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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.

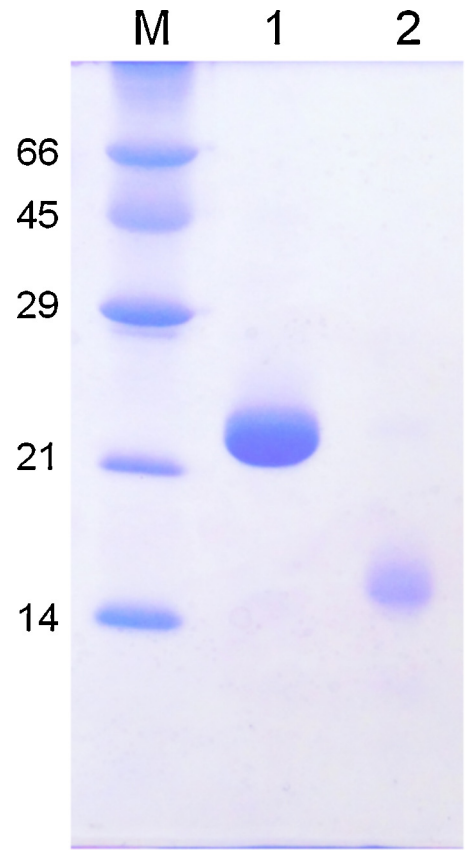

Fig. 1. SDS–PAGE (15%) of purified ELEL. (M) Molecular marker; (1) 40 μg of ELEL in the absence of reducing agents; (2) 40 μg of ELEL in the presence of β-mercaptoethanol.

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

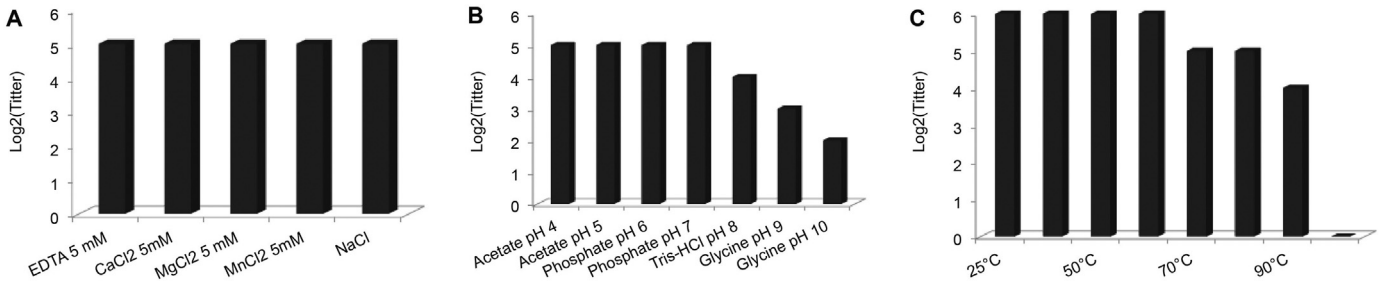

Fig. 2. Properties of the hemagglutinating activity of ELEL. Effect of divalent cations and EDTA (A), pH (B) and temperature (C) on the hemagglutinating activity of ELEL. Hemagglutinating activity was expressed in logarithm scale as units of titter.

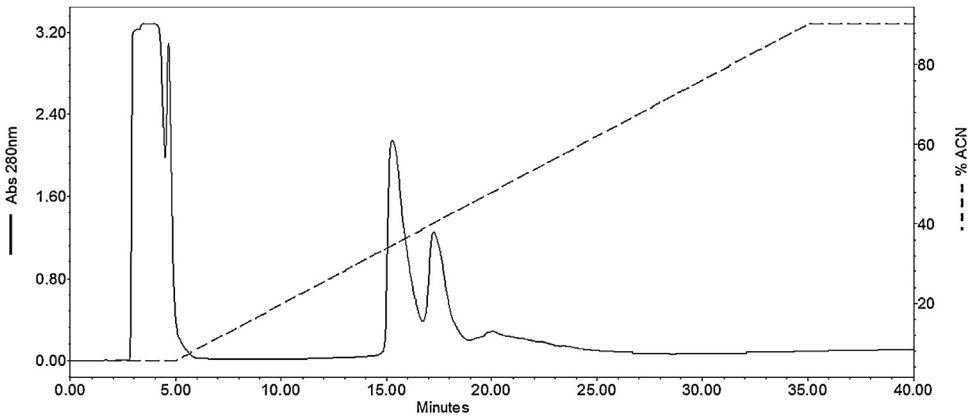

Fig. 3. Reverse phase chromatography of ELEL. Sephasil C-8 column was equilibrated and washed with 5% ACN in 0.1% TFA. Elution was performed with ACN gradient in 0.1% TFA. Flow rate was maintained at 1.0 mL/min.

