement was performed on a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) connected to a peltier with controlled temperature. ALL (0.2 mg mL^{-1} in 20 mM phosphate buffer, pH 7.0, containing 150 mM NaCl) was placed in a rectangular quartz cuvette with 0.5 mm path length. Spectra were acquired at a scan speed of 50 nm min^{-1} with a bandwidth of 1 nm. The acquisitions were performed at 190–250 nm (far UV). The analyses of secondary structure prediction were performed by DICHROWEB web server [27].

The thermodynamics parameters of ALL folding and unfolding in the presence and absence of lactulose, α-lactose and β-lactose were calculated by monitoring the *changes in ellipticity* at 205 nm as a function of temperature [28]. The lectin (8 μM) was submitted to temperatures ranging from 70 °C to 105 °C with a ramp rate of 3 °C min^{-1} and sampling at each 1 °C.

Ligand-induced changes in the molar *ellipticities* of the lectin at 363 K were employed to find the affinity constant (K*a*) for binding of carbohydrates to the lectin as proposed by Greenfield [28,29].

2.9. Antibacterial activity

2.9.1. Microorganisms and culture conditions

Antimicrobial activities were performed using *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 11303. Bacterial cells were grown on Tryptic Soy Agar medium (TSA; Himedia, India) for 24 h at 37 °C. To prepare the bacterial suspension, some isolated colonies of each bacterium were collected from the TSA plates and grown in Tryptic Soy Broth (TSB; Himedia, India) for 24 h at 37 °C under constant agitation. Cell suspensions were prepared in TSB at a cell density of 2×10^6 CFU mL^{-1}, unless otherwise stated.

2.9.2. Bacterial aggregation

S. aureus and *E. coli* and were grown in TSB at 37 °C for 24 h and harvested by centrifugation at 2000 × *g* for 10 min. Agglutination assays were performed as described by Melo et al. [30].

2.9.3. Effects of the lectin on planktonic cells

The effect of ALL on planktonic growth was determined by the broth microdilution method according to guidelines from the National Committee for Clinical Laboratory Standards, M7-A6 [31], with some modifications, as described by Vasconcelos et al. [32].

2.9.4. Biofilm formation assay

Bacterial biofilms were developed on sterile 96-well plates based on the microtiter plate test developed by Stepanovic et al. [33], with modifications. The assay was performed by the addition of 100 μL of each bacterium (2×10^6 CFU mL^{-1}) to each well with 100 μL of different concentrations of lectin (7.8–250 μg mL^{-1}). The microplates were incubated aerobically at 37 °C during 24 h in constant agitation for biofilm development. The biofilms were analyzed by crystal violet staining as an indicator of total biofilm biomass and by counting viable cells.

2.9.4.1. Biofilm mass quantification. Crystal violet staining was used as an indicator of bacterial biofilm biomass. After the growth of biofilm, the wells were washed with 200 μL of 150 mM NaCl to remove weakly adherent cells. Two hundred microliters of 99% methanol were added to each well for 15 min. Methanol was removed, and the plates were allowed to dry at 25 °C. Then, 200 μL of 0.1% (v/v) crystal violet were added, the excess of crystal violet was removed after 5 min, and the plates were washed twice with water. Finally, acetic acid 33% (v/v) was added. The optical density was measured at 590 nm (OD590) on a microtiter plate reader (SpectraMax® I3, Molecular Devices LLC, Sunnyvale, CA, USA).

2.9.4.2. Quantification of number of viable cells in the biofilms. The plates containing biofilm were washed twice with 200 μL of 150 mM NaCl, and biofilm suspensions were removed by sonication for 10 min. Serial decimal dilutions from the obtained suspensions were plated on TSA to verify the number of viable cells in the biofilms. After plating the serial dilution on TSA, plates were incubated at 37 °C in an aerobic incubator for 24 h prior to counting. The total number of colony forming units (CFU) (logCFU mL^{-1}) was determined.

2.9.5. Statistical analysis

Statistical analyses were performed by GraphPad Prism® version 5.0 from Microsoft Windows®. The data from all assays were compared using one-way analysis of variance (ANOVA), with Bonferroni *post hoc* test. The data were considered significant when $p < 0.05$.

3. Results

3.1. Purification of ALL

Crude extract of *A. lactuca* showed strong hemagglutinating activity against human and rabbit erythrocytes, both native and treated with proteases. After ammonium sulfate precipitation, hemagglutinating activity was concentrated in fraction F 0–70. This fraction was loaded onto guar gum column. The unbound proteins washed with TBS showed residual hemagglutinating activity, whereas the retained proteins eluted with glycine buffer showed strong activity against all erythrocytes tested (data not shown).

This procedure increased 110-fold the lectin's specific activity compared to the crude extract. ALL eluted from the column represented 76% of the total hemagglutinating present in the extract (Table 1).

3.2. Hemagglutinating activity and inhibition of ALL

ALL was able to agglutinate all tested erythrocytes with slight preference for untreated erythrocytes from rabbit.

Hemagglutinating activity of ALL was inhibited by the disaccharides α-lactose, β-lactose and lactulose with minimum inhibitory concentrations (MIC) of 25 mM, 50 mM and 50 mM, respectively. The glycoprotein PSM was the most potent inhibitor with MIC of 4 μg.mL^{-1} (Table 2).

The optimum pH for lectin activity was 7. At pH 5 and 6, the activity of ALL decreases, and at pH 4, it was almost entirely lost. Above pH 8, lectin activity was slightly reduced (Fig. 1A). The activity was unaltered until 60 °C. Above of 60 °C, it decreases, and ALL

Table 1
Purification procedure of the lectin from *Aplysina lactuca* (ALL).

Fraction	Protein total (mg)	HU mL^{-1}	Specific activity		Yield (%)	Purification (fold)
			(HU mg^{-1})	Total		
Crude extract	934	128	137	128,000	100	1
F0-70	523.9	1024	278	173,056	135	2
Guar gum	6.5	1024	15,058	98,304	76.8	110

Rabbit erythrocytes in native form were used.

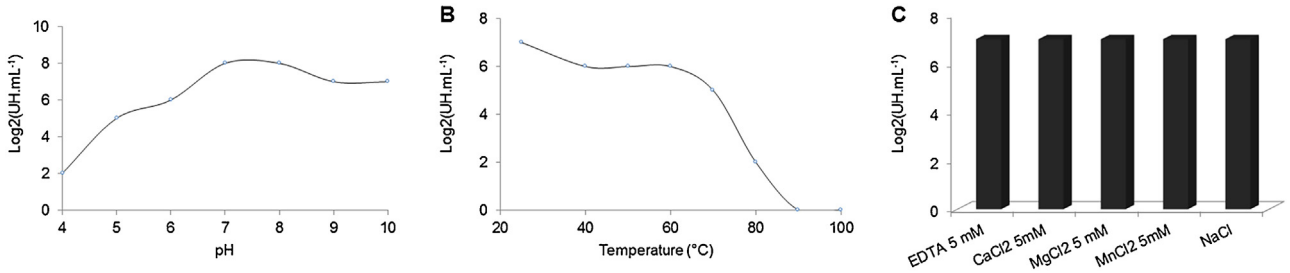

Fig. 1. Properties of the hemagglutinating activity of ALL. Effect of pH (A), temperature (B) and divalent cations and EDTA (C) on the hemagglutinating activity of ALL. Hemagglutinating activity was expressed in logarithm scale as units of titter. All assays were realized in duplicate.

Table 2
Inhibition of the hemagglutinating activity of ALL by sugars and glycoproteins.

Sugars	MIC[a]
α-D-Lactose	25 mM
β-D-Lactose	50 mM
α-D-Lactulose	50 mM
Glycoproteins	
Porcine Stomach Mucin	4 μg.mL^{-1}

The following sugars did not cause inhibition: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, D-maltose, D-raffinose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-thiogalactose, phenyl-β-D-galactopyranoside, 4-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-β-D-galactopyranoside, *O*-nitrophenyl-β-D-galactopyranoside.

[a] Minimum concentration of sugar required for inhibition.

was completely denatured at 90 °C (Fig. 1B). The presence of EDTA and $CaCl_2$ did not affect the activity of ALL (Fig. 1C).

3.3. Molecular mass and sugar content

SDS-PAGE of the pure lectin in the absence of β-ME showed one single band of 28 kDa, which was decomposed to a broad band of 19 kDa after treatment with reducing agent (Fig. 2). On gel filtration, ALL exhibited one single peak, corresponding to 94 kDa (Fig. 3).

ESI–MS deconvoluted spectra of intact lectin revealed one major ion of 31,810 ± 2 Da, with several low intensity ions around this value (Fig. 4A). When ALL was treated with IAA, no shift in mass spectrum was observed, indicating the absence of free-cysteines (data not shown).

On the other hand, when ALL was previously treated with DTT and incubated with IAA (CAM-ALL), two major ions were observed: 15,908 ± 2 Da (ALL-a) and 16,159 ± 2 Da (ALL-b) (Fig. 4B). When previously treated with β-ME and incubated with VP (PE-ALL), two other major ions were observed: 16,054 ± 2 Da and 16,302 ± 2 Da (Fig. 4C). The difference in molecular mass between CAM-ALL and PE-ALL suggests the presence of three cysteines, all of which are involved in disulfide bonds.

Intact ALL submitted to MALDI-ToF analysis (Fig. 5) revealed two major ions of 31,760 ± 2 Da (ALL-A) and 31,946 ± 2 Da (ALL-B).

In addition, ALL is not a glycoprotein, as observed in the Dubois assay.

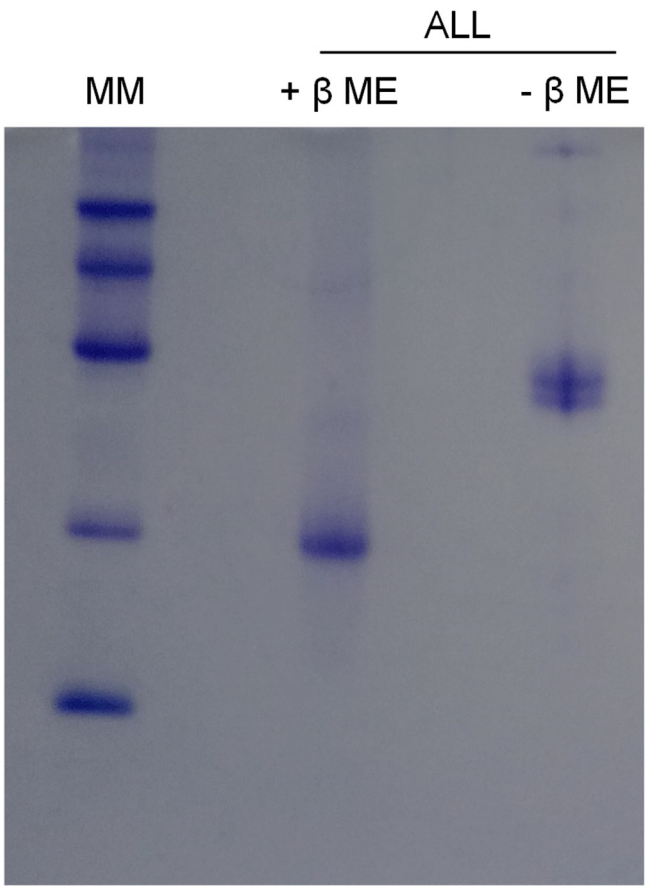

Fig. 2. SDS-PAGE of purified ALL. SDS-PAGE 15%. M) Molecular Marker. 20 μg of ALL were applied in the absence and presence of β-mercaptoethanol.

3.4. MS/MS

After in-gel digestion of ALL, eight tryptic peptides were identified and sequenced (Table 3). Together, these peptides represent 58% of the lectin's amino acid sequence. The partial amino acid sequence of ALL showed 42% of identity and 56% of similarity

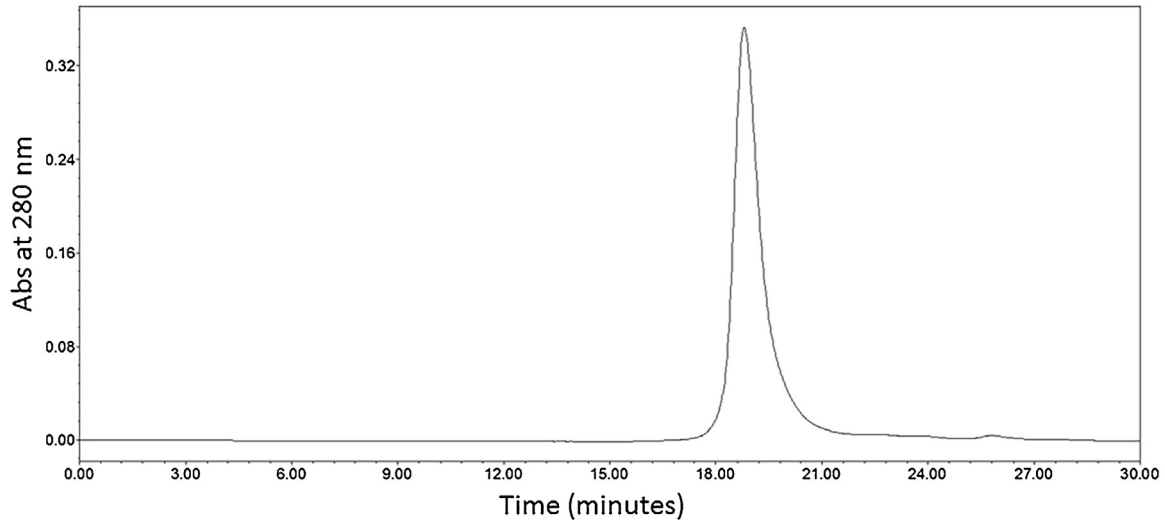

Fig. 3. Size exclusion chromatography of ALL. BioSuite 250 h SEC column (0.78 × 30 cm, 5 um particle size, Waters Corp.) was equilibrated and eluted with Tris-HCl, 50 mM, pH 7.2, containing NaCl 500 mM. Approximately 200 μg of ALL were loaded. UPLC operated at flow of 0.5 mL min^{-1}.

Table 3
Peptides originated by digestion of ALL with Trypsin.

m/z	Name	Sequence	Mass		Δ (Da)
			Observed	Calculated	
786.9293	T1	V[L/I]VSNVQNTVGTT[L/I]K	1571.8430	1571.8883	0.05
643.8115	T2	[L/I]ASESWNPARR	1285.6074	1285.6527	0.05
774.3490	T3	C[L/I]PEQE[L/I]YNVR	1546.6824	1546.7814	0.10
763.3114	T4	VQFDDQGYDAV[L/I]R	1524.6072	1524.7208	0.11
713.3212	T4′	VQFGNSGYDAV[L/I]R	1424.6268	1424.7048	0.08
502.9058	T5	TQFKEVATYTYR	1505.6940	1505.7515	0.06
443.2164	T6	[L/I]PVNTVSR	884.4172	884.5079	0.09
642.3108	T7	Q[L/I]QMVYFN[L/I]K	1282.6060	1282.6743	0.07

Table 4
Affinity constants and free energy of the binding of sugars to ALL at 363 K.

Sugar	K*a* (M^{-1})	α	$-\Delta G_F$(kJ mol^{-1})
Native	–	0.34	−366.1
α-D-lactose	12.5	0.44	−574.9
β-D-lactose	31.9	0.53	−805.5
D-Lactulose	145.5	0.66	−1463.7

α – Fraction folded at 363 K.
ΔG_F – Free energy of folding.

with the galactose-binding lectin isolated from *Axinella polypoides* (P28586.1), lectin I (Fig. 6).

3.5. CD

CD spectra of native ALL exhibited one minimum at 216 nm, suggesting a predominance of β-conformation in its secondary structure (Fig. 7). The prediction method CONTIN [34] indicated that the theoretical secondary structure consisted of 9% α-helix, 35% β-sheet, 23% β-turn and 33% coil.

Thermodynamic parameters of ALL, as determined by CD, revealed the binding constant to the ligands lactulose, α-lactose and β-lactose. The anomeric forms of lactose (α and β) showed close values of K*a*, whereas lactulose appears to be the ligand with which ALL has more affinity (Table 4).

3.6. Antibacterial activity and bacterial aggregation

ALL agglutinated both bacterial cells tested, *S. aureus* and *E. coli* (Fig. 8). On the other hand, the lectin was unable to inhibit the planktonic growth of both species (data not shown).

3.7. Effect of ALL on biofilm formation

ALL significantly reduced the biomass of both bacterial biofilms tested. Biofilm mass of *E. coli* was decreased in all tested concentrations (Fig. 9A). However, the effect on *S. aureus* biofilm showed a concentration-dependent behavior with activity in concentrations ranging from 250 to 15.6 μg mL^{-1} (Fig. 9B). Contact between ALL and both biofilms affected the number of viable cells at all concentrations tested (Fig. 9C and D).

4. Discussion

In this work, we reported the isolation and biochemical characterization of a new lectin from the marine sponge *A. lactuca*. ALL was purified by combining ammonium sulfate precipitation and affinity chromatography on guar gum column. Affinity chromatography on guar gum matrix has been employed to purify several galactophilic lectins, including HGA-2 from the sea cucumber *Holothuria grisea* and PFL from *Ptilota filicina* [23,30].

The genus *Aplysina* has already had two galactophilic lectins isolated from *A. lacunosa* and *A. archeri*. Both are glycoproteins formed by homotetramers with 16 kDa *per* subunit linked by weak interactions, and their hemagglutinating activity is Ca^{2+}-dependent [8].

ALL differs from other *Aplysina* lectins in some features. First, ALL is a trimer of dimers. Two monomers are linked by disulfide bond, and three dimers are linked by weak interactions to form a functional unit of six polypeptide chains. Sponge lectins have shown a wide range of oligomeric organization, including monomeric polypeptide chains, dimers linked by weak interactions, heterotrimers, tetramers linked by disulfide bonds, and large oligomers [4,10,19,35,36].

Second, ALL activity is independent from divalent ions. The presence of divalent ions in the binding site of invertebrate lectins is usual, and several lectins showed this characteristic. Only a few lectins from sponges are Ca^{2+}-dependent [4,8,10,14].

