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Structural characterization of coagulant *Moringa oleifera* Lectin and its effect on hemostatic parameters

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ABSTRACT

Lectins are carbohydrate recognition proteins. cMoL, a coagulant *Moringa oleifera* Lectin, was isolated from seeds of the plant. Structural studies revealed a heat-stable and pH resistant protein with 101 amino acids, 11.67 theoretical pI and 81% similarity with a *M. oleifera* flocculent protein. Secondary structure content was estimated as 46% α-helix, 12% β-sheets, 17% β-turns and 25% unordered structures belonging to the α/β tertiary structure class. cMoL significantly prolonged the time required for blood coagulation, activated partial thromboplastin (aPTT) and prothrombin times (PT), but was not so effective in prolonging aPTT in asialofetuin presence. cMoL acted as an anticoagulant protein on *in vitro* blood coagulation parameters and at least on aPTT, the lectin interacted through the carbohydrate recognition domain.

1. Introduction

Lectins are a group of proteins which recognize and bind mono- and oligosaccharides [1,2]. They can bind to carbohydrate moieties on the surface of erythrocytes and agglutinate the cells, without altering the carbohydrate structure [3]. Lectins exist in viruses and all forms of life, however the best known are extracted from plants, especially seeds, organ of stock, which is a major source to obtain these molecules [4]. Hundreds of lectins have been purified and their sugar specificities identified, which has enabled their development into powerful tools for the purification, separation, and structural analysis of glicoproteins [5], as well as recognition molecules inside cells, on cell surfaces, and in physiological fluids [6]. They have shown inhibitory activity against fungi and bacteria [7,8], insects [9], viruses [10] and cytotoxic effects against tumor cells lines [11].

Physical-chemistry characterization of lectins is important to explain the function in different biological processes [12]. Structural biology of macromolecules searches products potentially useful for solving biochemical problems and eventually develops new therapeutical agents [13]. To study structures of macromolecule, spectroscopic techniques such as circular dichroism (CD) is used, where optically active substances will absorb differently left and right circularly polarized light, and the difference in absorption of components is measured. The method has been extensively used to unravel secondary structure of proteins giving information on the effect of added ligands [14]. Proteins with high α-helical content shows CD spectrum in the far UV region as two negative CD bands around 208–210 nm and 222–228 nm as well as one positive band near 190–195 nm [15].

Moringa oleifera, known as horseradish or drumstick tree, is widely found throughout India, Asia, some parts of Africa and America, belonging to the Moringaceae family [16,17]. Its constituents such as leaf, flower, fruit and bark have been anecdotally used

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as herbal medicines in treatments for inflammation, paralysis and hypertension [18]. The seeds contain edible oils and water soluble substances (coagulant proteins) that can be used in drinking water clarification [19–22] or for treatment of selected dyeing effluents [23]. Lectins with coagulant properties were purified from *M. oleifera* seeds. Santos et al. [24] purified and partially characterized coagulant *M. oleifera* lectin, cMoL, the first lectin with coagulant properties for contaminants in water. cMoL is a basic protein, active at pH range 4.0–9.0 and complex sugar specificity which recognized mainly the glycoproteins azocasein and asialofetuin. WSMoL, Water-Soluble *M. oleifera* Lectin, was detected by Santos et al. [25] as an acid glycoprotein, with higher hemagglutination activity at pH 4.5, recognizing mainly fructose and porcine thyroglobulin. Ferreira et al. [26] referred that this lectin has coagulant activity and is a natural coagulant for contaminants in water, reducing turbidity and bacterial proliferation. WSMoL and cMoL showed insecticidal activity against *Aedes aegypti* [27] and *Anagasta kuehniella* [28], respectively. Katre et al. [29] reported the presence of a *M. oleifera* lectin, MoL, a homodimer with molecular mass of 14 kDa and subunits (7.1 kDa) linked by disulfide bond(s). MoL is also a glycoprotein with high stability and agglutinates human as well as rabbit erythrocytes.

cMoL is a protein with important biological activities [24,28], however its structural characterization was not completely elucidated. In this article, we report the primary structure, CD characterization and for the first time the anticoagulant properties of a coagulant lectin from *M. oleifera* on *in vitro* hemostatic parameters of human blood coagulation.

2. Materials and methods

2.1. Isolation of coagulant M. oleifera lectin (cMoL)

M. oleifera seeds were collected on the *campus* of Universidade Federal de Pernambuco, in Recife city, Northeast of Brazil. The extract, fraction and lectin were prepared according to Santos et al. [24], with modifications. Seeds were ground to flour, which was extracted with 0.15 M NaCl at room temperature (25 °C) for 6 h, and a saline extract was obtained. Proteins were precipitated with ammonium sulphate (60%) at room temperature for 4 h; the fraction obtained after centrifugation (12,000 × *g* for 20 min at 4 °C) was dialyzed against water and 0.15 M NaCl. The fraction was applied (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.1 M NaCl. cMoL was eluted with a saline gradient of 0.15, 0.3, 0.5 and 1 M NaCl. UV absorbance was used to monitor samples. cMoL active fractions eluted with 0.3 M NaCl were pooled, analyzed by HPLC and used in the experiments. cMoL protein concentration was evaluated according to Lowry et al. [30] using bovine serum albumin as standard at a range of 0–500 μg/mL and absorbance at 720 nm.