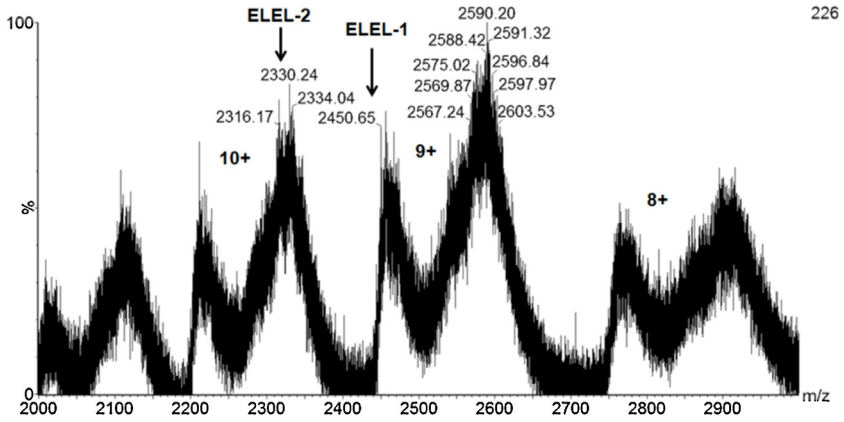

Fig. 4. Molecular mass determination of ELEL. Mass spectra of ELEL after RPC, using nano ESI infusion at 1 μL/min.

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

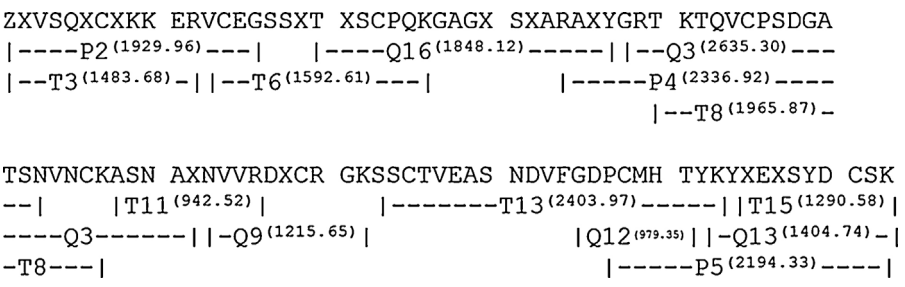

Fig. 5. Amino acid sequence of ELEL-1. Peptides originated by different digestions are represented by T (trypsin), Q (chymotrypsin) and P (pepsin). X represents Leucine or Isoleucine.