Finally, *A. archeri* lectin and ALL showed distinct specificity [8]. The former was inhibited by several galactosides, especially

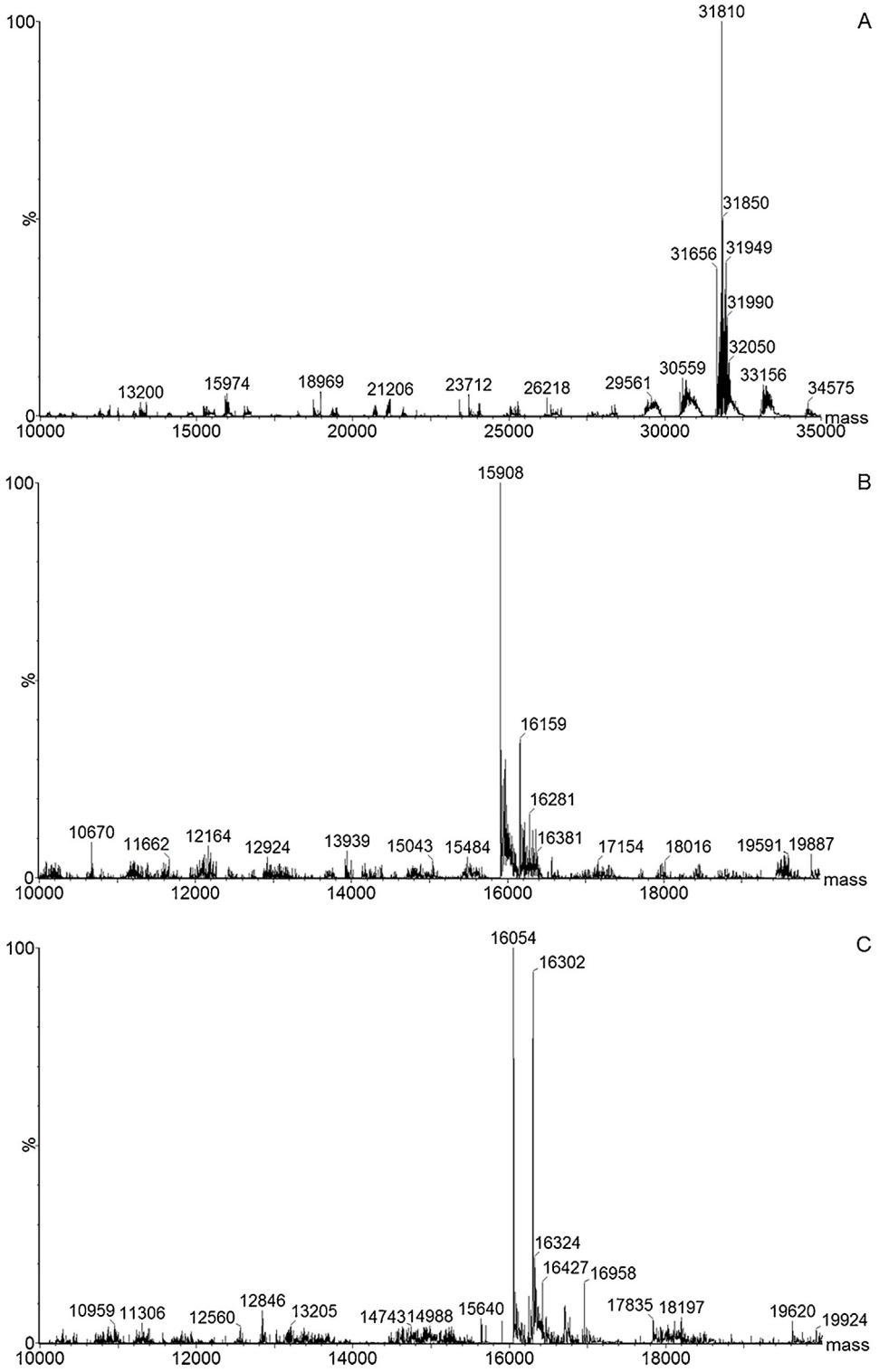

Fig. 4. Molecular mass determination of ALL by ESI–MS. A) Deconvoluted ESI mass spectra of ALL. The lectin (10 pmol μl^{-1}) was dissolved in a solution of ACN 50% containing 0.2% FA and infused into the NanoESI source coupled to an ESI-Q-ToF mass spectrometer. B) Deconvoluted mass spectra of CAM-ALL. C) Deconvoluted mass spectra of PE-ALL.

those that contain a terminal β-linked galactosyl, such as β-lactose, *N*-acetyllactosamine (lacNac) and digalactosyllactose, while thiol-β-D-galactose and β-methyl-D-galactopyranoside were unable to inhibit ALL activity. Indeed, ALL was isolated by guar gum from guar bean, a galactomannan composed of galactosyl linked (α1-6) to mannose backbone, and anomeric forms of the lactose were equally effective in the inhibition assay, which showed very similar K*a* values. Interestingly, these findings, when taken together, indicate that the specificity for terminal β-linked galactosyl is a characteristic that is not shared between *A. archeri* lectin and ALL.

However, ALL showed affinity for lactulose (β-linked galactosyl) and PSM, which presents T-antigen (Galβ1-3GalNac) in its composition, indicating that ALL is able to bind to β-galactosides, but not in an exclusive manner, and may also bind to α-galactosides, such as α-lactose and galactomannan.

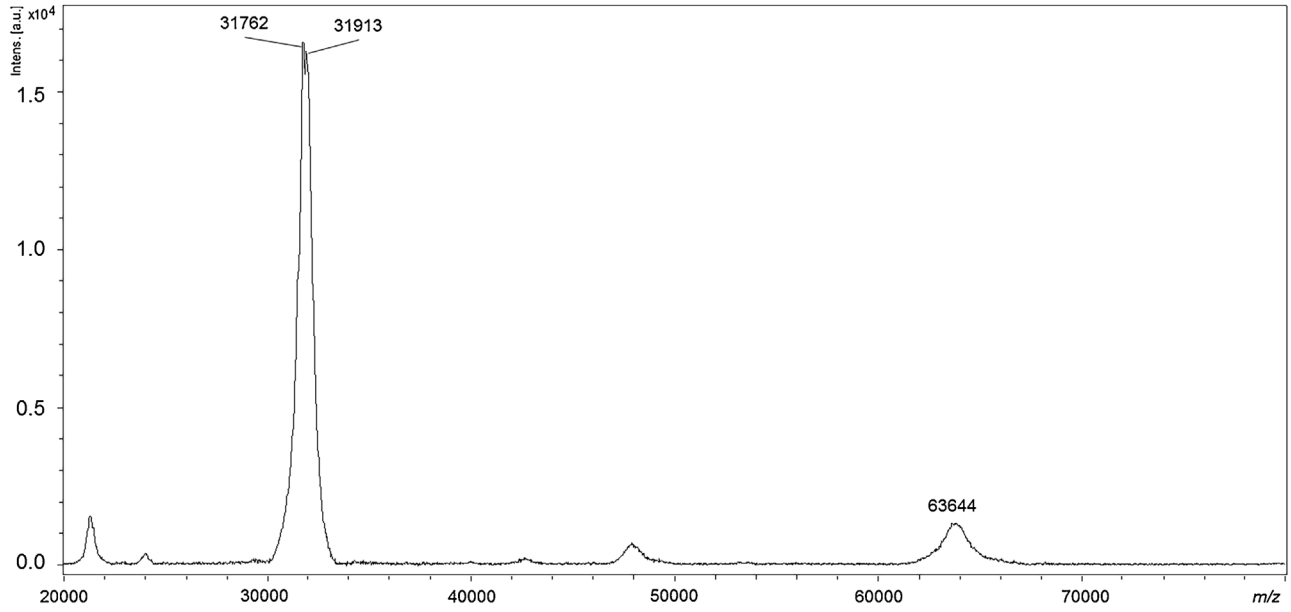

Fig. 5. Molecular mass determination of ALL by MALDI-ToF. The spectra were acquired in linear positive mode and processed with Flex Analysis 3.4 software.

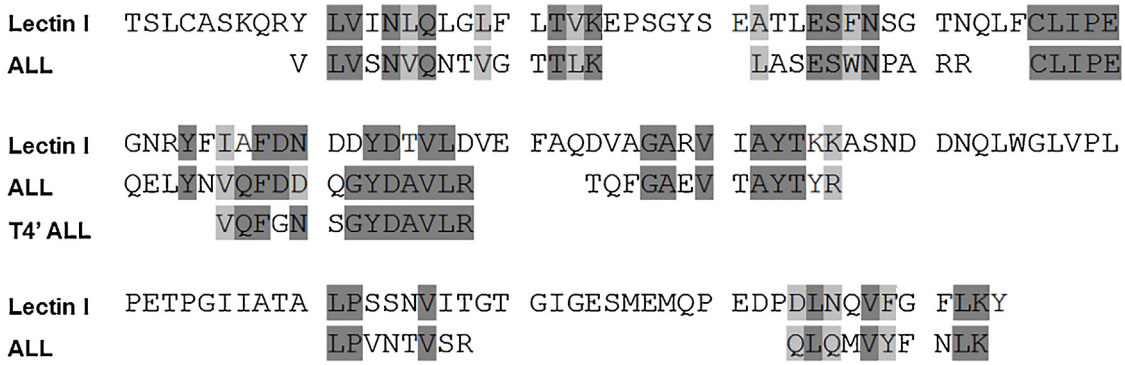

Fig. 6. Comparison of partial amino acid sequence of ALL and Lectin I from *Axinella polypoides*. Black boxes represent identical amino acids; gray boxes represent similar amino acids.

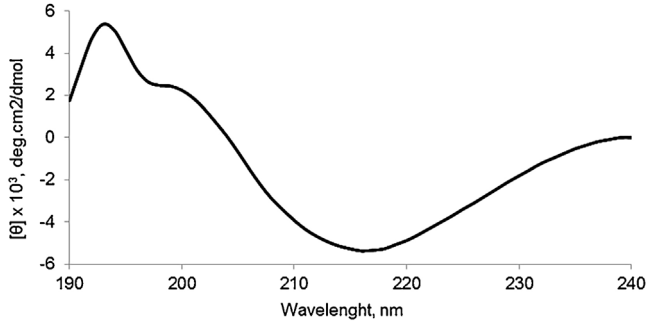

Fig. 7. CD spectra of ALL. Far-UV CD spectra (190–250 nm) of ALL. The cuvette path length was 0.05 cm; protein concentration was 0.2 mg mL^{-1} in PBS, pH 7. Eight acquisition were realized, an average value was calculated and plotted in the figure.

About 60% of the primary structure of ALL was obtained by amino acid sequencing of eight tryptic peptides. These peptides showed similarity with lectin I from *A. polypoides*. Indeed, ALL and lectin I shared some biochemical characteristics. For instance, both are non-glycosylated, their hemagglutinating activity is Ca^{2+}-independent, and their monomeric molecular masses are very close [17,37,38].

A. polypoides lectin I showed one intrachain disulfide bond between cysteines 4 and 46 [17]. ALL has three cysteines *per* subunit, and two of them seem to be involved in one intrachain disulfide bond. Since Cys^{46} appears conserved in ALL, it possible that disulfide bond 4–46 might present in ALL.

Furthermore, isolectins seem be present in both species. In *A. polypoides*, five lectins have been described. Lectins I–IV bind to galactose, whereas lectin V binds to hexuronic acids. Lectin II and V were identified in the first fifteen NH_2-terminal amino acids, and lectins I and II have similar molecular mass values (15,847 ± 10 Da and 16,228 ± 10 Da, respectively), sharing 65% of identity among I and II [17,38].

Like lectins I and II of *A. polypoides*, MS analyses of ALL revealed two distinct isolectins (A and B). When ALL was treated with reducing agents, followed by treatment with alkyl agents, subunits ALL-a and ALL-b were observed. Surprisingly, the combined molecular masses of the subunits did not correspond to the molecular mass of the intact isolectins. The deduced molecular masses of ALL-a and ALL-b are 15,737 ± 2 Da and 15,988 Da ± 2 Da, respectively, totaling hypothetical dimers of 31,474 Da (2x a), 31,725 Da (a + b) or 31,976 Da (2 × b), whereas molecular mass, as determined by MALDI-MS, was 31,760 ± 2 Da and 31,913 ± 2 Da for −A and −B, respectively. Interestingly, the average mass, as determined by ESI–MS, differs from both molecular mass, as determined by

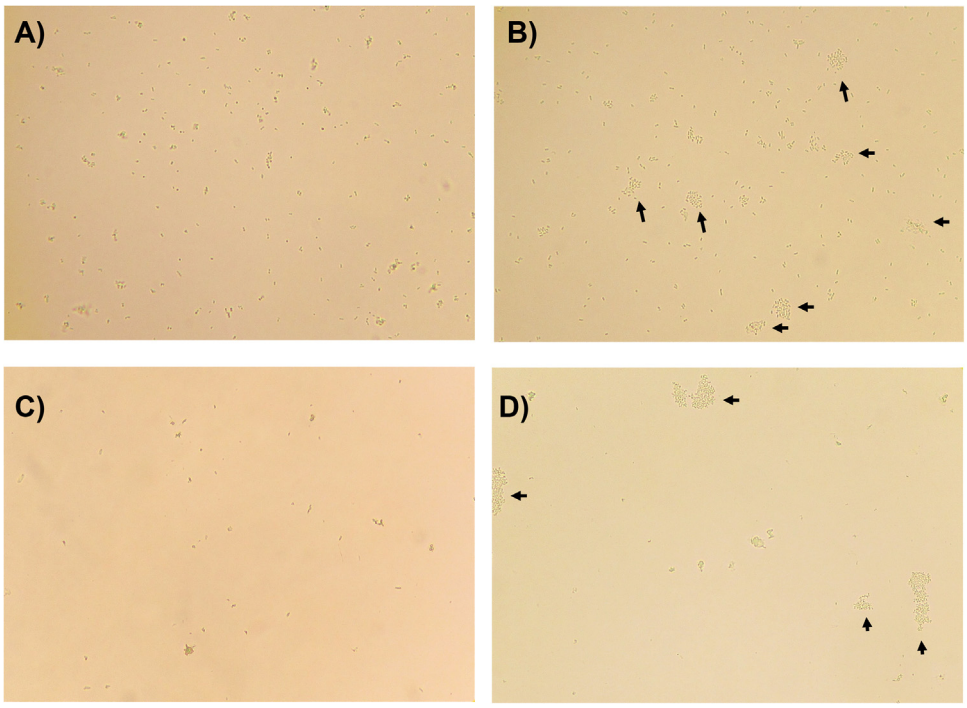

Fig. 8. Agglutination of bacteria by ALL. *S. aureus* incubated with TBS (A) and ALL (B); *E. coli* incubated with TBS (C) and ALL (D). Arrows indicate bacterial agglutination. Agglutinations were realized in duplicated.

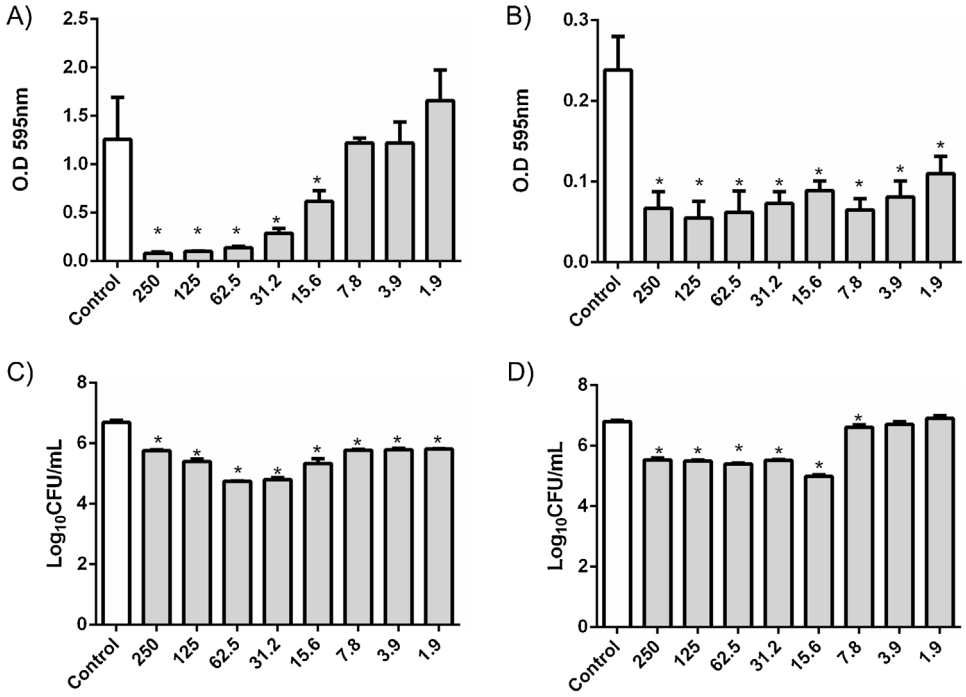

Fig. 9. Biofilm formation of S. aureus and E. coli in the presence of different concentration of ALL. (A and B) Total biomass and (C and D) number of viable cells present in the biofilms. (A) and (C) represents *S. aureus* and (B) and (D) E. coli biofilm. *$p < 0.05$ compared to control (corresponding to 0 μg/mL). Error bars display standard deviations (SD) of the means. The assays were repeated in triplicate on three different occasions.

MALDI-ToF, and molecular mass of the hypothetical dimers. The reason for this divergence is unclear, but it can be speculated that intrinsic properties of the analyte, as well as intrinsic properties of the instrument, may have contributed to these observations.

The major difference between lectins I and II of *A. polypoides* is the insertion of an alanyl-dipeptide in position 131–132 in lectin II. Therefore, lectin II is two amino acids longer than lectin I [38]. In ALL, peptides T4 and T4′ are quite different in their amino acid sequence, which may contribute to the difference between ALL-A and ALL-B.

Despite the common features, the differences between lectin I and ALL are remarkable. In lectin I, subunits are linked by weak interactions, and high affinity for non-reducing terminal β-linked galactosyl was observed [8,37].

Lectin I from *A. polypoides* was initially classified as a $(QxW)_3$-lectin, a family of lectins that included ricin [39]. Currently, ricin and other $(QxW)_3$-lectins are included in the R-type lectin family. Members of the R-type family present repeated domains similar to ricin and, of course, the $(QxW)_3$ motif (Hirabayashi, Dutta, & Kasai, 1998). However, the homology between lectin I and R-type lectins is weak since *Axinella* lectins display just one (QxW) motif, and the alignment of six other conserved amino acids is only achieved by inserting gaps [38].

Axinella lectins and ALL shared a certain level of similarity and, therefore, could be grouped into the same family. However, to accomplish this, the determination of the complete amino acid sequence of ALL is required.

The secondary structure of ALL was predominately formed by β-conformations. Several structural domains of proteins are formed by such predominance, some of which are present in lectins, such as *jelly roll* β-sandwich and β-trefoil, typical domains of L- and R-type lectins, respectively [40].

ALL has the ability to agglutinate Gram-positive and Gram-negative bacterial cells. Several sponge lectins may bind specific glycans on the cell surface of microorganisms, causing bacterial aggregation and/or exhibiting antimicrobial activities [15,41,42]. Moreover, some galactoside-specific lectins are able to cause agglutination of bacterial cells [10,30,43]. Several glycosylated structures present on the cell surface of Gram-positive and Gram-negative bacteria can be recognized by ALL, including peptidoglycan, capsular polysaccharides, teichoic acid and LPS. According to Gardères and coworkers [4], the ability of sponge lectins to bind specific carbohydrates in bacterial cells could potentially be used to develop new antimicrobial agents.

Sponge lectins have shown a large spectrum of antibacterial activities. For instance, the lectin isolated from *Suberites domuncula* displays antibacterial activity against *E. coli* and *S. aureus* [15]. According to Müller and coworkers [44], the *S. domuncula* lectin acts as an antibacterial molecule involved in immune defense. Interestingly, despite causing bacterial agglutination, ALL does not inhibit bacterial growth. Corroborating our results, Kazanjian and Fariñas [45] showed that the lectin from *A. lacunosa* did not exhibit any antibacterial activity on Gram-positive and Gram-negative bacteria. However, the aqueous extract of *A. lacunosa* was able to inhibit bacterial growth. The activity was attributed to the presence of secondary metabolites in the extract [45].

On the other hand, the lectin from *A. lacunosa* strongly reduced *S. aureus* and *E. coli* biofilm formation. Lectins isolated from marine organisms, such as algae marine lectins, have demonstrated the ability to inhibit bacterial biofilm formation [46]. Studies point out that bacterial aggregation may cause a decrease in the number of adherent cells, consequently inhibiting the formation of biofilm [47]. The effect of ALL on bacterial biofilm can be exploited since this could prevent the emergence of drug- resistant strains.

