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Molecular and functional characterization of a glycosylated Galactose-Binding lectin from *Mytilus californianus*

Efrén García-Maldonado, Patricia Cano-Sánchez, Alejandra Hernández-Santoyo*

Departamento de Química de Biomacromoléculas, Instituto de Química, Universidad Nacional Autónoma de México. Circuito Exterior, Ciudad Universitaria, Coyoacán, Cd. Mx. C.P. 04510, Mexico

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ABSTRACT

Lectins play crucial roles for innate immune responses in invertebrates by recognizing and eliminating pathogens. In this study, a lectin from the mussel *Mytilus californianus* (MCL) was identified and characterized. The lectin was purified by affinity chromatography in α-lactose-agarose resin showing an experimental molecular mass of 18000 Da as determined by SDS-PAGE and MALDI-TOF mass spectrometry. It was specific for binding D-galactose and *N*-Acetyl-D-galactosamine that contained carbohydrate moieties that were also inhibited by melibiose and raffinose. It had the ability to agglutinate all types of human erythrocytes, as well as rabbit red blood cells. Circular dichroism analyzes have indicated that this lectin possessed an α/β fold with a predominance of β structures. This was consistent with the structure of the protein that was determined by the X-ray diffraction techniques. MCL was crystallized in the space group $C2_1$ and it diffracted to 1.79 Å resolution. Two monomers were found in the asymmetric unit and they formed dimers in solution. The protein has shown to be a member of the β-trefoil family, with three sugar binding sites per monomer. In accord with fluorescence-based thermal shift assays, we observed that the MCL T_m increased about 10 °C in the presence of galactose. Furthermore, we have determined the complete amino acid sequence by cDNA sequencing. The gene had two ORF2 proteins, one resulting in a 180 residue protein with a theoretical molecular mass of 20227 Da, and another resulting in a 150 residue protein with a theoretical molecular mass of 16911 Da. The difference between the theoretical and experimental values was due to the presence of a glycosylation that was observed by the glycosylation assay. A positive microbial agglutination and a growth inhibition activity were observed against Gram-negative and Gram-positive bacteria. The *M. californianus* lectin is the fourth member of the recently proposed new family of lectins that have been reported to date, occurring only in mollusks belonging to the family Mytilidae. It is the first member to be glycosylated and with a strong tendency to form large oligomers.

1. Introduction

Lectins (carbohydrate-binding proteins) are well known to participate in the defense functions of invertebrates where they play an important role in the recognition of foreign particles. They have the ability to agglutinate red blood cells because they contain a CRD (Carbohydrate Recognition Domain) that reversibly binds the specific carbohydrate moiety that is present in those cells. Lectins have been isolated from diverse organisms and they have shown antitumor, antifungal, antibacterial, and antiviral activities [1–3]. Marine organisms have also been studied for potential sources of novel biologically active compounds, since they are in contact with an environment that has high concentrations of pathogenic fungi, bacteria, and viruses. As a result, much effort has been made in identifying and characterizing lectins from marine organisms. Lectins are classified according to different aspects, such as their carbohydrate specificity, their sources, their molecular structures, or their amino acid sequences. In particular, lectins that are derived from marine organisms can be classified into C-type lectins, F-type lectins, galectins, intelectins, and rhamnose-binding lectins [2,4,5]. A new family of lectins has been proposed as a consequence of the isolation of the first member from the mussel *Crenomytilus*

Abbreviations: CRD, Carbohydrate Recognition Domain; CGL, *Crenomytilus grayanus* lectin; MCL, *Mytilus californianus* lectin; MGL, *Mytilus galloprovinciallis* lectin; MTL, *Mytilus trossulus* lectin; RBC, Rabbit red blood cells.

* Corresponding author.
E-mail address: hersan@unam.mx (A. Hernández-Santoyo).

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grayanus (CGL), which binds specifically to α-D-galactose and *N*-acetyl-α-D-galactosamine and which their amino acid sequence does not match with the family of lectins that have been reported to date [6]. The second member of this novel Gal-specific lectin family was isolated from the bivalve *Mytilus galloprovincialis* (MGL) of which 3 isoforms have been reported when analyzing the gene [7,8]. The third member was isolated from the mussel *Mytilus trossulus* (MTL) [9]. All three lectins consist of 150 residues with three tandem-repeat domains of 50 amino acids each, having 83–84% homology with each other and possessing antibacterial and antifungal activity. In this work, we report on the isolation of the fourth member of this family, being isolated from the mussel *Mytilus californianus* (MCL), a native from Baja California, México.