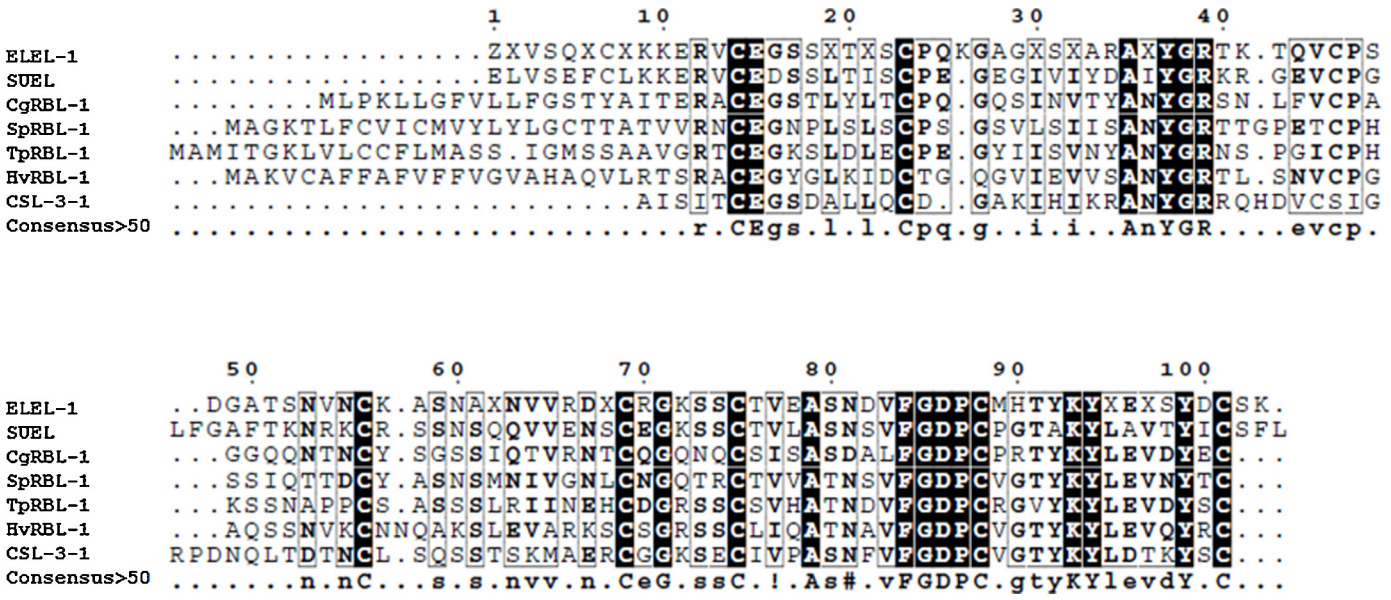

Fig. 6. Comparison of amino acid sequence of ELEL-1 to invertebrate RBLs and CSL-3. Multiple alignment among the amino acid sequence of the *E. lucunter* eggs lectin (ELEL-1), *A. crassispina* eggs RBL (SUEL), *Crassostreagigas* predicted RBL – domain 1(CgRBL-1), *Stroglyocentrotus purpureos* predicated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

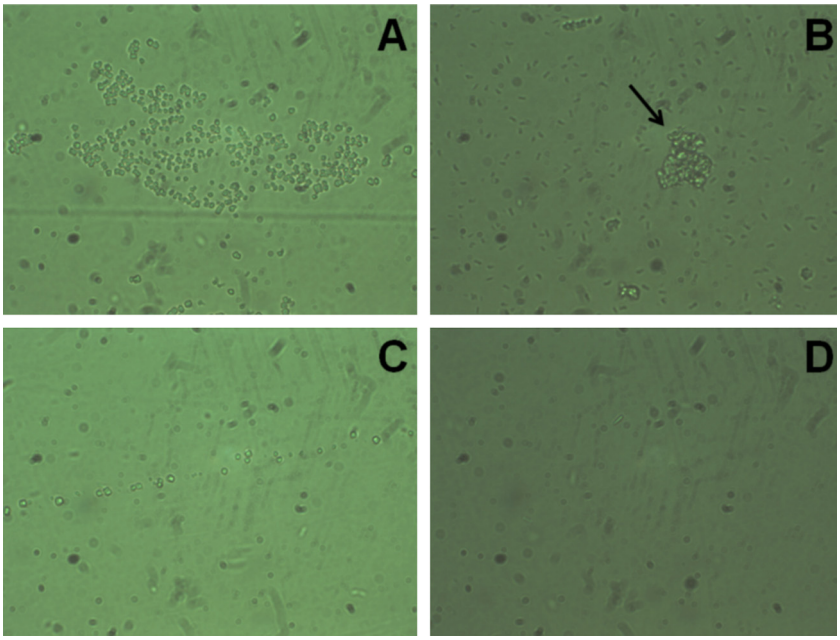

Fig. 7. Agglutination of bacteria by ELEL. (A) *S. aureus* incubated with TBS; (B) *S. aureus* incubated with ELEL. (C) *E. coli* incubated with TBS; (D) *E. coli* incubated with ELEL; scale bars indicate 4 μm.