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Artigo II

Purification, Biochemical Characterization, and Amino Acid Sequence of a Novel Type of Lectin from Aplysia dactylomela Eggs with Antibacterial/Antibiofilm Potential.

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ORIGINAL ARTICLE

Purification, Biochemical Characterization, and Amino Acid Sequence of a Novel Type of Lectin from *Aplysia dactylomela* Eggs with Antibacterial/Antibiofilm Potential

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Abstract A new lectin from *Aplysia dactylomela* eggs (ADEL) was isolated by affinity chromatography on HCl-activated Sepharose™ media. Hemagglutination caused by ADEL was inhibited by several galactosides, mainly galacturonic acid (Ka = 6.05 × 10^6 M^{-1}). The primary structure of ADEL consists of 217 residues, including 11 half-cystines involved in five intrachain and one interchain disulfide bond, resulting in a molecular mass of 57,228 ± 2 Da, as determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry. ADEL showed high similarity with lectins isolated from *Aplysia* eggs, but not with other known lectins, indicating that these lectins could be grouped into a new family of animal lectins. Three glycosylation sites were found in its polypeptide backbone. Data from peptide-N-glycosidase F digestion and MS suggest that all oligosaccharides attached to ADEL are high in mannose. The secondary structure of ADEL is predominantly β-sheet, and its tertiary structure is sensitive to the presence of ligands, as observed by CD. A 3D structure model of ADEL was created and shows two domains connected by a short loop. Domain A is composed of a flat three-stranded and a curved five-stranded β-sheet, while domain B presents a flat three-stranded and a curved four-stranded β-sheet. Molecular docking revealed favorable binding energies for interactions between lectin and galacturonic acid, lactose, galactosamine, and galactose. Moreover, ADEL was able to agglutinate and inhibit biofilm formation of *Staphylococcus aureus*, suggesting that this lectin may be a potential alternative to conventional use of antimicrobial agents in the treatment of infections caused by Staphylococcal biofilms.

Keywords Sea hare · Lectin · Galacturonic acid · Biofilm · Mass spectrometry

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Introduction

Lectins are sugar-binding proteins that possess the ability to decipher the glycocode. They have been found in all living organisms. In mollusks, lectins have been detected and purified from cephalopods, gastropods, and bivalves (Alpuche et al. 2010; Bulgakov et al. 2004; Fujii et al. 2011; Song et al. 2011). Among the prospected lectins representatives of some structural families are found, such as C-, F-, and R-types; galectins; and RBLs (Chen et al. 2011; Fujii et al. 2012; Naganuma et al. 2006; Pales-Espinosa et al. 2010; Song et al. 2011).

The presence of lectins in mollusks has been associated with hemolymph, mucus, hemocytes, and reproductive tissues (Chen et al. 2011; Gilboa-Garber et al. 1985; Ito et al. 2011; Zhang et al. 2014), and evidences suggest that they can act as pattern recognition receptors (PRRs), recognizing several pathogen-associated molecular patterns (PAMPs). In fact, lectin-coding genes have been found be upregulated after infection by pathogenic microorganisms (Chen et al. 2011; Mu et al. 2014). Moreover, some studies have explicitly demonstrated the antibacterial potential of lectins isolated from mollusks (Zheng et al. 2008; Takahashi et al. 2008; He et al. 2011).

Gastropoda is the most broadly distributed class of mollusk, and several lectins from gastropods have been isolated (Fujii et al. 2011; Gilboa-Garber et al. 1985; Kawsar et al. 2009). *Aplysia dactylomela* Rang, 1828 is a circumtropical aplysid gastropod and occurs worldwide in tropical to warm temperate waters (Matthews-Cascon et al. 2011). In the West Atlantic, this species is found in Florida, Mexico, the Caribbean, and Brazil. It is the biggest opisthobranch found in Northeastern Brazil and one of the main herbivores inhabiting consolidated intertidal bedrock. The egg mass of an *A. dactylomela* is a resistant gelatinous string that can reach 8 m in length. Egg mass color is variable, reaching tones of yellow, orange, and pink (Matthews-Cascon et al. 2011).

Currently, only a fetuin-binding lectin named dactylomelin-P has been isolated and characterized in some detail from the purple gland of *A. dactylomela* (Melo et al. 2000).

In the present work, we have isolated, characterized, and determined the primary structure and estimated the secondary structure of a new lectin from the eggs of *A. dactylomela*. Additionally, we created a 3D model and evaluated its antibacterial and antibiofilm potentials.

Material and Methods

Egg Collection and Extraction

Aplysia dactylomela eggs were collected during low tide in the intertidal zone at Pacheco Beach (3° 41′ 13.0″ S; 38° 38′ 21.7″ W), Caucaia, Brazil. Collections were authorized and certified by responsible environmental authorities (SISBIO ID: 33913-8).

Eggs were removed from stones and algae, washed with seawater, and transported to the lab. Immediately thereafter, eggs were crushed using mortar and pestle. Proteins from smashed eggs were extracted with ten volumes of 20 mM Tris-HCl, pH 7.6, containing 150 mM NaCl and phenylmethylsulfonyl fluoride (PMSF) 0.1 mM (Tris-buffered saline (TBS)). The mixture was then centrifuged at 8000×*g* for 20 min at 4 °C. The crude extract was stored at −20 °C until use. Protein content was determined by Bradford reagent, and BSA was used as protein standard (Bradford 1976).

Lectin Purification

The crude extract was applied to an HCl-activated Sepharose™ 4B column (1.0 × 8.0 cm), previously equilibrated with TBS. Unbound proteins were washed with the same buffer, and adsorbed proteins were recovered with TBS containing 200 mM α-lactose. Chromatography eluates were monitored by absorbance at 280 nm using a UV/Visible spectrophotometer. Retained fractions containing the purified lectin were exhaustively dialyzed against distilled water, freeze-dried, and stored at −20 °C until required.

Hemagglutinating Activity and Inhibition Assays

Hemagglutinating activity (HA) and inhibition assays were performed following pre-established methods (Sampaio et al. 1998), using human (A, B, and O) and rabbit erythrocytes, both treated with proteases and untreated.

The following sugars and glycoproteins were used in the inhibition assay: D-xylose, D-ribose, L-fucose, L-arabinose, L-rhamnose, D-galactose (Gal), D-mannose, D-glucose (Glc), D-glucosamine, D-galactosamine, *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), D-galacturonic acid, D-fructose, D-sucrose, D-melibiose, α-D-lactose, β-D-lactose, D-lactulose, D-maltose, D-raffinose, methyl-α-D-galactopiranoside, methyl-β-D-galactopiranoside, methyl-β-D-thiogalactose, phenyl-β-D-galactopiranoside, 4-nitrophenyl-α-D-galactopiranoside, 4-nitrophenyl-β-D-galactopiranoside, 2-nitrophenyl-β-D-galactopiranoside, O-nitrophenyl-β-D-galactopiranoside, and type 2 porcine stomach mucin (PSM).

Biochemical Characterization

The effects of pH, temperature, divalent cations, and EDTA on HA were determined as described by Sampaio et al. (1998). Molecular mass of the lectin was estimated by SDS-PAGE, as described by Laemmli (1970). LMW-SDS marker kit (GE Healthcare, IL, USA) was used as standard.

Native molecular mass was estimated by size exclusion chromatography (SEC) on BioSuite 250 HR SEC column (0.78 × 30 cm, 5-μm particle size, Waters Corp., MA, USA), coupled to an Acquity UPLC system (Waters Corp., MA, USA), which had been equilibrated with Tris-HCl 20 mM, pH 7.2, containing NaCl 500 mM. The column was previously calibrated with a mixture of standard proteins: conalbumin (75 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), ribonuclease A (14 kDa), and aprotinin (6.5 kDa).

Molecular Mass Determination

Molecular mass was determined on an AutoFlex III matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)/TOF mass spectrometer (Bruker Daltonics, Germany).

The samples were dissolved in deionized water at variable concentration, mixed (1:3 *v*/*v*) in an α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (5 mg/mL of CHCA on acetonitrile, water, and trifluoracetic acid, 50, 40, and 10% *v*/*v*), spotted onto a sample plate, and then dried at room temperature. The spectra were acquired in linear positive mode and were processed with Flex Analysis 3.4 software (Bruker Daltonics, Germany).

N-Terminal Sequencing

An aliquot of 15 μg of purified lectin was solubilized in 15 μL of acetonitrile 10% and centrifuged at 8000×*g* for 5 min at room temperature. The solution was applied into a glass fiber disk and submitted to N-terminal sequencing.

Automated Edman degradations were performed in the Shimadzu model PPSQ-31A protein sequencer (Shimadzu Corp., Japan). PTH-amino acids from the N-terminus sequence were separated on a 2.0 × 250 mm Wakosil ODS column (Wako Pure Chemical Corp., Osaka, Japan) connected to a model LC-20AT pump. The absorbance was detected at 269 nm with a UV–Vis SPD-20A detector.

Primary Structure Determination by Tandem Mass Spectrometry

Purified lectin was submitted to SDS-PAGE as described above. After staining, electrophoretic bands were excised using a plastic pipette tip. Spots were reduced with dithiothreitol, and the lectin was carboxamidomethylated (CAM-lectin) with iodoacetamide, following pre-established methods (Shevchenko et al. 2006).

Treated spots were digested with trypsin, chymotrypsin, and pepsin. Digestions with trypsin and chymotrypsin were performed in 50 mM ammonium bicarbonate at 1:50 *w*/*w* (enzyme/substrate). Digestion with pepsin was performed in 100 mM HCl at 1:50 *w*/*w* (enzyme/substrate). All digestions were maintained for 18 h and then stopped with 2 μL of 2% formic acid. Peptides were extracted from gel according to Shevchenko et al. (2006).

Peptide solution was loaded onto a C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system (Waters Corp., MA, USA). The eluates were directly infused into a nanoelectrospray source. Chromatography and mass spectrometry analysis were performed according to established methods (Carneiro et al. 2013).

Deglycosylation of tryptic peptides was carried out by Glycoprofile™ II, Enzymatic In-Solution N-Deglycosylation Kit (Sigma-Aldrich, St. Louis, MO, USA). First, the lectin (1 mg/mL) was solubilized in RapiGest™ SF surfactant 0.1% (Waters Corp., MA, USA), followed by digestion of CAM-lectin with trypsin 1:100 *w*/*w* (enzyme/substrate) for 18 h at 37 °C. RapiGest™ SF surfactant was pelleted by addition of TFA at final concentration of 0.2%.

Tryptic peptides were digested with peptide-N-glycosidase F (PNGase F) (1 U) (Sigma-Aldrich, St. Louis, MO, USA) for 18 h at 37 °C. Deglycosylated peptides were loaded onto a C-18 nanocolumn, and the eluates were directly infused into a nanoelectrospray source. The mass spectrometer was operated in positive mode with a source temperature of 373 K and a capillary voltage at 3.0 kV. MS/MS was performed according to the data-dependent acquisition (DDA) method. The lock mass used in acquisition was *m*/*z* 785.84 ion of the [Glu1] fibrinopeptide B. The selected precursor ions were fragmented by collision-induced dissociation (CID) using argon as the collision gas. All CID spectra were manually interpreted.

Circular Dichroism

Circular dichroism spectroscopic measurement was performed on a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) connected to a peltier with controlled temperature. The lectin (0.2 mg/mL in 20 mM phosphate buffer, pH 7.0, containing 150 mM NaCl) was placed in a rectangular quartz cuvette with 0.5-mm path length. Spectra were acquired at a scan speed of 50 nm/min with a bandwidth of 1 nm. The acquisitions were performed at 190–250 nm (far-UV) and different temperatures (20–80 °C) with eight accumulations per temperature. The analysis of structural data was performed by DICHROWEB web server (Whitmore and Wallace 2008).

For measurement at 250–330 nm (near-UV), lectin at 0.5 mg/mL was previously incubated with D-galactose, α-D-lactose, D-galactosamine, and galacturonic acid at 10 mM each and placed in a rectangular cuvette with 5-mm path length. Spectra were acquired as described above.

The thermodynamic parameters of lectin folding and unfolding in the presence and absence of ligands were calculated by monitoring the changes in ellipticity at 293 nm as a function of temperature (Greenfield 2007). The lectin (8 μM) was submitted to temperatures ranging from 70 to 95 °C with a ramp rate of 4 °C/min, and sampling was conducted at 1 °C each in the presence and absence of D-galactose, D-galacturonic acid, D-galactosamine, and α-lactose at 80 μM each.

As proposed by Greenfield (2006, 2007), ligand-induced changes in the molar ellipticities of the lectin at 368 K were employed to find the affinity constant (Ka) for binding of carbohydrates to the lectin.

Structural Modeling

Structural Prediction

The structural prediction of the lectin was performed through interplay among the MODELER (Webb and Sali 2014) suite, I-TASSER (Yang and Zhang 2015), and QUARK (Xu and Zhang 2013) servers.

Initially, the complete protein sequence (217 amino acids) was submitted to I-TASSER and QUARK servers for overall modeling. In parallel, three sequence fragments from the lectin (amino acid residues 1–70, 71–141, and 142–217) were also submitted to these servers in order to obtain the individual folding of each protein domain. The best models were selected based on their C- and TM-scores[2]. Afterwards, the selected models were used as input to the MODELER suite, v.9.16. For structural modeling of the lectin, MODELER default parameters were used, and sequence alignment corrections were manually edited.

Initially, 30 theoretical models were generated, which were ranked based on their discrete optimized protein energy (DOPE) scores (Shen and Sali 2006). The models were then selected and analyzed for their stereochemical properties with the Protein Structure Validation Suite (PSVS) server, which integrates consolidated validation platforms, such as Procheck (Laskowski et al. 1993) and Molprobity (Davis et al. 2004). Finally, the best model was submitted to ModRefiner (Xu and Zhang 2013) (http://zhanglab.ccmb.med.umich.edu/ModRefiner).

Binding Site Prediction

A meta-server approach (http://zhanglab.ccmb.med.umich.edu) using the COACH (Yang et al. 2013) server was used to identify the most reliable carbohydrate-binding site.

Molecular Docking Calculations

The structural basis for carbohydrate recognition was explored through molecular docking calculations. For this purpose, four carbohydrates previously analyzed on hemagglutination inhibition assays were selected, including D-galactose (CID: 6036), D-galactosamine (CID: 24154), D-galacturonic acid (CID: 439215), and α-lactose (CID: 84571). All carbohydrate structures were obtained from the PubChem Substance and Compound database (Wang et al. 2009).

Calculations were performed with AutoDock Vina, version 1.1.2 (Trott and Olson 2009). The Autodock graphical interface AutoDockTools, version 1.5.6, was used to keep polar hydrogens and add partial charges to the proteins and ligands using Kollman United charges (Morris and Huey 2009). The protein and carbohydrate ligands were treated as rigid and flexible molecules, respectively.

Initially, a blind docking strategy was applied using a search space defined by a 40 Å × 60 Å × 40 Å cube covering the whole protein surface. Then, a refined search was performed using a search space defined by a 20 Å × 20 Å × 20 Å cube centered on the spot previously predicted by the COACH server as the most suitable carbohydrate-binding site. For all calculations, exhaustiveness was set to 15, and all other parameters were used as default. For each docking, the ten top-ranked generations based on the predicted binding affinity (in kilocalories per mole) were analyzed. The solutions were first chosen based on the presence of important interactions commonly visualized for carbohydrate recognition by lectins, such as the stacking of pyranosidic rings against aromatic or aliphatic amino acid residues and the proper disposal of hydrogen bonds around hydroxyl groups (Wittmann and Pieters 2013). Since all selected ligands presented similar chemical structures, a common binding pattern was sought. The most suitable results were further ranked based on the theoretical binding energy, which was given as a negative score in kcal/mol.

Antibacterial Activity

Microorganisms and Culture Conditions

The assays to evaluate antimicrobial activity were performed on *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 11303. Bacteria were grown in trypticase soy agar medium (TSA; Himedia, India) and incubated at 37 °C for 24 h. After growth on the solid medium, some isolated colonies were removed, inoculated into 10 mL trypticase soy broth (TSB; Himedia, India), and incubated for 24 h at 37 °C under constant agitation. Prior to use, the cell density of each bacterial suspension was adjusted to a final concentration of 2×10^6 cells/mL.

Bacterial Aggregation Assay

After bacterial growth, bacterial suspensions were harvested by centrifugation at 2000×*g* for 10 min, washed twice with TBS/Ca^{2+}, suspended in TBS/Ca^{2+} containing formaldehyde 4% and then kept for 16 h at 4 °C. Afterwards, the cells were washed twice with TBS/Ca^{2+}, and suspended in the same buffer. Bacterial count was estimated by the serial dilution method, and optical density at 620 nm (OD_{620}) was maintained around 1.0. Bacterial agglutination was performed as described by Melo et al. (2014).

Effects of the Lectin on Planktonic Cells

The effects of lectin on planktonic cells were assessed by the broth microdilution method. Briefly, the lectin was diluted in 150 mM NaCl at concentrations ranging from 7.8 to 250 μg/mL. The assay was performed in 96-well polystyrene plates by addition of 100 μL of each bacterium at 2×10^6 cells/mL to each well. The microplates were then incubated at 37 °C for 24 h in constant agitation, and the optical density was measured at 620 nm using a microplate reader (SpectraMax® I3, Molecular Devices LLC, CA, USA).

Biofilm Formation Assay

The effects on biofilm formation were evaluated as proposed by Vasconcelos et al. (2014). Briefly, sterile 96-well polystyrene plates were prepared following the same steps as

previously established for planktonic cell growth. However, two plates were used for this purpose: one for biomass quantification by crystal violet and the other for enumeration of biofilm viable cells. In order to verify the role of the carbohydrate recognition domain (CRD) on antibiofilm activity exerted by the lectin, it was tested after previous incubation with 12.5 mM α-D-lactose. The influence of protein folding on antibiofilm activity was also investigated. For this purpose, the lectin was tested after denaturation by heating 1 h at 100 °C.

Biofilm Mass Quantification Quantification of biofilm mass was determined by crystal violet staining. After biofilm development, the content of each well was removed, and the wells were washed twice with 200 μL of 150 mM NaCl to remove weakly adherent cells. For fixation of biofilms, 200 μL of 99% methanol were added to each well. After 15 min, the methanol was removed, and the plates were allowed to dry at 25 °C. Then, 200 μL of crystal violet were added to each well. After 5 min, the excess of crystal violet was removed, and the plates were washed twice with water. Finally, 200 μL of acetic acid (33%, *v*/*v*) were added to wells to dissolve the crystal violet bound to the biofilm mass, and the absorbance was measured at 590 nm (OD_{590}) using a microplate reader (SpectraMax® I3).

Quantification of Number of Biofilm Viable Cells In order to determine the number of viable cells, the wells were washed twice with 150 mM NaCl, and then 200 μL of the same solution were added to each well containing biofilms. The plates were placed in an ultrasonic bath for 10 min to release biofilm-entrapped cells. Serial decimal dilutions from the obtained suspensions were plated on TSA to allow viability determination. The plates were then incubated for 24 h at 37 °C, and the number of colony forming units per milliliter (CFU/mL) was determined.

Statistical Analysis

Statistical analyzes were performed by GraphPad Prism® version 5.0 from Microsoft Windows®. The data from all assays were compared using one-way analysis of variance (ANOVA), with Bonferroni post hoc test. The data were considered significant when $p < 0.05$.