2.2. Reversed-phase HPLC

cMoL was subjected to reverse-phase column C4 on HPLC system (Shimadzu) for purity analysis. The column was equilibrated with solvent A [0.1% (v/v) trifluoroacetic acid (TFA) in H_2O] and eluted using solvent B (90% acetonitrile in 0.1% TFA) in a linear gradient, where B = 5% when t = 0 min, B = 5% at t = 5 min, B = 100% at t = 60 min, B = 0% when t = 65 min. The elution profile was monitored at 215 and 280 nm.

2.3. Hemagglutination activity (HA)

Glutaraldehyde-treated rabbit erythrocytes were obtained as described by Bing, Weyand, and Stavinsky [31]. The lectin (50 μL) was serially two-fold diluted in microtiter V-plates containing 0.15 M NaCl before addition of 50 μL 2.5% (v/v) suspension of treated rabbit erythrocytes. The results were read after about 45 min when the control, containing only erythrocytes fully precipitated, appeared as a dot at the bottom of the well. HA (inverse of the titer) was defined as the highest sample dilution showing full hemagglutination [4,32].

2.4. Primary sequence determination

Edman degradation [33] was performed with an automatic gas-phase sequencer (492cLC; *Applied Biosystems*) using conditions recommended by the manufacturer. Samples (0.8 mg/mL) for sequencing were reduced in 200 μL of 0.25 M Tris–HCl buffer, pH 8.5 containing 6 M guanidine–HCl, 1 mM EDTA and 5 mg of DTT, and alkylated with iodacetamide [34]. Then, the protein was separated by reversed phase cromatography HPLC. The similarity of sequences was searched using the BLAST protein sequence database [35] and the sequences were aligned with the MULTALIN program [36]. Theoretical pI was calculated by ExPASy ProtParam tool through the primary sequence of the protein.

2.5. Spectroscopic measurements

CD data were recorded on a Jasco J-810 spectropolarimeter. Samples were placed in a 0.5 mm path length circular quartz cuvette. Lectin concentration was 0.2 mg/mL in phosphate–borate–acetate (PBA) buffer for measurements in the far UV region (250–190 nm), as an average of 8 scans. Data were expressed in terms of mean residue ellipticity [θ]. The secondary structure estimative was calculated by deconvoluting the CD spectrum using the CDPro software package, which contains three CD analysis programs, CONTINLL, SELCON3 and CDSSTR [37]. The three programs were used with a reference protein set consisting of 56 proteins, thus increasing the reliability of deconvolution. Results were expressed as a mean of the three programs. CDPro package also contains the Cluster program that was used to determine the tertiary structure class of cMoL [38]. To study the pH effect on cMoL, the protein (0.2 mg/mL) was incubated in phosphate–borate–acetate buffer (PBA), 10 mM, for 10 min, at pH values of 2.0, 4.0, 6.0, 7.0, 8.0, 10 and 12. The temperature effect on cMoL secondary structure was also analyzed. Protein samples were heated at 40, 60, 80 and 100 °C for 30 min and at 100 °C for 1 h. CD measurements were recorded as described above.

2.6. Determination of activated partial thromboplastin time – aPTT and prothrombin time – PT

aPTT and PT were determined in a semi-automated coagulometer BFT II (Dade Behring). Total plasma was obtained by centrifugation of human blood samples at 1726 × *g*, for 15 min (25 °C). PT assay control was made with 50 μL of saline and 50 μL of plasma, incubation for 60 s, with subsequent addition of 100 μL of reagent (Thromborel S-Dade Behring). cMoL in different concentrations (3.0, 15, 30, 37.5, 45 and 60 μg/mL) in 50 μL was incubated (60 s) with 50 μL of plasma, followed by subsequent addition of 100 μL of reagent. aPTT assay control was made with 50 μL of saline, 50 μL of plasma and 50 μL of aPTT reagent (Dade actin activated cephaloplastin reagent-Dade Behring), incubation for 120 s and subsequent addition (50 μL) of 0.025 M calcium chloride. cMoL (3.0, 15, 30, 37.5, 45 and 60 μg/mL) was incubated for 120 s with 50 μL of plasma and 50 μL of the aPTT reagent, followed by addition of 50 μL of 0.025 M calcium chloride. The blood coagulation assays were also performed with cMoL (50 μL) inhibited by glycoprotein. cMoL was previously incubated with asialofetuin (0.5 mg/mL) for 15 min e then the assay proceeded as described above. Each assay was made in duplicate and results were expressed as average of