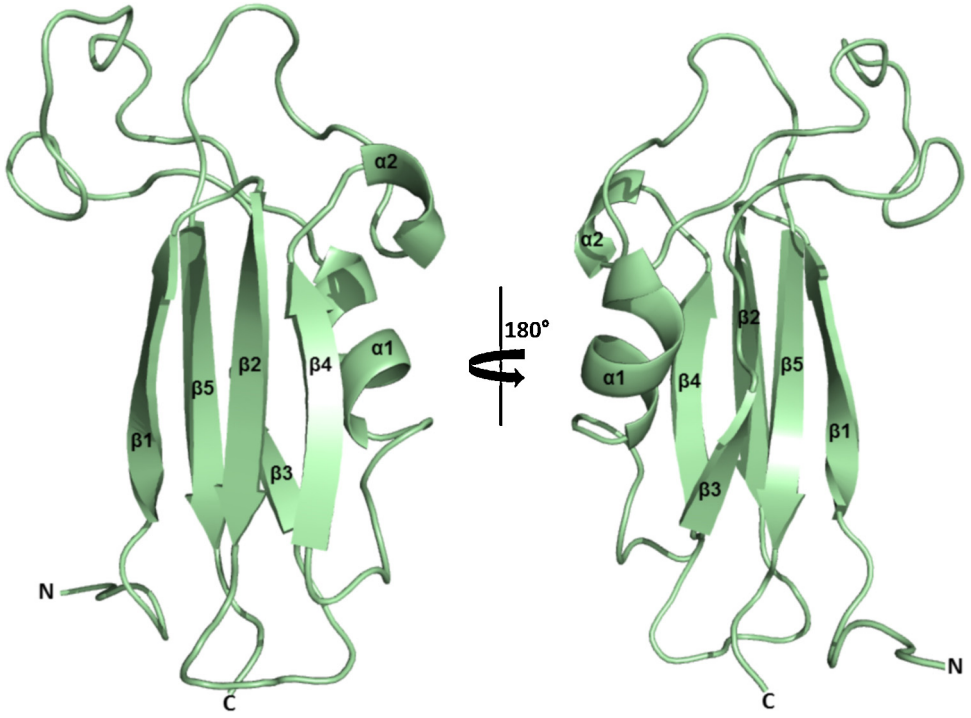

Fig. 8. Three dimensional structure model of ELEL-1. β-sandwich structure composed of two β-sheets: posterior (β1, β2 and β4 strands) and frontal (β3 and β5 strands).