Results

Lectin Purification

Crude extract of *A. dactylomela* eggs showed an evident and strong HA against human and rabbit erythrocytes. The HA induced by the treatment with extract was inhibited by the monosaccharide D-galactose and the disaccharide α-D-lactose. Affinity chromatography on HCl-activated Sepharose™ 4B was performed, and a novel lectin from sea hare eggs was successfully purified after elution with 0.2 M lactose (Fig. 1). In order to simplify the nomenclature, the new lectin was termed ADEL (derived from *A. dactylomela* eggs lectin). It was purified eight times and represents 81% of the total activity of the crude extract (Table 1).

Hemagglutinating Activity and Inhibition Assays

ADEL showed agglutinating activity in all tested erythrocytes in native and protease-treated forms. The lectin showed a slight preference for trypsin-treated rabbit erythrocytes (data not shown).

Several galactose-related carbohydrates showed inhibitory activity at distinct levels, with HA promoted by ADEL (Table 2). Galacturonic acid was the most potent inhibitor, followed by D-galactosamine and α-methyl-D-galactopyranoside. The

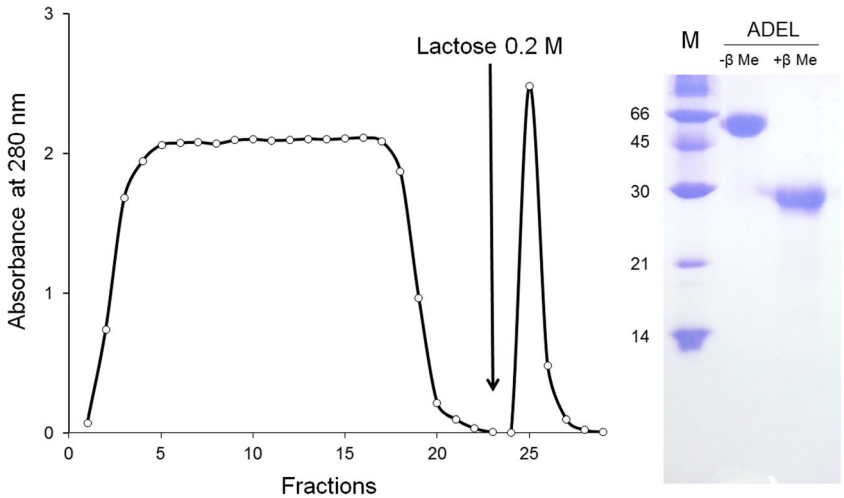

Fig. 1 ADEL purification. Affinity chromatography on HCl-activated Sepharose™ column (1.0 × 8.0 cm). The column was equilibrated and washed with TBS. Retained fractions were eluted with 0.2 M lactose in TBS. Approximately 20 mL of the crude extract were applied. Flow was 1 mL/min. SDS PAGE 15%. *M* molecular marker. ADEL in absence and presence of β-mercaptoethanol

Table 1 ADEL Purification

Fraction	Protein total (mg)	Titer (HU/mL)	Specific activity (HU/mg)	Total	Yield (%)	Purification (fold)	MAC[a] (μg/mL)
Crude extract	19.4	64	355	6887	100	1	2.80
ADEL	2.0	256	2844	5688	81	8	0.35

[a] Minimal concentration of lectin able to cause hemagglutination

minimum inhibitory concentrations (MIC) for these sugars were 0.2, 5, and 5 mM, respectively.

Biochemical Characterization

HA promoted by ADEL was optimal at pH 6 and 7. The lectin was stable in temperatures lower than 60 °C, and a complete loss of activity was achieved at 80 °C. EDTA and $CaCl_2$ did not affect lectin activity (data not shown).

SDS-PAGE of ADEL resulted in 60 and 29 kDa bands in the absence and presence of β-mercaptoethanol, respectively, suggesting that ADEL is a dimeric protein formed by two identical polypeptide chains linked by a disulfide bond (Fig. 1). Native molecular mass estimated by SEC was 14 kDa, suggesting an anomalous behavior of the lectin in gel filtration (Fig. 2).

MALDI-TOF mass spectrometric analysis showed two distinct ions: $[M + 1H]^{1+}$ at *m/z* 57,228 and $[M + 2H]^{2+}$ at *m/z* 28,613, which agrees with the molecular mass estimated by SDS PAGE (data not shown).

The first 29 amino acid residues of ADEL were identified by amino acid sequencing. The N-terminal sequence ^{1}DPDKCKTIRVESWSYKYAEKVVEDASYVL^{29} showed high similarity with a galacturonic acid-binding lectin from *Aplysia kurodai*.

Table 2 Inhibition of the hemagglutinating activity of ADEL by sugars and glycoproteins

	MIC
Sugar	
D-galactose	12.5 mM
α-methyl-D-galactopyranoside	10 mM
β-methyl-D-galactopyranoside	5 mM
D-galactosamine	5 mM
N-acetyl-D-galactosamine	>100 mM
Phenyl-β-D-galactopyranoside	6.25 mM
Phneyl-β-D-thiogalacopyranoside	>50 mM
4-nitrophenyl-α-D-galactoside	10 mM
4-nitrophenyl-β-D-galactoside	5 mM
2-nitrophenyl-β-D-galactoside	10 mM
O-nitrophenyl-β-D-galactoside	10 mM
D-galacturonic acid	0.2 μM
L-rhamnose	100 mM
L-arabinose	100 mM
α-lactose (galβ(1➔4)α-glc)	12.5 mM
β-lactose (galβ(1➔4)β-glc)	6.25 mM
Lactulose (galβ(1➔4)fru)	6.25 mM
Melibiose (galα(1➔6)glc)	6.25 mM
Rafinose (galα(1➔6)glcα(1➔4)fru)	6.25 mM
Glycoprotein	
PSM type III	0.125 mg/mL

Amino Acid Sequencing by MS/MS

The primary structure of ADEL was determined by overlapping of amino acid sequences of peptides sequenced by MS/MS deposited in Uniprot under accession number C0HK25. The amino acid sequence of ADEL consists of 217 residues, including 11 half-cystines involved in at least five intrachain disulfide bonds and one interchain disulfide bond (Fig. 3). According to the DiANNA 1.1 web server (Ferre and Clote 2005), cysteines are paired in the following form: ^{5}Cys-Cys^{187}, ^{42}Cys-Cys^{68}, ^{61}Cys-Cys^{77}, ^{114}Cys-Cys^{135}, and ^{142}Cys-Cys^{206}, whereas ^{172}Cys is involved in maintenance of the dimer. The theoretical isoeletric point was 5.41.

Theoretical molecular mass for the amino acid sequence totaled 24,450 Da. However, this value is lower than the molecular mass determined by MALDI-TOF. This divergence is most likely a result of glycosylation in the polypeptide chain.

ADEL has four glycosylation sites, of which three contain attached glycans, as indicated by tryptic glycopeptides (Table 3). These peptides showed differences in their observed and calculated molecular mass. These differences were attributed to glycans with distinct masses.

To confirm the existence of glycans attached to the polypeptide backbone, a deglycosylation assay was performed. Peptides presented in Table 3 were already deglycosylated after treatment with PNGase F. For instance, peptide T-9 at *m/z* 1076.45 has a sequence of ^{118}SFGFYNQNSTVWVNHGCR^{136}, and its calculated molecular mass was 2171.95, whereas mass observed by MS was 3226.25. The difference between observed and calculated mass was 1054.3, corresponding to the glycan with composition $HexNAc_2Hex_4$. After PNGase F digestion, T-9 showed *m/z* 726.31 and observed molecular mass of 2172.9, indicating the release of the glycan (Fig. 4).

The RADAR tool (http://www.ebi.ac.uk/Tools/pfa/radar/) was employed to detect the presence of three tandem-repeat domains along the polypeptide chain, which share distinct levels of identity (Fig. 5). Domain A (2-70) has identity of 63 and 55% to domain B (74-144) and C (152-214), respectively, whereas domain B showed identity of 49% to domain

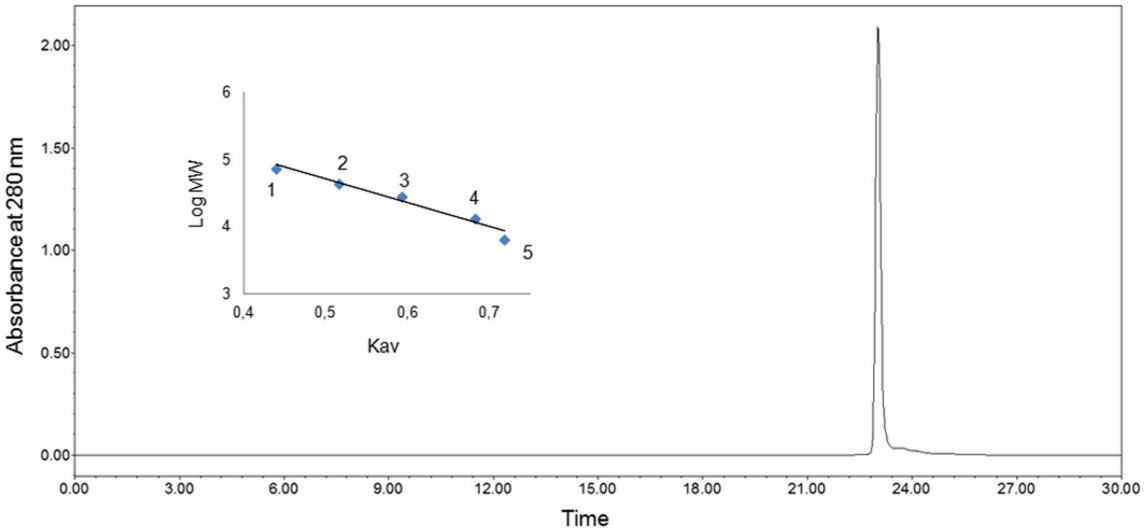

Fig. 2 Size exclusion chromatography of ADEL. The column BioSuite 250 HR SEC (0.78 × 30 cm, 5-um particle size, Waters Corp.) was equilibrated and eluted with Tris-HCl, 50 mM, pH 7.2, containing NaCl 500 mM. Approximately 200 μg of ADEL were loaded. UPLC operated at flow of 0.5 mL/min

C. Furthermore, ADEL presented microheterogeneities in three positions: ^{4}K/M, ^{53}D/A, and ^{199}A/T.

ADEL showed high sequence similarity with AKL (*A. kurodai* eggs lectin, GenBank: AB968312.1) and with a hypothetical lectin isoform X1 (NCBI RefSeq XP_005101558.1) from *Aplysia californica* (Fig. 6).

Circular Dichroism

Spectra of native ADEL in far-UV exhibited minimum absorption at 215 nm and maximum at 202 and 229 nm, suggesting a predominance of β-conformation in its secondary structure (Fig. 7a).

According to the CONTIN prediction method (Van Stokkum et al. 1990), available online as part of the server DICROWEB (http://dichroweb.cryst.bbk.ac.uk), the theoretical secondary structure of ADEL consisted of 4% α-helix, 44% β-sheet, 21% turn, and 32% coil.

Changes in the secondary structure of ADEL were examined by monitoring modifications in CD spectra as a function of temperature. Between 20 and 70 °C, no significant changes in secondary structure were observed, but after heating at 80 °C, some modifications occurred in maximum and minimal absorptions, indicating loss of secondary structure (Fig. 7b).

The spectra acquired in near-UV in the presence of some ligands revealed mild changes in tertiary structure of the lectin in comparison with native form (Fig. 7c). D-galactose (10 mM) caused a slight variation in tertiary structure of ADEL, while galacturonic acid (10 mM) exhibited strong effect on ADEL conformation.

Thermodynamics parameters of ADEL folding and unfolding in the presence and absence of carbohydrates are

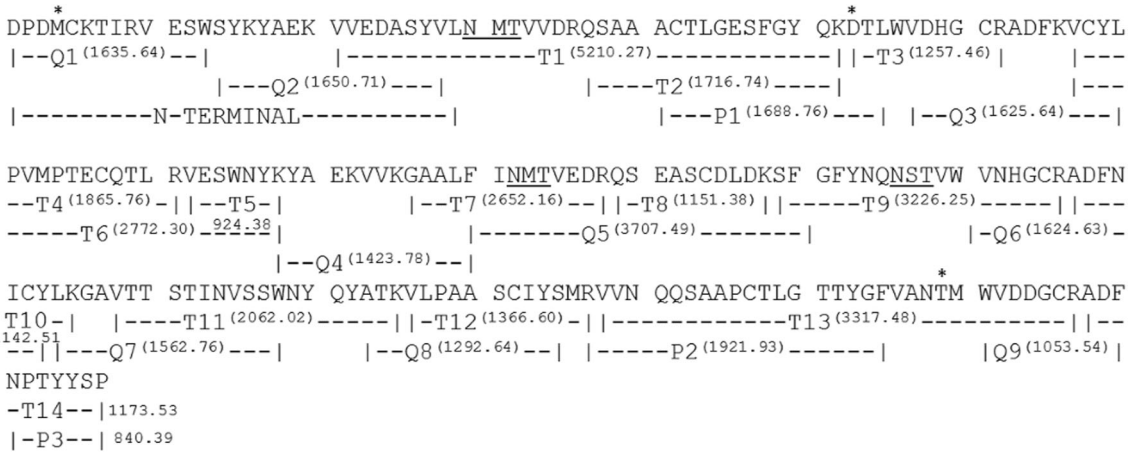

Fig. 3 Amino acid sequence of ADEL. Primary structure of ADEL was determined by overlapping of peptides obtained by digestions with trypsin (T), chymotrypsin (Q), and pepsin (P). *Asterisks* represent sequence microheterogeneites. Molecular mass determined by mass spectrometry are in parenthesis. Leucine and Isoleucine were assigned according with similarity between ADEL and AKL

Table 3 Amino acid sequence and mass differences of glycopeptides of ADEL

Peptide	*m/z*	Amino acid sequence	Molecular mass		Δ	Glycan prediction	Glycan mass
			Observed	Calculated			
T-1	1303.57	^{21}VVEDASYVLN**N**MTVVDRQSAAACTLGESFGYQK	5210.27	3507.66	1702.61	$HexNAc_2Hex_8$	1702.58
T-7	1246.05	^{96}GAALFI**N**MTVEDR	2490.10	1435.71	1054.39	$HexNAc_2Hex_4$	1054.37
T-7.1	1165.04	^{96}GAALFI**N**MTVEDR	2328.07	1435.71	892.36	$HexNAc_2Hex_3$	892.31
T-7.2	1327.09	^{96}GAALFI**N**MTVEDR	2652.16	1435.71	1216.45	$HexNAc_2Hex_5$	1216.42
T-9	1076.45	^{119}SFGFYNQ**N**STVWVNHGCR	3226.25	2171.95	1054.30	$HexNAc_2Hex_4$	1054.37
T-9.1	1400.59	^{119}SFGFYNQ**N**STVWVNHGCR	4198.66	2171.95	2026.70	$HexNAc_2Hex_{10}$	2026.68
Q-5	1236.83	^{100}FI**N**MTVEDRQSEASCDLDKSF	3707.49	2491.10	1216.39	$HexNAc_2Hex_5$	1216.42

N Asn residue that bound *N*-linked glycans

presented in Table 4. As demonstrated in the hemagglutinating assay, galacturonic acid is the most important ligand of ADEL; the interaction between galacturonic acid and ADEL shows Ka = 1.5 × 10^7 M^{-1}. Furthermore, the presence of galacturonic acid increases ADEL Tm by 9 K in comparison to apo-lectin (data not shown).

ADEL Structural Prediction and Docking Calculation

Prior to molecular modeling, the ADEL sequence was used as input data for a PSI-BLAST (Ding et al. 2014) search against the protein data bank (PDB) to identify suitable template structures (http://blast.ncbi.nlm.nih.gov/Blast.cgi). No significant sequence

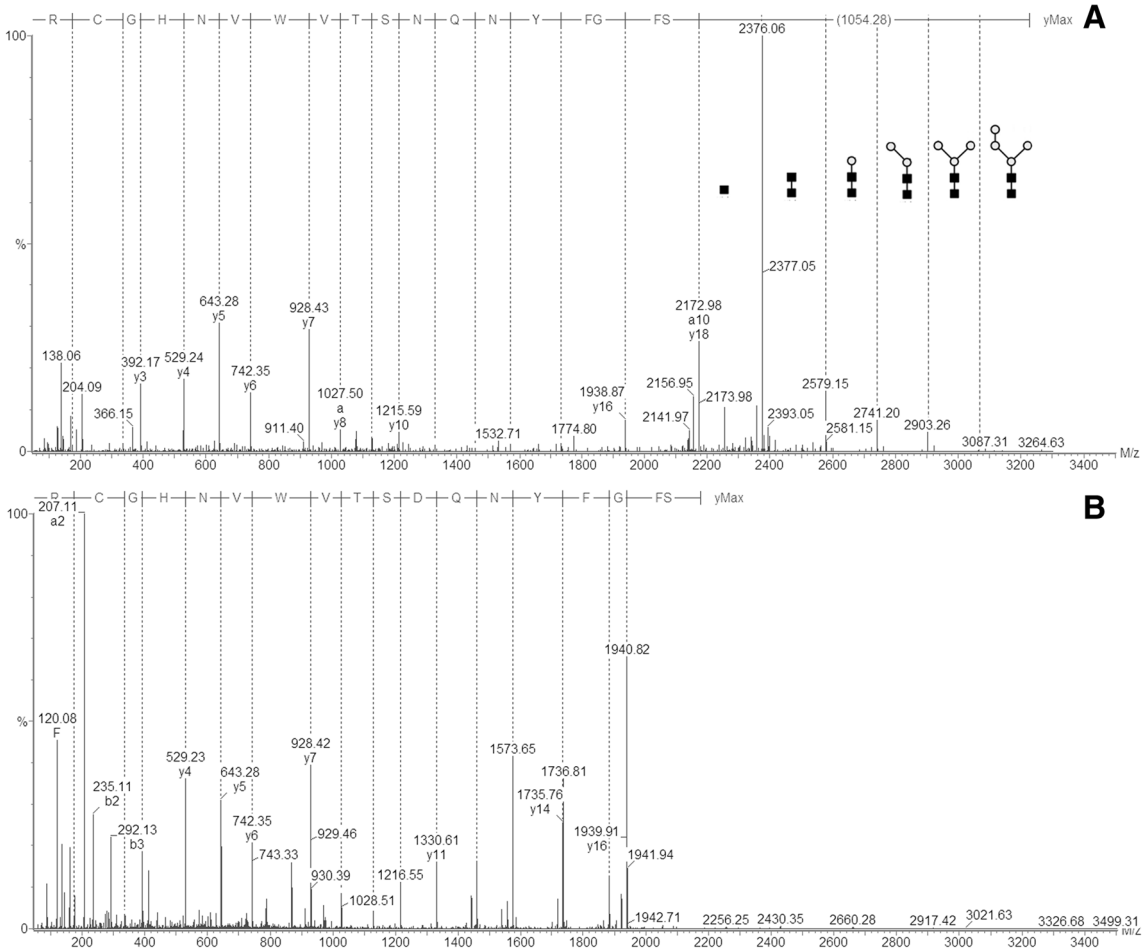

Fig. 4 Glycosylated peptide of ADEL. Mass spectra of the ion [M + 2H]2+ at *m/z* 1246.05. N-acetyl-hexosamines and hexoses are represented by *squares* and *circles*, respectively (**a**). Deglycosylated peptide of ADEL, after PNGase F digestion (**b**)

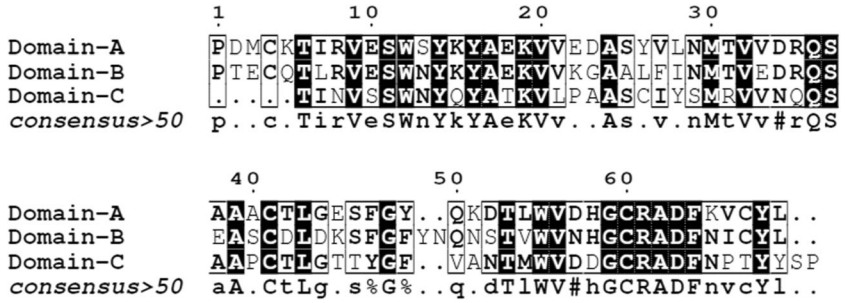

Fig. 5 Alignment of the repeated domains of ADEL. Alignment realized by ESPript 3.0. *Black* and *white boxes* represent identical and non-identical amino acids, respectively. Consensus >50 represents amino acids present in at least 50% of the aligned sequences

similarity was found to any experimentally determined structure. Based on that, interplay between different modeling servers and software was performed to produce the most reliable structural prediction of ADEL.