2. Methods

2.1. Isolation and the purification of the MCL lectin

The mussels (*Mytilus californianus*) were obtained from Ensenada Baja California, México. The tissues were homogenized with 0.05 M TBS buffer (50 mMTris-HCl, 150 mMNaCl, pH 7.4) containing 2 mM PMSF. The homogenate was centrifuged at 10,000 × *g* for 1 h at 4 °C and the supernatant was dialyzed against the TBS buffer. The supernatant was loaded onto an α-lactose-agarose column (1 × 5 cm) that had been previously equilibrated with the TBS buffer. The specifically bound fraction was eluted with the TBS buffer containing 0.2 M D-galactose. The homogeneity of the purified fraction was determined by polyacrylamide gel electrophoresis under denaturing conditions and mass spectrometry. The latter was performed by mixing the lectin with sinapinic acid in 30% acetonitrile, together with 70% water and 0.1% trifluoroacetic acid. This was analyzed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry when using a Microflex Bruker spectrometer. The protein concentration was determined according to the BCA method [10], using bovine serum albumin as a protein standard. The purified protein was dialyzed against a 0.05 M TBS buffer, pH 7.4.

2.2. Assays of hemagglutination activity

The hemagglutinating activity of the MCL was tested by using rabbit red blood cells (RBC) that were prepared when using the Fragkiadakis protocol [11] in 96-Well Microtiter U Plates (NUNC™). This was achieved according to a 2-fold serial dilution procedure with 2% (w/v) of human type A, B or O and rabbit formalinized erythrocytes that were suspended in phosphate-buffered saline (PBS; 0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.4). Then 25 μL of a 2-fold dilution of purified lectin in the TBS buffer was mixed with 25 μL of 2% rabbit erythrocytes. The mixture was incubated at 37 °C for 30 min. The hemagglutinating titer was reported as being the inverse of the last dilution with an agglutinating activity.

2.3. Inhibition of the hemagglutination activity

The sugar specificity of the lectin was tested by inhibiting the hemagglutinating activity with fifty simple sugars and oligosacharides. The carbohydrates were 2-fold serial diluted in Microtiter U Plates with the PBS buffer. The MCL was incubated at room temperature for 1 h with different concentrations of the inhibitors before the addition of 2% rabbit formalinized erythrocytes in suspension and the plates were incubated at 37 °C. The results were expressed as the minimal concentration of carbohydrates that inhibited the hemagglutination.

2.4. pH and temperature effects

The pH dependence of the lectin was determined by dialyzing the samples with the universal Britton-Robinson buffer 0.04 M [12] when using different pH values (3–10) and this was incubated for 1 h at 25 °C. Subsequently, this was dialyzed against the TBS buffer and the agglutinating activity of the samples was determined by using the rabbit formalinized erythrocytes. Later, the MCL was dialyzed against an optimum pH and then they were incubated for 1 h at different temperatures (4 °C - 60 °C). After this, the agglutinating activity was measured at 25 °C.

2.5. Monitoring of the MCL aggregation by gel filtration chromatography

The purified lectin was applied to a size exclusion Bio SEC-5 (4.6 mm × 150 mm x 5 μm, *Agilent Technologies, Germany*) column connected to an HPLC system (HP Agilent 1100 series, Agilent Technologies, Germany). The column was equilibrated with the TBS buffer and the flow rate was set at 1.0 mL/min. The standards for the mass calibration were tetrameric glucose isomerase (M_r 173 kDa), bovine serum albumin (M_r 66 kDa), Thaumatin (M_r 22 kDa), Lysozyme (M_r 14 kDa), Ubiquitin (M_r 8 kDa) and Hevein (M_r 4.8 kDa).

2.6. Differential scanning fluorimetry

Using fluorescence-based thermal shift assays [13], we studied the effects of pH and galactose on the stability of the MCL. The assays were performed with 20 μL of the MCL containing SYPRO Orange dye (Invitrogen, Life Technologies, USA) in a Britton-Robinson buffer with different pH values (3–10) for the pH studies and with 250 mM of Galacose. The fluorescence was recorded with an qPCR instrument (Life Technologies, USA) while the temperature was increased in continuous steps from 25 °C to 95 °C.

2.7. Circular dichroism

The secondary structure of the MCL was measured by using a Jasco J-700 spectropolarimeter equipped with a Peltier temperature control system. The MCL was dialyzed against a 10 mM phosphate buffer at pH 7.4 and then filtered. The far-UV spectra were collected from 190 nm to 260 nm in a 1 cm path-length cell. Three scans were averaged in order to obtain the final spectra of the protein at a concentration of 0.13 mg/mL. The mean residue ellipticity was measured as a function of the wavelength. The secondary structure contents of the spectra were determined by using the Provencher and Glockner method that is included in the Dichroweb online server [14].

2.8. RNA extraction

The total RNA was extracted from the powdered liquid nitrogen frozen tissues of the *M. californianus* by using TRIzol (SIGMA) according with the manufacturer's protocol.

2.9. Isolation of the cDNA and the encoding of the MCL lectin

The cDNA was synthesized from 2.0 μg of total RNA by using a Protoscript II RT-PCR kit (New England BioLabs, Ipswich, Massachusetts, USA), in accordance with the manufacturer's recommendations. One-twentieth of the RT reaction was used in the PCR assays. The degenerated primers were designed from the sequenced regions of the protein that were obtained from the sequenced analyzes. From the N-terminus, the sequence MYFQFDV,