[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

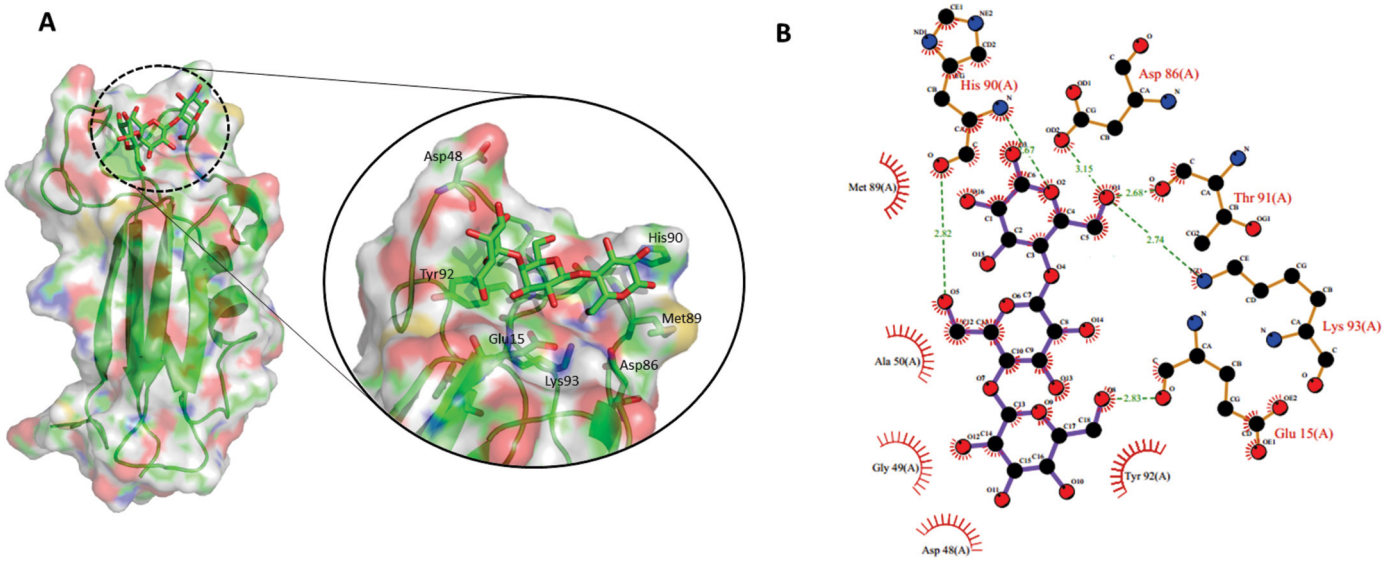

Fig. 9. Molecular docking of ELEL-1 with Gb_3. (A) Representation of the eletrostatic surface area of the carbohydrate recognition domain of ELEL-1 complexed with Gb_3. (B) Prediction of H-bonds and van der Waals interactions in the carbohydrate recognition domain. Distances in Å.

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.

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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

References

[1] H. Tateno, A. Saneyoshi, T. Ogawa, K. Muramoto, H. Kamiya, M. Saneyoshi, J. Biol. Chem. 273 (1998) 19190–19197.
[2] H. Tateno, Biosci. Biotechnol. Biochem. 74 (6) (2010) 1141–1144.
[3] T. Ogawa, M. Watanabe, T. Naganuma, K. Muramoto, J. Amino Acids (2011) 1–20.
[4] T. Shirai, Y. Watanabe, M. Lee, T. Ogawa, K. Muramoto, J. Mol. Biol. 391 (2009) 390–403.
[5] M. Hosono, K. Ishikawa, R. Mineki, K. Murayama, C. Numata, Y. Ogawa, Y. Takayanagi, K. Nitta, Biochim. Biophys. Acta 1472 (3) (1999) 668–675.
[6] M. Hosono, S. Sugawara, T. Tastuda, T. Hikita, J. Kominami, S. Nakamura-Tsuruta, J. Hirabayashi, S.M.A. Kawsar, Y. Ozeki, S. Hakomori, K. Nitta, Fish Physiol. Biochem. 39 (6) (2013) 1619–1630.
[7] J. Nosek, A. Krajhanzl, J. Kocourek, Histochemistry 79 (1984) 131–139.
[8] A. Krajhanzl, A. Danisova, J. Kocourek, P. Pancoska, in: T.C. Bog-Hansen, E. van Driesche (Eds.), Lectins Biology, Biochemistry, and Clinical Biochemistry, vol. 4, de Gruyter, Berlin–New York, 1985.
[9] N. Franchi, F. Schiavon, M. Carletto, F. Gasparini, G. Bertoloni, S.C.E. Tosatto, L. Ballarin, Immunobiology 216 (2011) 725–736.
[10] H. Tateno, T. Ogawa, K. Muramoto, H. Kamiya, M. Saneyoshi, Biosci. Biotechnol. Biochem. 66 (3) (2002) 604–612.
[11] N. Shiina, H. Tateno, T. Ogawa, K. Muramoto, M. Saneyoshi, H. Kamiya, Fish. Sci. 68 (2002) 1352–1366.
[12] T. Naganuma, T. Ogawa, J. Hirabayashi, K. Kasai, H. Kamiya, K. Muramoto, Mol. Divers. 10 (4) (2006) 607–618.
[13] H. Sasaki, K. Aketa, Exp. Cell. Res. 135 (1) (1981) 15–19.
[14] Y. Ozeki, T. Matsui, M. Suzuki, K. Titani, Biochemistry 30 (1991) 2391–2394.
[15] F. Gasparini, N. Franchi, B. Spolaore, L. Ballarin, Dev. Comp. Immunol. 32 (2008) 1177–1191.
[16] M. Wisshak, L. Tapanila, Current Developments in Bioerosion, Erlangen Earth Conference Series, Springer-Verlag, Berlin, 2008.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248–534.
[18] A.H. Sampaio, D.J. Rogers, C.J. Barwell, Phytochemistry 48 (5) (1998) 765–769.
[19] U.K. Laemmli, Nature 227 (1970) 680–683.
[20] R.M. Zacharius, T.E. Zell, J.H. Morrison, J.J. Woodlock, Anal. Biochem. 30 (1969) 148–152.