The overall starting ADEL structure was obtained by I-TASSER. However, several poorly modeled patches were identified since no template structures were available on PDB. To overcome this issue, the structural predictions of each individual ADEL domain performed by QUARK were used to fill the structural gaps. The selected structures were used by MODELER to produce a complete ADEL model, with 99.1% of residues placed in allowed regions (90.7% on favorable regions) of the Ramachandran plot (Supplementary Fig. 1).

The obtained ADEL model (217 amino acids) possesses two independent domains connected by a short loop (Phe100-Val105), which were named domains A (Asp1-Leu99) and B (Glu106-Pro217) (Fig. 8a). Domain A is composed of a flat three-stranded and a curved five-stranded β-sheet, while domain B presents a flat three-stranded and a curved four-stranded β-sheet. According to I-TASSER, the crystal structure of the polymeric immunoglobulin receptor ectodomain from the rainbow trout *Oncorhynchus mykiss* (PDB 5F1S) is the structure most closely related to ADEL on PDB, despite its low sequence identity.

The predicted binding site is located at the interface between domains A and B, mainly built by residues Lys6, Ile8, Tyr17, Glu106, Asp107, Gln109, and Arg207 (Fig. 8b).

Refined docking calculations resulted in an increasing order of theoretical binding energy among the analyzed carbohydrates, as follows: D-galactose < D-galactosamine < α-lactose < D-galacturonic acid (Table 5). Docking calculations also revealed a conserved binding pattern for the analyzed monosaccharides over the ADEL carbohydrate-binding site. It is possible to observe that the side chains of residues Lys6 and Ile8 act to stabilize the pyranosidic ring of the monosaccharides through hydrophobic stacking interactions. On the other hand, the hydroxyl groups of these ligands establish an

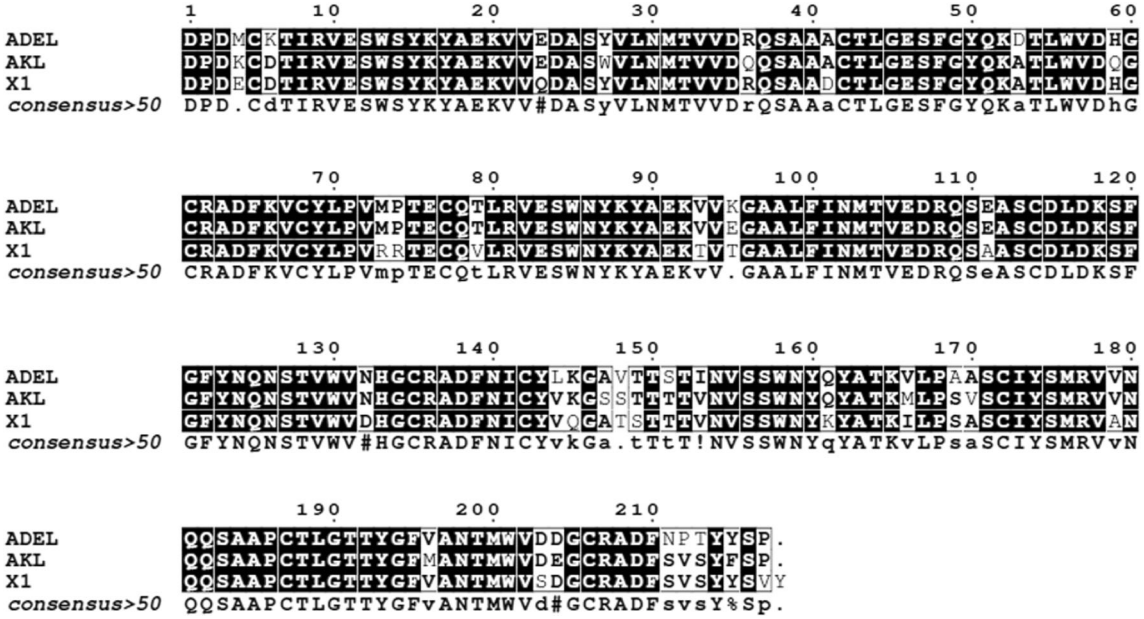

Fig. 6 Alignment of ADEL and other *Aplysia* eggs lectins. Amino acid sequence of ADEL was aligned with *Aplysia kurodai* eggs lectin (AKL) and a putative lectin from *Aplysia californica* (isoform X). Alignment realized by ESPript 3.0. *Black and white boxes* represent identical and non-identical amino acids, respectively. Consensus >50 represents amino acids present in at least 50% of the aligned sequences

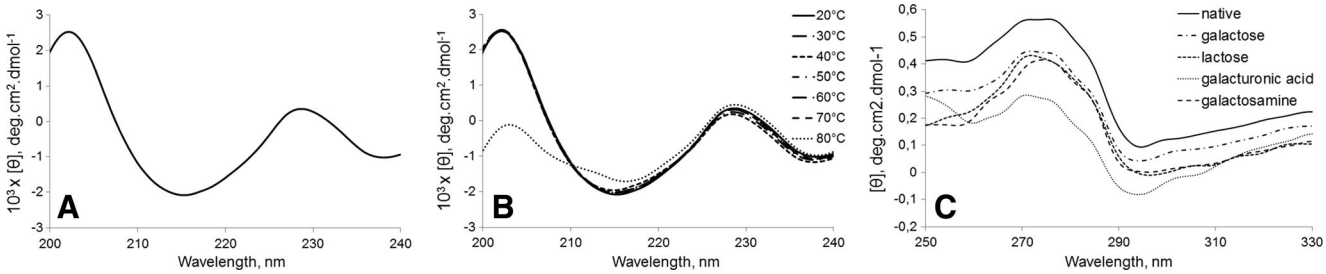

Fig. 7 CD spectra of ADEL. **a** Far-UV CD spectra (190–250 nm) of ADEL. The cuvette path length was 0.05 cm; protein concentration was 8 μM in PBS, pH 7. **b** Far-UV CD spectra of ADEL in different temperatures. **c** Near-UV CD spectra (250–330 nm) of ADEL with some ligands. The cuvette path length was 0.5 cm; protein concentration was 20 μM in PBS, pH 7

intricate hydrogen bond pattern with the main chain carbonyl groups from residues Tyr17 and Asp107 and with the side chain polar groups from residues Glu106, Gln109, and Arg207 (Fig. 9 and Table 5).

In the case of α-lactose, the binding pattern seems to be slightly different. The pyranosidic rings from the D-galactose and D-glucose residues are stabilized not only through hydrophobic interactions with residues Lys6 and Ile8, but also with the side chain of residue Thr7. Further, the hydrogen-binding pattern also changes to accommodate this bulkier ligand (Fig. 9d and Table 5). It is worth mentioning that the ADEL carbohydrate-binding site is disposed in a groove between domains A and B surrounded by other solvent-exposed charged and hydrophobic amino acid residues that may comprise extended sites for the anchorage of complex ligands.

Agglutination and Antibacterial Activity

ADEL agglutinated *S. aureus* cells, but it was not able to agglutinate *E. coli* (Figure 10). Despite its agglutinating activity, ADEL was not able to inhibit the growth of either species (data not shown).

Effects of ADEL on Biofilm Formation

The effect of ADEL on the prevention of biofilm formation was assessed by two different assays: determination of biofilm mass and enumeration of biofilm viable cells. The treatment of *S. aureus* biofilms with ADEL reduced biomass by almost 40%. The effect was seen in all concentrations evaluated (Fig. 11a). Regarding the number of biofilm-entrapped viable cells, ADEL reduced *S. aureus* viability mainly at higher concentrations (250–62.5 μg/mL) (Fig. 11b). Interestingly, the biofilm formation of *E. coli* was not affected (Fig. 11).

The antibiofilm activity on *S. aureus* was abolished when ADEL was denatured by heating at 100 °C. On the other hand, treatment with α-D-lactose neither interfered with biofilm formation nor did it inhibit the antibiofilm activity of ADEL (data not shown).

Table 4 Affinity constants and free energy of the binding of sugars to ADEL at 358 K

Sugar	$10^{-5} \times Ka$ (M^{-1})	α	$-\Delta G_F$ (kJ mol^{-1})	T_M (K)
Native	–	0.338	361.5	356
α-D-lactose	0.4	0.594	1060	359
D-galactose	6.1	0.788	2697.5	361
D-galactosamine	6.8	0.793	2775	361
D-galacturonic acid	150	0.873	4989.2	365

α fraction folded at 358 K, ΔG_F free energy of folding, T_M unfolding transition

Discussion

A new lectin from eggs of the sea hare *A. dactylomela* was successfully isolated and purified by affinity chromatography using Sepharose™ chromatography media. The use of Sepharose™ for purification of galactophilic lectins is a classical method, and several lectins have been successfully isolated by this procedure (Gasparini et al. 2008; Jimbo et al. 2000; Moura et al. 2006; Vasta and Marchalonis 1986). The activation of Sepharose™ by HCl constitutes an essential step to expose galactosides of the internal matrix, as long as these residues are available for specific lectin interaction.

The literature reports three lectins isolated from reproductive tissues of sea hares, which were isolated from *Aplysia depilans* (AGL) and *A. kurodai* (AKL-1 and AKL-2). AGL and AKL-1 are dimeric proteins with 30 kDa per subunit linked by disulfide bonds, as is ADEL. AKL-2 is also a dimeric protein, but with 16 kDa per subunit (Gilboa-Garber et al. 1985; Kawsar et al. 2009, 2011).

The literature shows that AKL-1, AKL-2, and AGL all have a strong affinity for galacturonic acid (Gilboa-Garber et al. 1985; Kawsar et al. 2009, 2011). ADEL also presents high specificity for the same carbohydrate. In fact, the specificity of lectins from sea hare eggs is a characteristic that deserves closer attention based on the fact that the species are spread far apart, but share the same sugar specificity.

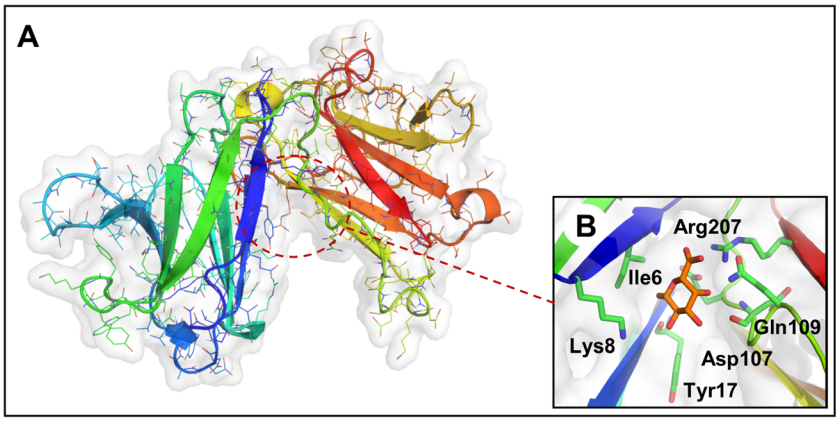

Fig. 8 ADEL overall structure. **a** Monomer structure of ADEL represented as *colored cartoon* and *lines with a transparent surface* delineating the protein shape. The carbohydrate-binding site location is highlighted by a *red dashed circle*. **b** Carbohydrate-binding site representation, which shows the coordination of D-galacturonic acid by the main site residues (represented as *green sticks*)

Thus, galacturonic acid should be considered as one of the preferred targets for the sea hare eggs lectins.

In addition to galacturonic acid, ADEL was also inhibited by D-galactose and its derivatives. ADEL showed slight preference by β-galactosides, thus suggesting the involvement of the hydroxyl at C-1 in the interaction between lectin and sugar. For instance, methyl-β-D-galactopyranoside and 4-nitrophenyl-β-D-galactopyranoside are two times more effective than methyl-α-D-galactopyranoside and 4-nitrophenyl-α-D-galactopyranoside, respectively. Moreover, β-lactose was more effective in inhibiting ADEL than α-lactose. The presence of substituents at C-1, such as methyl, phenyl, and nitrophenyl, increases inhibitory efficiency when compared to D-galactose. However, methyl-β-D-thiogalactopyranoside

Table 5 Comparative analysis based on docking calculations of the main interactions and bond distances involved in the coordination of the selected carbohydrate ligands by ADEL. The theoretical binding energies are presented at the bottom. The selected cutoff for hydrogen bonds and hydrophobic interactions were 3.5 and 4.5 Å, respectively

ADEL		Galacturonic acid		Lactose		Galactosamine		Galactose	
Atom	Residue	Atom	Distance (Å)	Atom	Distance (Å)	Atom	Distance (Å)	Atom	Distance (Å)
CE	Lys6	C1	4.5	C1 (Glc)	4.5	C1	4.2	C1	4.2
O	Thr7	–	–	O4 (Gal)	2.7	–	–	–	–
		–	–	O6 (Gal)	2.8	–	–	–	–
CG1	Ile8	C1	4.1	C2 (Glc)	4.1	C1	4.0	C1	4.0
O	Tyr17	O1	2.8	O1 (Glc)	3.2	O1	2.7	O1	2.7
		O2	3.0	O2 (Glc)	3.1	N	3.1	N	3.1
OE1	Glu106	O4	3.1	O1 (Glc)	3.5	O4	3.0	O4	3.0
		O5	3.1	–	–	O5	3.4	O5	3.4
		–	–	–	–	–	3.0	O6	3.1
O	Asp107	O3	2.8	O6 (Glc)	2.8	O3	2.8	O3	2.8
		O4	2.9	–	–	O4	3.2	O4	3.2
OE1	Gln109	O7	3.0	O2 (Gal)	2.8	–	–	–	–
NE2	Gln109	O7	3.3	O1 (Gal)	3.2	–	–	–	–
		–	–	O2 (Gal)	3.3	–	–	–	–
		–	–	O6 (Glc)	3.0	–	–	–	–
NH1	Arg207	O6	3.2	–	–	O6	3.0	O6	2.9
NH2	Arg207	O4	3.1	–	–	O4	3.0	O4	3.0
		O6	3.3	–	–	O6	3.1	O6	3.1
		O7	3.0	–	–	–	–	–	–
Total energy		−5.6 kcal/mol		−5.4 kcal/mol		−5.2 kcal/mol		−5.1 kcal/mol	

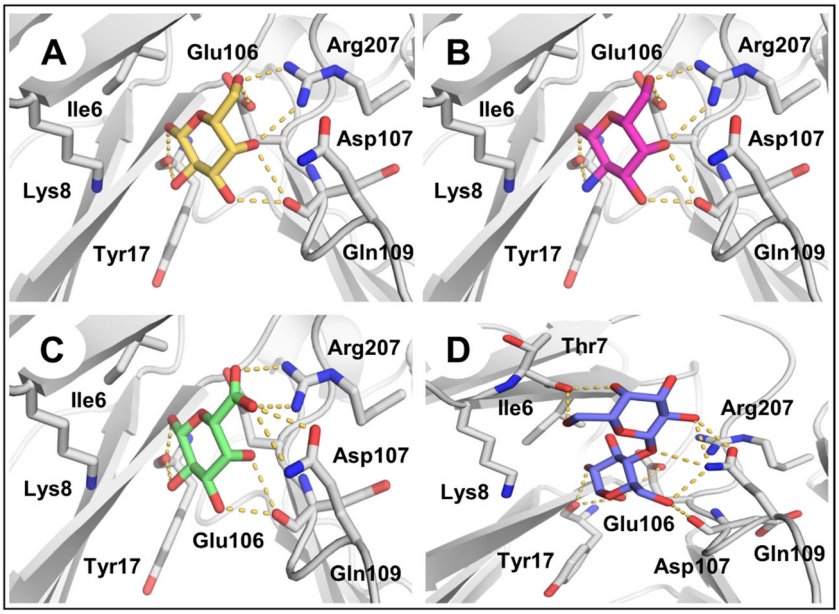

Fig. 9 Ligand coordination at the carbohydrate-binding site. **a** Coordination of D-galactose, represented as *yellow sticks*. **b** Coordination of D-galactosamine, represented as *magenta sticks*. **c** Coordination of D-galacturonic acid, represented as *green sticks*. **d** Coordination of α-lactose, represented as *purple sticks*. The protein backbone and the main amino acid residues involved in carbohydrate coordination by ADEL are represented as *gray cartoon* and *sticks*, respectively. Hydrogen bonds are represented as *yellow dashes*. A few interactions were omitted for the purpose of clarity (see Table 5)

showed no inhibitory effect, indicating steric hindrance, most likely from the presence of a thiol group. Although C-1 seems to be an important recognition point, a similar grade of inhibition by melibiose (Galα1➔6Glc), α-D-lactose (Galβ1➔Glc), and raffinose (Galα1➔6Glcα1➔4Fru) indicates that C-1 cannot be considered as a key point in the recognition by ADEL.

Since D-glucose, L-fucose, and D-mannose are not recognized by ADEL, the orientation of hydroxyls at C-2 and C-4 seems to be more important than that at C-1. The axial orientation of C-2 and equatorial orientation of C-4 present in D-galactose seem to be essential for interaction. ADEL was inhibited to a greater degree by D-galactosamine compared to D-galactose, and it was not inhibited by GalNAc, indicating that a small uncharged substituent at C-2 (i.e., amine) increases affinity, whereas a charged substituent (i.e., acetyl) abolishes it.

The importance of C-5 was indicated by strong inhibition by the presence of a hydroxymethyl group in D-galacturonic acid when compared to the inhibitory effect produced by D-galactose, L-arabinose, and L-rhamnose, even though the first two sugars show the same orientation of hydroxyls at C-2, C-3, and C-4. The presence of the hydroxymethyl group strongly improves lectin-sugar binding since galacturonic acid is 6×10^5 times more effective than D-galactose.

The glycoprotein PSM has a terminal GalNAc and D-galactose as internal residue (Slomiany and Meyer 1972),

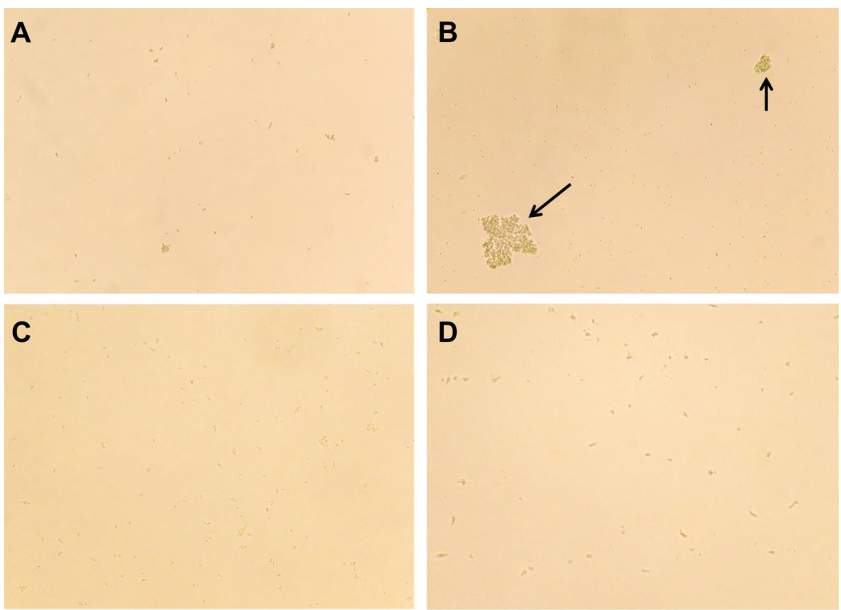

Fig. 10 Agglutination of bacteria by ADEL. *S. aureus* incubated with TBS (**a**) and ADEL (**b**); *E. coli* incubated with TBS (**c**) and ADEL (**d**). *Arrows* indicate bacterial agglutination

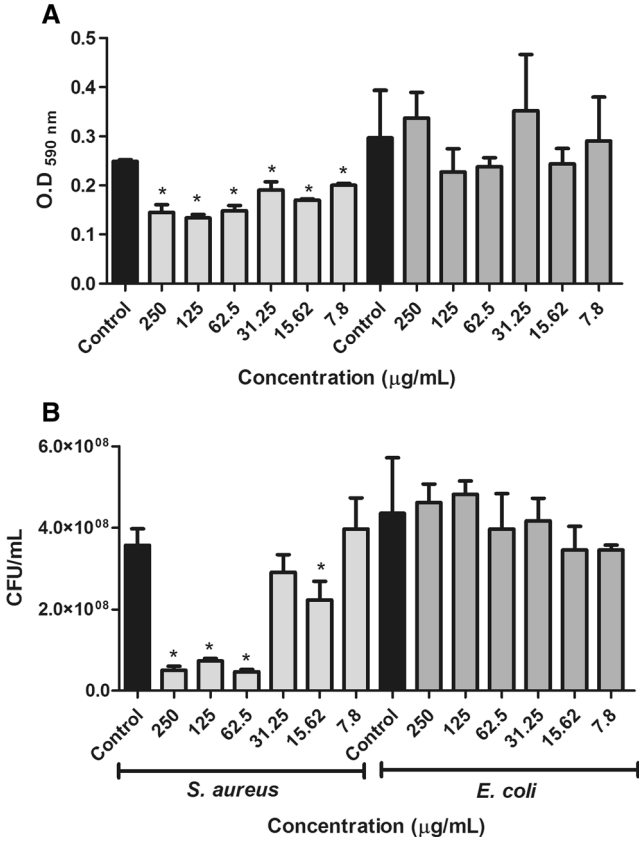

Fig. 11 Biofilm formation of *S. aureus* and *E. coli* in the presence of ADEL. **a** Biofilm biomass and **b** number of viable cells present in the biofilms. *$p < 0.05$ compared to control

and it exhibited inhibitory effect on ADEL. Like other sea hare lectins, ADEL was inhibited by small amounts of PSM (Kawsar et al. 2009). The presence of T-antigen (Galβ1➔3GalNAc) in PSM could account for the inhibitory effect based on the strong recognition of T-antigen by AGL (Gilboa-Garber and Sudakevitz 2001).

Consistent with data from the hemagglutination inhibition assay, CD spectrum of ADEL acquired in the near-UV spectral region revealed that the interaction with galacturonic acid plays an important role in ADEL conformation. D-galactose, D-galactosamine, and α-D-lactose also exerted some influence on the tertiary structure of ADEL, but to a lesser extent. These sugars showed distinct Ka values in the following order: galacturonic acid > D-galactosamine > D-galactose > α-D-lactose.

Curiously, the order of affinity seen in molecular docking was different in comparison to that observed in CD and HA inhibition. In the computational approach, galacturonic acid remains a sugar with higher binding energy, but α-D-lactose comes in second, followed by D-galactosamine and D-galactose.

In addition to clarifying the interactions between ADEL and sugars, CD measurement was also used to estimate secondary structure. According to the CD spectra, ADEL has a high content of β-conformations (sheets and turns), a characteristic of many lectins (Vasta et al. 2004). Indeed, the 3D model of ADEL is predominantly composed of β-structures, but interestingly, it presents a singular structure for lectins.

Animal lectins present a wide structural diversity. For instance, galectins have a typical jelly roll β-sandwich conformation (Vasta et al. 2004). The C-type lectin domain (CTLD) consists of a double loop stabilized by two disulfide bonds highly conserved in the base of the second loop (Zelensky and Gready 2005), while R-type lectins showed a β-trefoil (Uchida et al. 2004). However, none of these folds resembles the ADEL model. Obviously, for a more accurate comparison, the determination of three-dimensional structure of ADEL by experimental approaches is required.

ADEL, *A. kurodai* egg lectin and AGL are independent of divalent ions (Gilboa-Garber et al. 1985; Kawsar et al. 2009). AKL-1 and AGL are glycoproteins (Gilboa-Garber et al. 1985; Kawsar et al. 2009). In addition to evidence that AKL and AGL are glycoproteins, our study could provide accurate glycan structures of ADEL by MS analysis. ADEL is also a glycoprotein with three distinct glycans attached to three distinct N-glycosylation sites.

N-glycosylation is one of the most common post-translational modifications (PTMs) present in proteins, and several functions are attributed to its presence, including correct protein folding, protein localization, and regulation of protein degradation. Glycosylation events typically occur in the endoplasmatic reticulum (ER), and oligosaccharide maturation occurs in the Golgi apparatus (Ghazarian et al. 2011). According to the type of carbohydrate attached to polypeptide chain, N-oligosaccharides can be classified into three groups: high mannose, complex, and hybrid oligosaccharides (Kukuruzinska and Lennon 1998). Generally, all N-glycans share a common pentasaccharide core: Manα3(Manα6)Manβ4GlcNAcβ4GlcNAc. Our findings indicated that this precursor is also present in the ADEL structure.

In the first N-glycosylation site of ADEL (^{30}NMT), an oligosaccharide of 1702.61 Da was found with mass corresponding to the pentasaccharide precursor (892 Da) with addition of five hexose residues (810 Da). In the second site (^{102}NMT), molecular mass of the oligosaccharide ranges from 892.36 to 1216.45 Da, corresponding to the pentasaccharide precursor alone, and the precursor with two hexose residues (324 Da), respectively. Finally, the third site (^{126}NST) has an oligosaccharide of 1054 Da that consists of the precursor and one hexose residue.

All oligosaccharides found in ADEL consist of a pentasaccharide precursor increased by some hexose residues. Among N-glycan groups, the only one that contains only hexoses, besides the precursor, is the high-mannose oligosaccharide.

Therefore, MS analysis strongly suggests that all oligosaccharides attached to the ADEL polypeptide chain are high-mannose-type oligosaccharides. To the best of our knowledge, this is the first time that glycan composition has been characterized for a lectin from opisthobranch (Gastropoda).

Although several efforts were made to remove oligosaccharides attached to ADEL, they were unsuccessful.

Digestion of ADEL by PNGase F showed no effect, since no shift was observed in SDS PAGE and MS (data not shown). We speculate that the native folding of ADEL was maintained, even when using heat treatment and surfactant reagents during digestion. However, PNGase F could digest tryptic peptides of ADEL. All glycosylated peptides were digested by PNGase, indicating that no deoxyhexoses are attached to chitobiose precursors.

Besides glycosylation, specificity for carbohydrates, divalent ion independence, and quaternary arrangement, ADEL shares a high level of sequence similarity with AKL-1. ADEL showed 91 and 88% of identity and 97 and 93% of similarity with AKL-1 (GenBank: AB968312.1) and isoform X1 of *A. californica* (NCBI RefSeq XP_005101558.1), respectively.

Lectins isolated from the *Aplysia* genus showed no similarity to other known lectins, but the presence of a repeated domain in their structures is a common property of certain families of lectins. In particular, lectins that showed repeated domain in their primary structures and shared similarity of sequence with B-chain of Ricin were grouped in the R-type lectin family (Hirabayashi et al. 1998).

Lectins from sea hare eggs showed low similarity with Ricin, did not share conserved residues, and had no β-trefoil domain typical of R-type lectins, according to structural modeling. Thus, ADEL, AKL, and isoform X1 could not be grouped in this family, but could be grouped in a new family of lectins considering their similarities of sequence and biochemical characteristics. However, further experiments should be performed to better characterize these lectins.

ADEL was effective in agglutinating planktonic cells and inhibiting biofilm formation by *S. aureus* ATCC 25923. In fact, several galactophilic lectins can agglutinate bacterial cells (Carneiro et al. 2015; Melo et al. 2014; Moura et al. 2006), recognize glycans on the cell surface and distinguish Gram-positive from Gram-negative bacteria (Sun et al. 2007; Moura et al. 2006). The surface of Gram-positive bacteria is constituted by several structures containing carbohydrates, such as peptidoglycan, capsular polysaccharides, and teichoic acid (Weidenmaier and Peschel 2008), which may serve as possible binding sites for ADEL.

Lectins from marine organisms have been exploited for several biological applications, including antimicrobial activity (Moura et al. 2006; Roué et al. 2012; Cheung et al. 2015). Although some studies have focused on the antimicrobial potential of marine lectins, only a few are directly related to their effect on pathogenic biofilms. In the present work, we also evaluated the antibacterial potential of ADEL on planktonic growth and biofilm formation by *S. aureus*. ADEL does not inhibit bacterial growth, but rather inhibits biofilm formation. According to Liljemark et al. (1981), the formation of bacterial aggregates can cause a decrease in the number of adherent cells, which may explain why ADEL does not inhibit bacterial growth, but is, instead, effective on biofilm formation. Similar results were obtained by Vasconcelos et al. (2014), using plant and red algae lectins on Gram-positive and Gram-negative bacteria, as well as on yeasts biofilms.

In order to investigate the involvement of CRD in antibiofilm activity, the lectin was preincubated with α-D-lactose at MIC (12.5 mM). Interestingly, the activity was not abolished. This is not surprising since differences in affinity can be achieved between monosaccharides and structured glycans. Therefore, it is possible that ADEL has a higher affinity to complex glycans located on the *S. aureus* surface. Indeed, lectins can preferentially recognize carbohydrate complexes (Ghazarian et al. 2011). Furthermore, the inhibition of biofilm formation by ADEL is dependent on its structural integrity, in particular because denaturation by heating inhibited its antibiofilm activity.

Biofilms are described as structured communities of surface-associated microbial cells enclosed in a self-produced polymeric matrix (Flemming and Wingender 2010). Since biofilms are associated with increased resistance to conventional antimicrobial agents, ADEL could be considered as an alternative to preventing biofilm formation.

In addition, the antibiofilm activity exhibited by ADEL allows us to speculate about its physiological roles. For instance, *Aplysia* eggs are deposited on the surface of stones and algae where they remain for a few days until hatching. During incubation, eggs are exposed to several biological stresses, including bacterial infection. Therefore, the endogenous production of ADEL may be induced by the presence of pathogenic bacteria, and the lectin might act in the defense of eggs.

In conclusion, we herein report a novel type of lectin isolated from *A. dactylomela*, which is highly specific for galacturonic acid and has three high-mannose oligosaccharides attached to its polypeptide chain. Moreover, ADEL can inhibit biofilm formation by *S. aureus*. Thus, ADEL could be considered as an alternative to conventional antimicrobial therapy used to combat Staphylococcal biofilms.

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Artigo III

L-rhamnose-binding lectin from eggs of the *Echinometra lucunter*: amino acid sequence and molecular modeling

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L-rhamnose-binding lectin from eggs of the *Echinometra lucunter*: Amino acid sequence and molecular modeling

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ABSTRACT

An L-rhamnose-binding lectin named ELEL was isolated from eggs of the rock boring sea urchin *Echinometra lucunter* by affinity chromatography on lactosyl-agarose. ELEL is a homodimer linked by a disulfide bond with subunits of 11 kDa each. The new lectin was inhibited by saccharides possessing the same configuration of hydroxyl groups at C-2 and C-4, such as L-rhamnose, melibiose, galactose and lactose. The amino acid sequence of ELEL was determined by tandem mass spectrometry. The ELEL subunit has 103 amino acids, including nine cysteine residues involved in four conserved intrachain disulfide bonds and one interchain disulfide bond. The full sequence of ELEL presents conserved motifs commonly found in rhamnose-binding lectins, including YGR, DPC and KYL. A three-dimensional model of ELEL was created, and molecular docking revealed favorable binding energies for interactions between ELEL and rhamnose, melibiose and Gb_3 (Galα1-4Galβ1-4Glcβ1-Cer). Furthermore, ELEL was able to agglutinate Gram-positive bacterial cells, suggesting its ability to recognize pathogens.

1. Introduction

Rhamnose-binding lectins (RBLs) belong to a group of lectins that bind specifically to L-rhamnose and α-galactose, rather than β-galactosides, and do not require divalent cations or thiol groups for their hemagglutinating activity [1,2]. Many RBLs are composed of repeated carbohydrate recognition domains (CRD), containing about 95 amino acid residues each and a unique α/β fold with long structured loops important for monosaccharide recognition [3,4]. These lectins share four conserved disulfide bonds and two conserved motifs: -ANYGR(TD)- in N-terminal and -DPCX(G)T(Y)KY(L)- in C-terminal, which are involved in the primary recognition of ligands [4–6].

The biological function of RBLs is not yet known. However, it is likely that these lectins play a role in the production of reactive oxygen species and phagocytosis, regulation of carbohydrate metabolism, prevention of polyspermy and self-defense [7–9]. Members of the RBL family act as pattern recognition receptors (PRRs), recognizing various pathogen-associated molecular patterns (PAMPs). RBLs can recognize lipopolysaccharides and lipoteichoic acid, constituents of cell wall of Gram-positive and Gram-negative bacteria, respectively, thus acting as opsonins [10–12].

Structurally, RBLs can be divided into five subfamilies. Type I possesses three repeated domains, and Type II is composed of two repeated domains with an extra domain. Type III and Type IV have two repeated domains, but they have different sugar specificity. Finally, Type V has only one domain able to form a homodimer with a disulfide bond between subunits [3].

Most RBLs studied have been found in fish eggs and ovaries. However, the first RBL described, the sea urchin eggs lectin, or SUEL, was purified from eggs of the sea urchin *Anthocidaris crassispina* [13,14]. Also, RBLs have been described in colonial ascidians and

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bivalves [12,15]. Interestingly, SUEL is unique member of the Type V subfamily.

The rock boring sea urchin *Echinometra lucunter* Linnaeus is commonly found throughout the Caribbean Sea and South America coast. It commonly occurs on coral reefs and shallow rock areas where depths reach 2 m. Great populations of *E. lucunter* can contribute greatly to the breakdown of coral reefs as a consequence of their burrowing behavior. *E. lucunter* is therefore considered a pest in many countries, including Brazil [16].

In this work, we report the purification, amino acid sequencing and structure prediction of a new RBL from *E. lucunter* eggs, named ELEL (*E. lucunter* eggs lectin). Based on structural data, the interaction of ELEL with carbohydrates was tested by molecular docking.

2. Materials and methods

2.1. Animal collection and extract preparation

Specimens of the rock boring sea urchin *E. lucunter* were collected at Pacheco Beach, Caucaia, Ceará State, Brazil. The animals were transported to the laboratory in a thermal box containing sea water.

E. lucunter eggs were obtained by coelomic injection of 0.5 M KCl. The eggs were dejelled by several washes in acidic seawater, pH 5.0. Dejelled eggs were defatted by treatment with cold acetone. After three exchanges of acetone, eggs become colorless.

Colorless eggs were suspended in five volumes of TBS (Tris-buffered saline, 150 mM NaCl, 0.1 mM PMSF, 50 mM Tris–HCl, pH 7.6) and sonicated in ice 10 times at 70 W for 50 s at intervals of 1 min using a Bandelin SONOPULS HD 2070 sonicator. The mixture was centrifuged at 5000 × *g* for 20 min at 4 °C. The supernatant was named crude extract and was assayed for hemagglutinating activity and protein concentration [17].

2.2. Purification of E. lucunter eggs lectin

The crude extract was loaded on a lactosyl-agarose column (1.0 cm × 3.0 cm), previously equilibrated with TBS. The column was washed with the same buffer at a flow rate of 1 mL/min until the column effluents showed absorbance of less than 0.01 at 280 nm. The adsorbed lectin was eluted with 0.3 M L-rhamnose in TBS. 1-mL fractions were manually collected, dialyzed and freeze-dried until use.

2.3. Hemagglutinating activity and hemagglutination inhibitory assay

Lectin activity was estimated by hemagglutinating activity against a solution of 3% rabbit and human erythrocytes (A, B and O) in native form and treated with proteases. The hemagglutination tests were performed in microtiter plates with V-bottom wells using the two-fold serial dilution method [18].

A hemagglutination inhibition assay was performed using the standard procedure [18]. The following carbohydrates and glycoproteins were used: D-fructose, D-galactose, D-glucose, D-mannose, L-rhamnose, methyl-α-D-galactopyranoside, methyl-α-D-galactopyranoside, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-sucrose, D-melibiose, α-lactose, β-lactose, orosomucoid, ovomucoid, thyroglobulin and porcine stomach mucin (PSM). The initial concentrations of the inhibitors were 100 mM for sugars and 2 mg/mL for glycoproteins.

The effects of pH, temperature, EDTA and divalent cations on lectin activity were evaluated as described by Sampaio et al. [18].

2.4. Molecular mass and sugar content of ELEL

Molecular mass of ELEL under denaturing condition was estimated by SDS–PAGE in the presence and absence of β-mercaptoethanol, followed by staining with Coomassie Brilliant Blue, as described by Laemmli [19]. LMW-SDS Marker kit (GE Healthcare) was used as the standard (phosphorylase b (Mr: 97,000), albumin (Mr: 66,000), ovalbumin (Mr: 45,000), carbonic anhydrase (Mr: 30,000), trypsin inhibitor (Mr: 20,100) and α-lactalbumin (Mr: 14,400).

Glycoproteins in SDS–PAGE were stained with periodic acid-Schiff (PAS), as described by Zacharius et al. [20]. Neutral sugar content in ELEL was evaluated by phenol-sulfuric acid, using lactose as the standard [21].

The relative mass of native ELEL was estimated by gel filtration on a BioSuite 250 5 μm HR SEC (0.78 cm × 30 cm) column coupled to the H-Class Bio Acquity UPLC System (Waters Corp.). The column was equilibrated with 50 mM Tris–HCl, pH 7.2, containing 500 mM NaCl, and calibrated with conalbumin (75 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa).

The molecular mass of ELEL was determined by ElectroSpray Ionization-Mass Spectrometry (ESI-MS). Purified lectin (6 mg/mL) was solubilized in 8 M Urea and submitted to reverse phase chromatography (RPC) on a Sephasil Peptide C-8 10/250 column coupled to the H-Class Bio Acquity UPLC System (Waters Corp.). The column was equilibrated with 5% ACN in 0.1% trifluoracetic acid (TFA) and eluted with a gradient of acetonitrile (ACN) in 0.1% TFA. Fractions containing ELEL were collected and directly infused into a nanoelectrospray source coupled to a Synapt HDMS ESI-Q-ToF mass spectrometer (Waters Corp.). The instrument was calibrated with [Glu1]- Fibrinopeptide B fragments. Mass spectra were acquired by scanning at *m*/*z* ranging from 1000 to 4000 at 5 scans/s. The mass spectrometer was operated in positive mode, using a source temperature of 363 K and capillary voltage at 3.2 kV. Data collection and processing were controlled by Mass Lynx 4.1 software (Waters).