[21] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[22] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, Nat. Protoc. 1 (6) (2006) 2856–2861.
[23] A.A. Melo, R.F. Carneiro, W.M. Silva, R.M. Moura, G.C. Silva, O.V. Sousa, J.S. Saboya, K.S. Nascimento, S. Saker-Sampaio, C.S. Nagano, B.S. Cavada, A.H. Sampaio, Int. J. Biol. Macromol. 64 (2014) 435–442.
[24] Y. Zhang, BMC Bioinformatics 23 (2008) 9–40.
[25] A.W. Schüttelkopf, D.M.F. van Aalten, Acta Crystallogr. D 60 (2004) 1355–1363.
[26] R. Thomsen, M.H. Christensen, J. Med. Chem. 49 (2006) 3315–3321.
[27] A. Roy, A. Kucukural, Y. Zhang, Nat. Protoc. 5 (2010) 725–738.
[28] H. Tateno, T. Ogawa, K. Muramoto, H. Kamiya, T. Hirai, M. Saneyoshi, Biosci. Biotechnol. Biochem. 65 (2001) 1328–1338.
[29] H. Tateno, T. Ogawa, K. Muramoto, H. Kamiya, M. Saneyoshi, Biosci. Biotechnol. Biochem. 66 (2002) 1356–1365.
[30] H. Sakai, K. Edo, H. Nakagawa, M. Shinohara, R. Nishitsutsuji, K. Ohura, Int. Aquat. Res. 5 (12) (2013) 1–10.
[31] Y.W. Lam, T.B. Ng, Protein. Expres. Purif. 26 (3) (2002) 378–385.
[32] Y. Watanabe, N. Shiina, F. Shinozaki, H. Yokoyama, J. Kominami, S. Nakamura-Tsuruta, J. Hirabayashi, K. Sugahara, H. Kamiya, H. Matsubara, T. Ogawa, K. Muramoto, Dev. Comp. Immunol. 32 (2008) 487–499.
[33] M. Hosono, H. Kawauchi, K. Nitta, Y. Takayanagi, H. Shiokawa, R. Mineki, K. Murayama, Biol. Pharm. Bull. 16 (1993) 1–5.
[34] M. Okamoto, S. Tsuitsui, S. Tasumi, H. Suetake, K. Kikuchi, Y. Suzuki, Biochem. Biophys. Res. Co. 333 (2005) 463–469.
[35] I. Vakonakis, T. Langenhan, S. Prömel, A. Russ, I.D. Campbell, Structure 16 (2008) 944–953.
[36] L.C. McCaughey, R. Grinter, I. Josts, A.W. Roszak, K.I. Waløen, R.J. Cogdell, J. Milner, T. Evans, S. Kelly, N.P. Tucker, O. Byron, B. Smith, D. Walker, PLoS Pathog. 10 (2) (2014) e1003898.
[37] T. Terada, Y. Watanabe, H. Tateno, T. Naganuma, T. Ogawa, K. Muramoto, H. Kamiya, Biochim. Biophys. Acta 1770 (2007) 617–629.
[38] M. Jimbo, R. Usui, R. Skai, K. Muramoto, H. Kamiya, Comp. Biochem. Physiol. B 147 (2) (2007) 164–171.
[39] T. Mori, N. Kiyokawa, Y.U. Katagiri, T. Taguchi, T. Suzuki, T. Sekino, N. Sato, K. Ohmi, H. Nakajima, T. Takeda, J. Fujimoto, Exp. Hematol. 28 (2000) 1260–1268.
[40] Y.U. Katagiri, T. Mori, H. Nakajima, C. Katagiri, T. Taguchi, T. Takeda, N. Kiyokawa, J. Fujimoto, J. Biol. Chem. 274 (1999) 35278–35282.
[41] O. Kovbasnjuk, R. Mourtazina, B. Baibakov, T. Wang, C. Elowsky, M.A. Choti, A. Kane, M. Donowitz, PNAS 102 (52) (2005) 19087–19092.
[42] S. Sugawara, M. Hosono, Y. Ogawa, M. Takayanagi, K. Nitta, Biol. Pham. Bull. 28 (2005) 434–441.
[43] Y. Watanabe, H. Tateno, S. Nakamura-Tsuruta, J. Kominami, J. Hirabayashi, O. Nakamura, T. Watanabe, H. Kamiya, T. Naganuma, T. Ogawa, R.J. Naudé, K. Muramoto, Dev. Comp. Immunol. 33 (2009) 187–197.
[44] T.B. Ng, Y.W. Lam, N.Y.S. Woo, Vet. Immunol. Immunop. 94 (3) (2003) 105–112.