2.5. Primary structure determination by tandem mass spectrometry (MS/MS)

ELEL was submitted to SDS–PAGE as described above. After staining, ELEL spots were excised, reduced with dithiothreitol (DTT), and carboxyamidomethylated with iodoacetamide (IAA), as described by Shevchenko et al. [22].

Treated spots were subjected to digestion with the following enzymes: trypsin, chymotrypsin and pepsin. Digestions with trypsin and chymotrypsin were realized in 50 mM ammonium bicarbonate at 1:50 w/w (enzyme/substrate). Digestion with pepsin was performed in 0.1 M HCl at 1:50 w/w (enzyme/substrate). All digestions were maintained at 37 °C for 16 h.

The digestions were stopped with 2 μL of 2% formic acid (FA). The peptides were extracted from the gel conform, as described by Shevchenko et al. [22]. Two microliters of the peptide solution were loaded onto a C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system (Waters Corp.). The column was equilibrated with 0.1% FA and eluted with an ACN gradient in 0.1% FA. The eluates were directly infused into a nanoelectrospray source. The mass spectrometer was operated in positive mode with a source temperature of 373 K and a capillary voltage at 3.0 kV. LC–MS/MS was performed according to the data-dependent acquisition (DDA) method. The lock mass used in acquisition was *m*/*z* 785.84 ion of the [Glu1]- Fibrinopeptide B. The selected precursor ions were fragmented by collision-induced dissociation (CID) using argon as collision gas. All of the CID spectra were manually interpreted. A search for similar sequences was performed using the online tool

BLASTp. Leucine and isoleucine were assigned according to their similarity with other lectins.

2.6. Agglutination of bacterial cells

Escherichia coli and *Staphylococcus aureus* were grown in nutrient broth at 37 °C for 24 h and harvested by centrifugation at 2000 × *g* for 10 min. Bacterial count was calculated by the serial dilution method, and absorbance at 625 nm was maintained around 1.0. Agglutination assays were performed as described by Melo et al. [23].

2.7. Molecular modeling by homology of ELEL

The structures were predicted through the homology molecular modeling server I-TASSER [24], a free online program that allows a protein structure to be fully modeled by homology. The template used to obtain the model of ELEL-1 was the crystal structure of the rhamnose-binding lectin CSL-3 (PDB ID: 2ZX2), which had the highest degree of identity among the primary structures aligned with ELEL-1. All model quality parameters were analyzed at the end of computational simulation.

2.8. Molecular docking

The model ELEL-1 structure obtained by structural homology was used for all docking simulations. The three-dimensional structures of potential ligands were drawn using the online software PRODRG [25]. The carbohydrates used were L-rhamnose and melibiose and a glycolipid (globotrioseceramide; Galα1-4Galβ1-4Glcβ1-Cer). Molecular docking analysis was performed with Molegro using the MolDock method [26]. MolDock is based on a search algorithm combining differential evolution with a cavity prediction algorithm. The program takes hydrogen bond directionality into account as an additional term in the docking scoring function. A re-ranking procedure was added to increase docking accuracy. A MolDock Score (MDS) was calculated using the scoring function. Grid resolution was 0.30 Å with radius of 15 Å. The search algorithm used was MolDock Optimizer with default settings. The number of runs was 10, and the maximum number of interactions was 2000. The population size and maximum number of poses were 200 and 10, respectively. Protein–ligand interaction energy was expressed in the form of the MDS in arbitrary units. A more negative value reflects a stronger interaction. The MDS was calculated with the following equation: $MDS = E_{inter} + E_{intra}$, where E_{inter} is the ligand–protein interaction energy [26].

$$E_{inter} = \sum_{i \in ligand} \sum_{j \in ligand} \left[E_{PLP}(r_{ij}) + 332.0 \frac{q_j q_j}{4 r_{ij}^2} \right]$$

3. Results

3.1. Purification of ELEL

The crude extract of *E. lucunter* eggs showed strong hemagglutinating activity against rabbit and human erythrocytes. After affinity chromatography on the lactosyl-agarose column, unadsorbed material was devoid of hemagglutinating activity, whereas adsorbed proteins, recovered with 0.3 M L-rhamnose, concentrated most of the hemagglutinating activity of the crude extract.

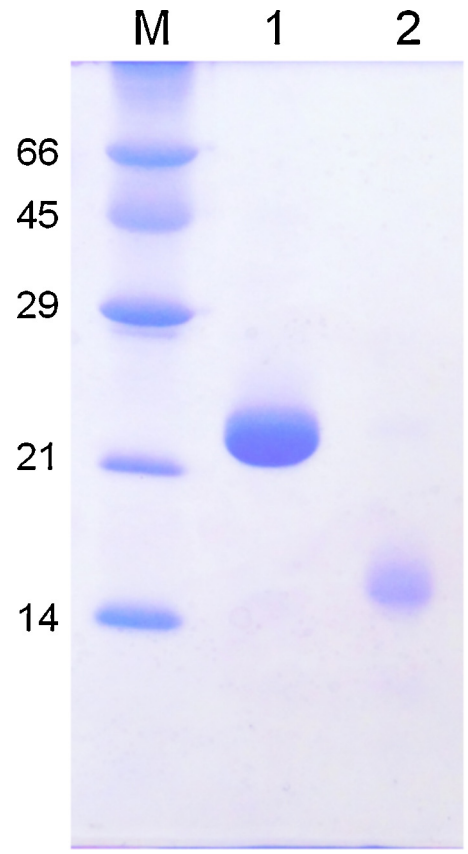

Fig. 1. SDS–PAGE (15%) of purified ELEL. (M) Molecular marker; (1) 40 μg of ELEL in the absence of reducing agents; (2) 40 μg of ELEL in the presence of β-mercaptoethanol.

SDS–PAGE analysis of the adsorbed proteins revealed one unique band of 22 kDa and 14 kDa in the absence and presence of reducing agents, respectively (Fig. 1). *E. lucunter* eggs lectin (ELEL) appears to be a dimeric protein formed by two subunits linked by a disulfide bond. ELEL was purified 28 times and represented 70% of the total hemagglutinating activity of the extract (Table 1).

3.2. Sugar binding specificity of ELEL

ELEL was able to agglutinate human and rabbit erythrocytes with a slight preference for rabbit erythrocytes treated with trypsin. Hemagglutinating activity of ELEL was, however, inhibited by several sugars, including galactose, lactose, rhamnose and melibiose (Table 2). L-rhamose was the most potent inhibitor for ELEL with minimum inhibitory concentration (MIC) of 0.4 mM. Glycoproteins, such as mucin and thyroglobulin, showed no inhibition at concentrations up to 2 mg/mL.

Table 1
Purification procedure of ELEL.

Fraction	Protein total (mg)	HU/mL	Specific activity		Yeld (%)	Purification (fold)	MAC[a] (μg/mL)
			(HU/mg)	Total			
Crude extract	80.9	32	19.7	1600	100	1	50
Affinity column	2	32	561.4	1120	70	28.4	1.8

[a] Minimum concentration able to cause hemagglutination.

Table 2
Inhibition of the hemagglutinating activity of ELEL by sugars and glycoproteins.

Sugars	MIC*
D-Arabinose	–
L-Fucose	–
D-Galactose	12.5 mM
D-Glucose	–
D-Mannose	–
L-Rhamnose	0.39 mM
N-acetyl-D-galactosamine	–
N-acetyl-D-glucosamine	–
N-acetyl-D-mannosamineD-Xylose	– –
Methyl-α-D-galactopyranoside	12.5 mM
Methyl-β-D-galactopyranoside	12.5 mM
D-Maltose	–
D-Sucrose	–
α-D-Lactose	1.56 mM
β-D-Lactose	1.56 mM
α-D-Melibiose	0.78 mM
Glycoproteins	
Orosomucoid	–
Thyroglobulin	–
Ovomucoid	–
Porcine stomach mucin	–

* Minimum concentration of sugar required for inhibition.

3.3. Effects of pH, temperature and divalent cations

Divalent cations and EDTA did not affect ELEL activity (Fig. 2A). The optimum pH for ELEL activity was 7. Below this value, the activity was stable up to pH 4. Above pH 7, hemagglutinating activity decreased until it was abolished at pH 10 (Fig. 2B). ELEL showed relative thermal stability, maintaining its total activity up to 60 °C. After that, activity decreased slightly until it was entirely lost at 100 °C (Fig. 2C).

3.4. Molecular mass and sugar content

The molecular mass of native ELEL was estimated to be 26 kDa by size exclusion chromatography. RPC revealed heterogeneities in ELEL preparations. Two isolectins (ELEL-1 and ELEL-2) could be partially separated by RPC on a C8 column (Fig. 3). On ESI-MS, ELEL-1 showed a molecular mass of 22,091 ± 2 Da, whereas ELEL-2 showed a molecular mass of 23,286 ± 2 Da (Fig. 4). These ions represent dimeric forms of ELEL-1 and ELEL-2. Estimated mass of the monomeric forms of ELEL-1 and ELEL-2 were 11,045 ± 2 Da and 11,643 ± 2 Da, respectively.

No sugar contents were found in ELEL by the phenol-sulfuric acid method, and no coloration was observed when SDS–PAGE was incubated with periodic acid Schiff. These results indicated that ELEL is not a glycoprotein.

3.5. Primary structure of ELEL

Because of the low yield of isoform 2 in soluble form, only ELEL-1 was sequenced. Its N-terminal was blocked and thus could not be determined by Edman degradation. Peptides sequenced by MS/MS, corresponding to the N-terminal region, presented a blocked terminal amino group, with all peptides having pyroglutamic acid as the first residue in this region, whereas the unblocked peptides had glutamic acid in the N-terminal (Supplementary table).

De novo sequencing revealed a polypeptide chain of 103 residues with a combined molecular mass of 11,047 Da. This value is in good agreement with molecular mass determined by ESI-MS (11,045 ± 2 Da).

The amino acid sequence of ELEL-1 is shown in Fig. 5. Nine half-cysteines were found; eight appear to be involved in intrachain disulfide bonds, whereas one half-cysteine forms a disulfide bond between two subunits to link the homodimer. No glycosylation site was found. Microheterogeneities were observed in four positions: 26 (K/R); 64 (N/Q); 91 (H/L); and 99 (T/S).

A homolog search with BLASTp demonstrated that the ELEL-1 sequence was significantly similar to RBLs of other invertebrates (Fig. 6), such as sea urchin eggs lectin from *A. crassispina* (61%), predicted RBL from the sea urchin *Strongylocentrotus purpuratus* (45%), RBL from the Pacific oyster *Crassotrea gigas* (47%) and predicted RBL from *Hydra vulgaris* (48%). Moreover, ELEL-1 showed relative identity with CSL3 (43%), an RBL isolated form chum salmon eggs (*Oncorhynchus keta*).

3.6. Agglutination of bacterial cells

ELEL (100 μg/mL) could agglutinate Gram-positive formalin-killed *S. aureus*, but was not able to agglutinate Gram-negative *E. coli* (Fig. 7).

3.7. Molecular modeling by homology of ELEL-1

The model of ELEL-1 resulted in a C-score of 1.56. A high-quality model is expected to have strongly positive C-scores (−5 to 2). TM-score of 0.93 ± 0.06 and RMSD of 1.2 ± 1.2 Å indicate that the model obtained by homology showed excellent quality [24,27]. The ELEL-1 model consists of 103 amino acids folded as a β-sandwich. The structure has two anti-parallel β-sheets with two (β2 and β4) and three (β1, β3, and β5) strands, two helices (α1–2), and six loop regions (Fig. 8), from which loops 1, 5 and 6 comprise residues that are part of the CRD.

3.8. Molecular docking

Based on hemagglutinating assays, the interaction between ELEL-1 and mono- (L-rhamnose) and disaccharide (melibiose) was calculated. We also determined the interaction with the glycolipid

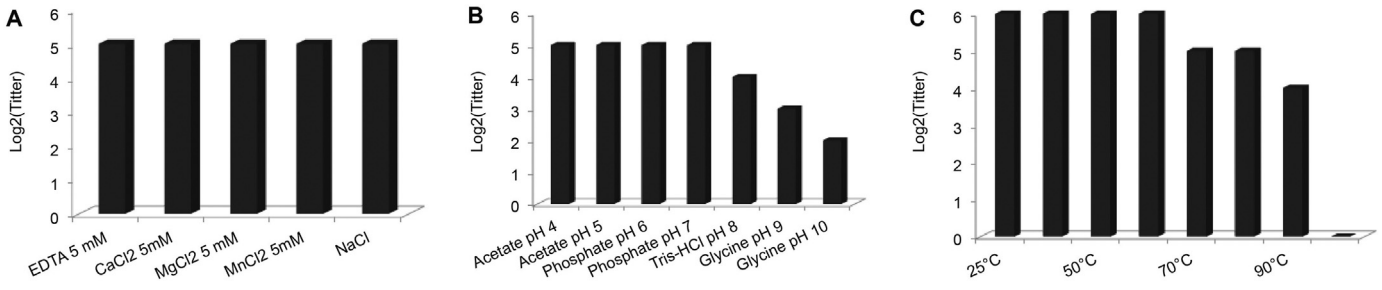

Fig. 2. Properties of the hemagglutinating activity of ELEL. Effect of divalent cations and EDTA (A), pH (B) and temperature (C) on the hemagglutinating activity of ELEL. Hemagglutinating activity was expressed in logarithm scale as units of titter.

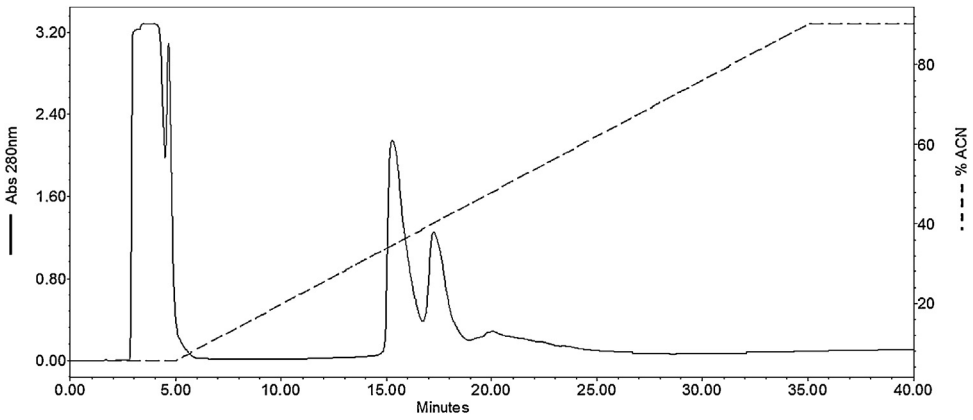

Fig. 3. Reverse phase chromatography of ELEL. Sephasil C-8 column was equilibrated and washed with 5% ACN in 0.1% TFA. Elution was performed with ACN gradient in 0.1% TFA. Flow rate was maintained at 1.0 mL/min.

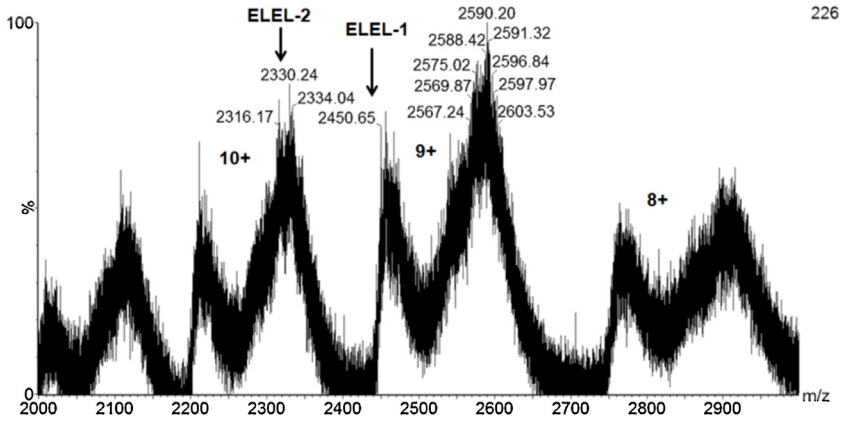

Fig. 4. Molecular mass determination of ELEL. Mass spectra of ELEL after RPC, using nano ESI infusion at 1 μL/min.

Gb_3 (Galα1-4Galβ1-4Glcβ1-Cer) present on the surface of both macrophages and some tumor cells.

Carbohydrates were anchored in the region corresponding to CRD of ELEL-1 and presented scores of −54.94 MDS and −112.74 MDS for L-rhamnose and melibiose, respectively.

ELEL-1 molecular docking performed with Gb_3 showed favorable binding energy with MDS at −143.99 (Fig. 9A) and revealed that Gb_3 interacts with amino acids Glu15, Asp48, Gly49, Ala50, Asp86, Met89, His90, Trh91, Tyr92, and Lys93 through hydrogen bonds and van der Waals interactions (Fig. 9B).

4. Discussion

Since Ozeki and coworkers reported the first known RBL [14], several RBLs have been isolated and characterized from roes and ovaries of teleosts [1,11,28,29] and marine invertebrates [12,15,30]. In this work, we have isolated an L-rhamnose-binding lectin, named ELEL, from the eggs of the rock boring sea urchin *E. lucunter* by affinity chromatography on lactosyl-agarose. Since all lectin activity presented in the crude extract was totally recovered by adsorption onto immobilized lactose, the use of lactosyl-agarose to purify the lectin proved to be successful. Affinity chromatography is a standard procedure for isolation of RBLs, such as lectins from *Pteria penguin* [12], *Ctenopharyngodon idellus* ovaries [31], *Plecoglossus altivelis* eggs [32], *Silurus asotus* roe [33], and *Botryllus schlosseri* [15].

Actually, the protein isolated from *E. lucunter* eggs is a mixture of two isolectins. MS analysis revealed a slight difference between the molecular masses of ELEL-1 and ELEL-2. The microheterogeneities found in four positions of the amino acid sequence of ELEL could be attributed to inter-individual variations. Unfortunately, these small differences could not be detected by SDS–PAGE or size

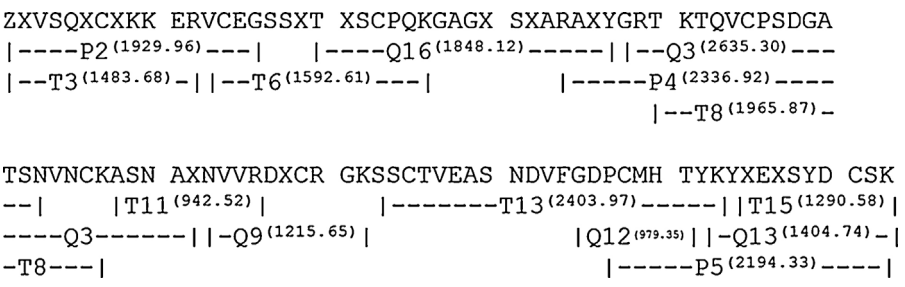

Fig. 5. Amino acid sequence of ELEL-1. Peptides originated by different digestions are represented by T (trypsin), Q (chymotrypsin) and P (pepsin). X represents Leucine or Isoleucine.

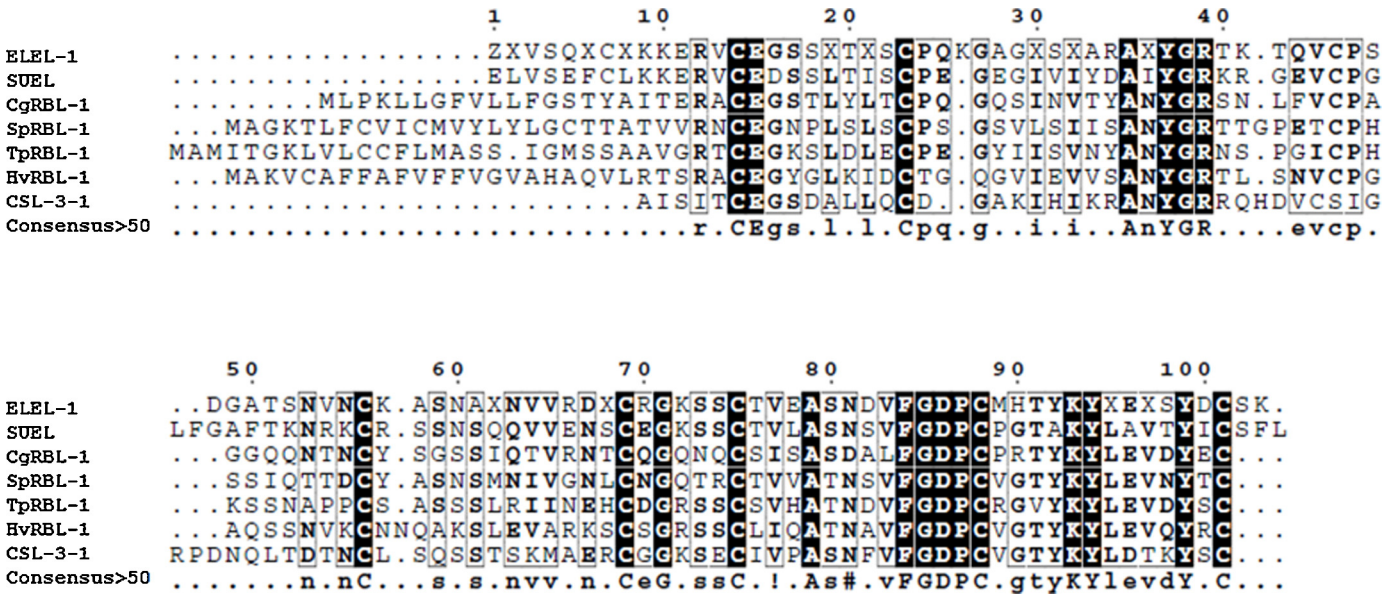

Fig. 6. Comparison of amino acid sequence of ELEL-1 to invertebrate RBLs and CSL-3. Multiple alignment among the amino acid sequence of the *E. lucunter* eggs lectin (ELEL-1), *A. crassispina* eggs RBL (SUEL), *Crassostreagigas* predicted RBL – domain 1(CgRBL-1), *Stroglyocentrotus purpureos* predicated RBL – domain 1 (SpRBL-1), *Toxopneustes pileolus* RBL – domain 1 (TpRBL-1), *H. vulgaris* predicated RBL – domain 1 (HvRBL-1) and chum salmon RBL – domain 1 (CSL-3-1).

exclusion chromatography, nor could a successful separation of ELELs be performed by RPC. Therefore, in biological assays and biochemical characterization, ELEL preparations were considered a unique protein.

The presence of isoforms in RBL preparations is relatively common and has already been described in the Japanese catfish *S. asotus* [33] and the ponyfish *Leiognathus nuchalis* [34]. In the colonial ascidian *B. schlosseri*, five transcripts were identified by cDNA cloning, and four of these five isolectins were effectively identified in affinity chromatography preparations. *B. schlosseri* isolectins (BrRBLs) only differ by a few amino acids; therefore, such as ELELs, BrRBLs could not be separated by analytical techniques, such as SDS–PAGE and size exclusion chromatography [15].

ELEL-1 is a typical RBL since its sequence contains two typical motifs present in the most known RBLs: -ANYGR(TD)- in N-terminal and -DPCX(G)T(Y)KY(L)- in C-terminal. The current classification of RBLs suggests that ELEL-1 is grouped in V-type subfamily, because of its quaternary organization as homodimer linked by disulfide bond. Besides ELEL-1, only SUEL is grouped in V-type subfamily [3].

In our modeling, the three-dimensional structure of ELEL-1 revealed a β-sandwich fold. This folding is found in two structures of L-rhamnose-binding lectins deposited in the PDB: CSL-3 (PDB ID: 2ZX2) [4], which was used as a template, and mouse lectin latrophilin-1 (PDB ID: 2JXA) [35]. Interestingly, a D-rhamnose-binding lectin, pyocin L1 of *Pseudomonas aeruginosa* (PDB ID: 4LED)

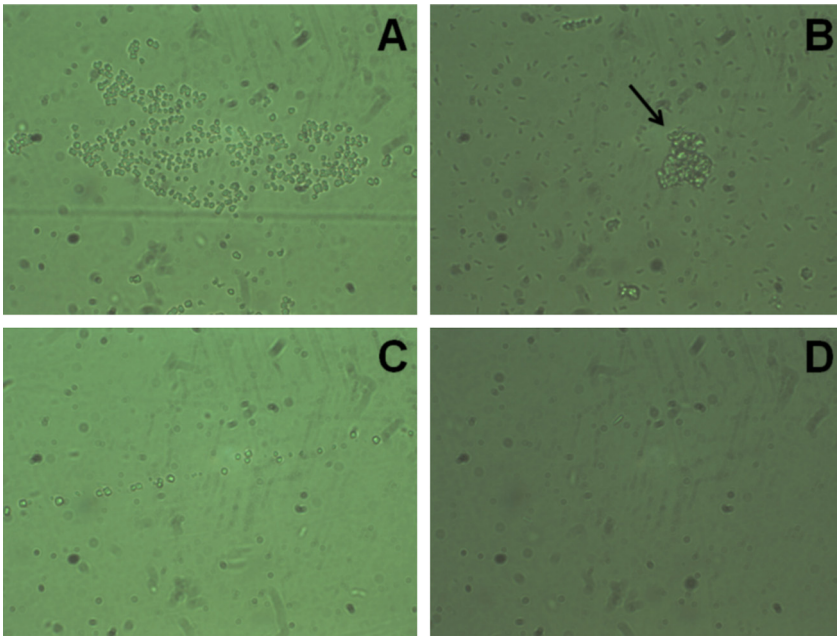

Fig. 7. Agglutination of bacteria by ELEL. (A) *S. aureus* incubated with TBS; (B) *S. aureus* incubated with ELEL. (C) *E. coli* incubated with TBS; (D) *E. coli* incubated with ELEL; scale bars indicate 4 μm.

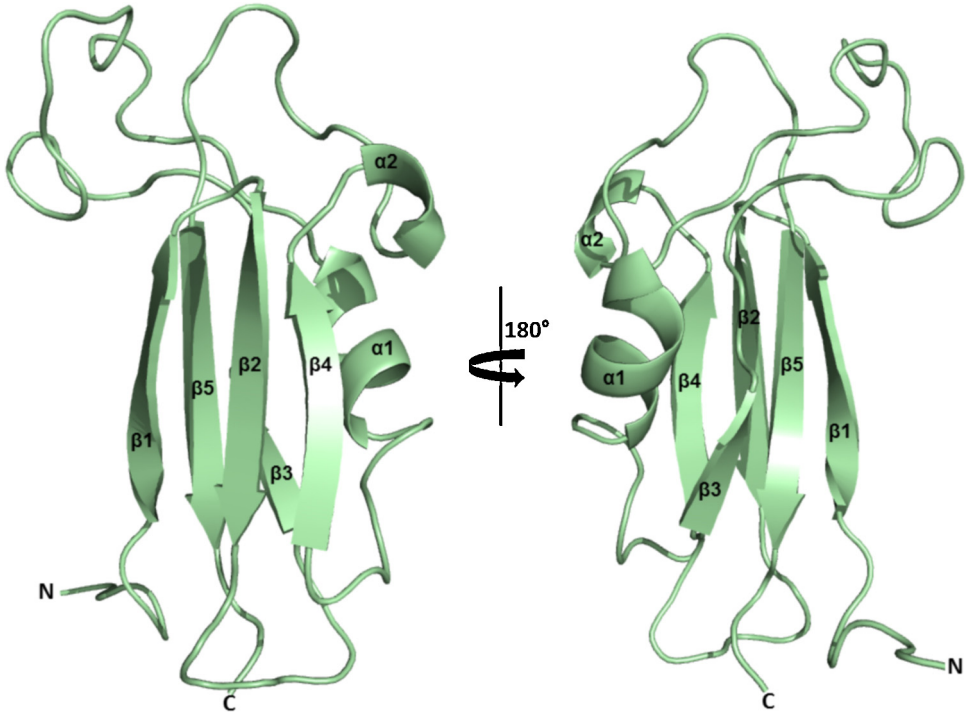

Fig. 8. Three dimensional structure model of ELEL-1. β-sandwich structure composed of two β-sheets: posterior (β1, β2 and β4 strands) and frontal (β3 and β5 strands).

[36], possesses a β-prism-II domain, which is different from ELEL-1, CSL-3 and latrophilin-1, indicating that the difference in the recognition of rhamnose configurations (D and L) by lectins can be related to a domain that characterize their three-dimensional structures.

ELEL was very stable when it was submitted to a wide variation of pH and temperature, as expected by their high contents of half-cysteine residues. Nine half-cysteines were found in ELEL. Curiously, once ELEL was reduced (DTT) and alkylated (IAA) before digestions, some cysteines were not found in its carboxyamidomethylated form, as might be expected. For instance, peptide at *m*/*z* 955.39 corresponds to sequence ^{41}TKTQVCPSDGATSNVNCK^{58} and has a determined molecular mass of 1908.78 Da, whereas peptide at *m*/*z* 983.93 has a determined molecular mass of 1965.87 Da and the same sequence. The difference of 57 Da apparently results from carboxyamidomethyl missing in one of the two cysteine residues in peptide at *m*/*z* 955.39. Moreover, other peptides were found without carboxyamidomethylated cysteines, indicating that different cysteines have different reducing potential resulting from localization and/or neighborhood of the residue.

RBLs typically have eight cysteines paired in four disulfide bonds: Cys (1)-Cys(3), Cys (2)-Cys (8), Cys (4)-Cys (7), and Cys (5)-Cys (6) [37]. All cysteines present in ELEL are aligned with

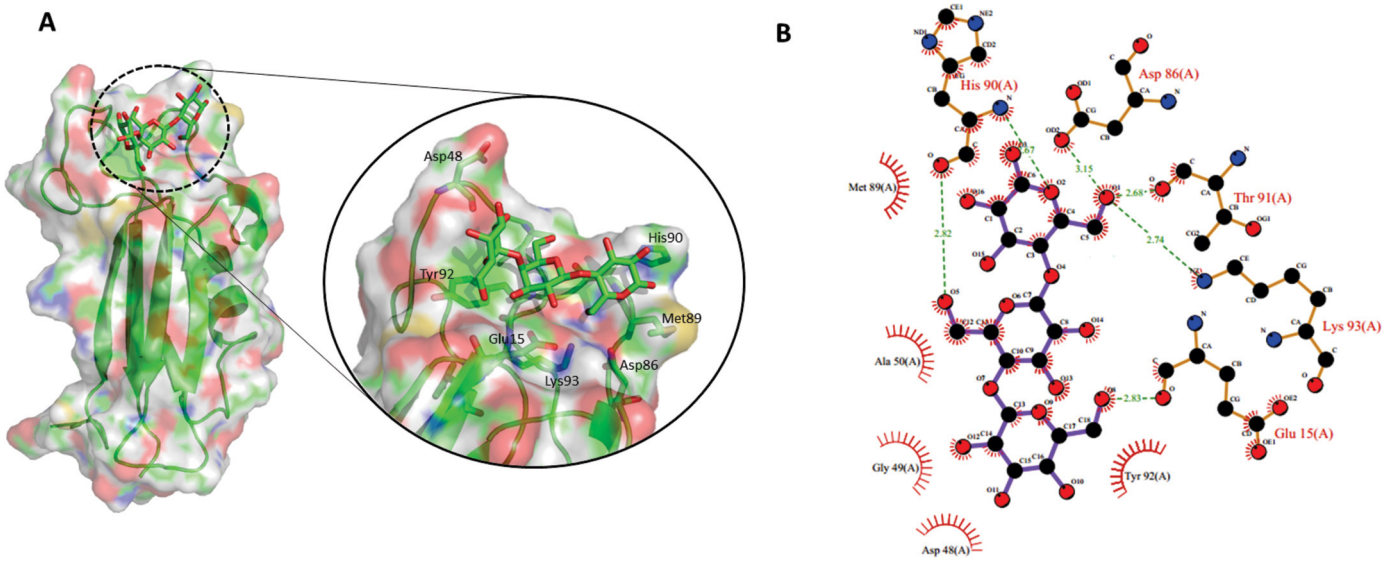

Fig. 9. Molecular docking of ELEL-1 with Gb_3. (A) Representation of the eletrostatic surface area of the carbohydrate recognition domain of ELEL-1 complexed with Gb_3. (B) Prediction of H-bonds and van der Waals interactions in the carbohydrate recognition domain. Distances in Å.

corresponding residues of the other RBLs, except Cys in position 7. ^{7}Cys is also present in SUEL, which, similar to ELEL, is a homodimer linked by a disulfide bond [14]. Thus, ^{7}Cys seems be involved in the maintenance of dimers.

ELEL was not inhibited by glycoproteins, such as CSLs [11]. However, hemagglutinating activity of ELEL was inhibited by saccharides with the same orientation of hydroxyl groups at C2 and C4 in the pyranose ring, such as L-rhamnose, melibiose and, to a lesser degree, galactose. Because glucose, arabinose, L-fucose and mannose showed no inhibitory activity against ELEL, the configuration at C-4 is primordial for recognition.

The difference in structure between L-rhamnose and D-mannose can be seen when comparing the symmetrical ring structures where, in Haworth projection, the hydroxyl group at C-4 in D-mannose is below the plane of the ring (equatorial), while L-rhamnose is above it (axial). The configuration at C-3 and C-5 of the pyranose ring is also important based on the lower inhibition potency observed for melibiose, lactose, galactose and methyl-galactoside in comparison to L-rhamnose. The failure of GalNAc to inhibit agglutination may have resulted from the presence of an acetamide group linked to C-2 of the pyranose ring. On the other hand, since galactose and α-methyl galactoside showed very similar MIC values, substituent groups in other positions, i.e., methyl in C-1, did not affect inhibition and are, therefore, unimportant in the binding of the sugars to the lectin. Since α- or β-galactosides, in general, produced similar levels of inhibition, results showed that ELEL does not differentiate between them. Methyl-α-D-galactoside had the same inhibitory effect as methyl-β-D-galactoside, as did α-D-lactose and β-D-lactose.

Several RBLs showed inhibition results similar to ELEL. For example, the lectins isolated from chum salmon eggs (CSL1, CSL2 and CSL3) were inhibited by L-rhamnose, melibiose, raffinose and, to a lesser extent, galactose, but they were not inhibited by lactose [11]. BrRBLs demonstrated better inhibition by melibiose, rhamnose and galactose, as well as raffinose, but, again, to a lesser extent [15]. Like ELEL, the lectin isolated from penguin wing oyster (PPL) was not inhibited by GalNAc. Furthermore, PPL showed a strong affinity for terminal Galβ1-4GlcNAc and β-lactose [12]. In contrast to ELEL, the RBL isolated from the skin mucus of ponyfish showed strong affinity for GalNAc [34], and RBLs isolated from *Tribolodon brandti* eggs, TBL 1 and TBL 2, were not inhibited by galactose and melibiose, respectively [38].

Although RBLs typically recognize L-rhamnose, a few RBLs do not [12]. Curiously, L-rhamnose is an uncommon sugar present in vertebrates or invertebrates, suggesting an exogenous impact on the recognition of L-rhamnose by RBLs. Several RBLs are able to recognize Globotriaosylceramide (Gb_3: Galα1-4Galβ1-4Glcβ1-Cer). Gb_3 is a common epitope located in glycolipid membrane [39,40]. Molecular simulations suggest that ELEL-1 recognizes Gb_3 in a manner similar to that of CSL3. In accordance with our results, the amino acids involved in Gb_3 recognition by ELEL-1 correspond to the carbohydrate-binding site in CSL-3 structure [4].

Gb_3 is a glycolipid antigen highly expressed in metastatic colon cancer [41]. The interaction between ELEL and Gb_3, as indicated by *in silico* experiments, makes this lectin a potential tool in the identification of cancer metastasis. Indeed, RBLs can trigger apoptosis in cell lines containing Gb_3. For instance, CSL-3 showed cytotoxicity to Gb_3-displayng Caco-2 human colorectal adenocarcinoma cells in a dose-dependent manner, while no effects were observed in tumor cell lines lacking Gb_3 [4]. It has been reported that RBLs also bind endogenously to globotriaosylceramide and that the interaction between RBLs and Gb_3 results in the induction of proinflammatory cytokines and activation of the immune system [32,42,43]. Thus, boosting immune response seems to be an important endogenous function of the RBLs.

As shown in the present study, RBLs also play an important role in combating pathogens. More specifically, since different RBLs can agglutinate bacterial cells, they seem to act as nonself recognition molecules [3,11,44]. The agglutination of bacterial cells most likely results from RBL binding to lipopolysaccharides and lipoteichoic acid, as described by Tateno and coworkers [10,29]. Like RBLs isolated from *P. penguin* and *B. schlosseri*, ELEL was also able to agglutinate bacterial cells. However, unlike PPL and BsRBL, ELEL only agglutinated the Gram-positive bacterium *S. aureus*, whereas PPL agglutinated both Gram-positive and Gram-negative bacteria, while BsRBL agglutinated only Gram-negative bacteria [12,15].

In conclusion, we isolated a Type V L-rhamnose-binding lectin from eggs of the sea urchin *E. lucunter*. ELEL has carbohydrate specificity, amino acid sequence and three-dimensional structure similar to other rhamnose-binding lectins, characteristics which would qualify it as a new member of the RBL superfamily. Furthermore, *in silico* experiments suggest that ELEL recognizes Gb_3 what makes this protein a potential biotechnological tool. However, future assays should be performed to evaluate the real activity of ELEL against malignant cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2015.03.072

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5. CONCLUSÃO

Neste trabalho lectinas com afinidade por galactose e seus derivados foram isoladas de três diferentes táxons de invertebrados marinhos, demonstrando o potencial destes organismos em fornecer moléculas de interesse biotecnológico.

Interessantemente, as três lectinas apresentaram estruturas primárias completamente distintas, indicando que a especificidade neste caso não está associada a sequência de aminoácidos. As três lectinas apresentaram algum efeito sobre células bacterianas e/ou biofilmes, estando o DRC envolvido nas respectivas atividades, o que indica que a afinidade por galactose pode ter surgido para o reconhecimento de bactérias patogênicas ou mesmo simbiontes.

Conclui-se, portanto, que a afinidade por galactosídeos em diferentes táxons de invertebrados marinhos evoluiu de maneira convergente. Lectinas com esta afinidade podem estar associadas ao reconhecimento de microrganismos e, consequentemente, desencadear efeitos antibacterianos e antibiofilme.