Short communication

Isolation of a seed coagulant *Moringa oleifera* lectin

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A B S T R A C T

In this work hemagglutinating activity (HA) was investigated in distinct *Moringa oleifera* tissue extracts. A new lectin from seeds (cMoL) was purified and characterized; hemagglutinating and coagulating activities were evaluated. HA was detected in 0.15 M NaCl extracts from flowers and rachis inflorescence (5%, w/v), seeds, leaves, fundamental tissue of stem and steam bark (10%, w/v). cMoL isolated after saline extraction and guar gel column chromatography was active at pH range 4.0–9.0 agglutinating erythrocytes from rabbit and human blood types. Extracts of tissues and cMoL activities were carbohydrate inhibited; azocasein and asialofetuin abolished cMoL HA. The lectin was thermostable at 100 °C during 7 h. Polyacrylamide gel electrophoresis under reduced conditions revealed a main polypeptide band of 26.5 kDa; native basic cMoL was detected as a unique band. Seed lectin preparations and cMoL showed coagulant activity, similar to aluminium sulphate, the coagulant most widely used in water treatment.

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1. Introduction

Lectins are hemagglutinating proteins that exist in virus and all forms of life but most known are extracted from plants, especially from seeds [1], a storage organ, which is one of the main sources to obtain these molecules. In general, other plant tissues contain lower amount of lectins; also, not necessarily identical in structure or carbohydrate specificity to seed lectins [2]. These proteins interact selectively and reversibly with residues of specific sugars [3] and, structural characteristics result in the use of lectins for therapeutic applications [4] and biotechnological purposes [5]. Cytotoxic effects revealed by mitogenic, antitumoral, antimicrobial, and insecticide activities have been found in lectins [2].

Different protein purification protocols have been used to isolate lectins from the same tissue. In general, the first step involves preparation of extracts in saline or buffer solution [6]. From the crude extract, the protein can be fractionated through methods such as selective precipitation with salts at different concentrations. The isolation techniques used include ion exchange [7], gel filtration [8] and affinity [9] chromatography. However, due to the lectin ability for recognizing carbohydrates, affinity chromatography in columns containing polysaccharides [10] or glycoproteins [11] has been the most commonly used technique [4].

Polymers have been applied in coagulation/flocculation processes for water purification for decades [12]. Natural coagulants of vegetable origin were used in water treatment before the advent of synthetic chemicals like aluminium and ferric salts [13]. Cactus (*Cactaceous opuntia*) is a natural macromolecular coagulant and the turbidity removal efficiency could reach 94%, the optimum pH was about 10 and temperature had slight influence on the coagulation effect [14]. *Cactus latiforia* and seeds of *Prosopis juliflora* act as natural coagulants and produced comparable turbidity removals in water with high (100–200 NTU) and low (30–40 NTU) initial turbidity [15].

Moringa oleifera (Lam), Moringaceae family, has been used in the Philippine diet; leaves, flowers and green pods are edible as a human nutritious vegetable [16]. In Malaysia, the young tender pods are cut into small pieces and added to curries [17]. Several properties have been identified in different parts of *M. oleifera*: the leaf extract is a potential source of antioxidants [18]; hypocholesterolemic effects have also been found in leaf extract [19] and fruits [20]; plant seeds contain hypotensive activity [21], strong antioxidant activity and chelating property against arsenic toxicity [22]. Moringa seed coagulant properties have been used to water treatment [23,24]. Shelled *M. oleifera* seeds have the property to decontaminate arsenic from water and can be used as domestic and environment-friendly safe technology [25].

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In this report hemagglutinating proteins were identified in *M. oleifera* tissue extracts and a seed coagulant lectin isolated by affinity chromatography was partially characterized.

2. Materials and methods

2.1. Extract preparations

Flowers, inflorescence rachis, seeds, leaf tissue, fundamental tissue of stem and steam bark of *M. oleifera* Lam were collected in Recife city, Northeast of Brazil. A sample of the collected material is archived as voucher specimen number 63184, IPA, at the herbarium "Dárdano de Andrade Lima" (Empresa Pernambucana de Pesquisa Agropecuária, Recife, Brazil). Tissue flours of *M. oleifera* were extracted with 0.15 M NaCl for 6 h at room temperature and resulted in saline extracts from flowers and inflorescence rachis (E1 and E2, 5%, w/v), as well as seeds, leaves, fundamental tissue of stem and steam bark (E3, E4, E5 and E6, 10%, w/v).

2.2. Saline fractionation

Proteins of E3 were precipitated using 0–60% ammonium sulphate fractionation for 4 h at room temperature. The 0-60F was dialyzed with distilled water (two changes) and 0.15 M NaCl overnight.

2.3. Hemagglutinating activity

HA was performed in microtiter plates according to Correia and Coelho [26]. Lectin preparations (50 μL) were serially two-fold diluted in 0.15 M NaCl before addition of 50 μL 2.5% (v/v) suspension of rabbit glutaraldehyde treated erythrocytes or fresh human erythrocytes. Titer was expressed as the highest dilution exhibiting hemagglutination. Specific HA (SHA) was defined as the ratio between the titer and protein concentration (mg/mL).

2.4. Lectin isolation

The 0-60F was chromatographed (10 mg of protein) on a guar gel column (10.0 cm × 1.0 cm) previously equilibrated with 0.15 M NaCl (20 mL/h flow rate). The bound HA was eluted with 1.0 M NaCl (cMoL).

2.5. Effect of different pH, temperatures and cations on HA

The pH effect was evaluated with cMoL (50 μL) diluted in 10 mM citrate–phosphate buffer (pH 3.0–6.5), 10 mM sodium phosphate buffer (pH 7.0–8.0) or 10 mM Tris–HCl buffer (pH 8.5–9.0). Lectin thermal stability was evaluated with previously heated cMoL (30–90 °C, up to 30 min and subsequently 100 °C, every 30 min, up to 7 h). Assays for divalent cation requirements were performed by dialysis with 5 mM EDTA (16 h, 4 °C) and 0.15 M NaCl (6 h, 4 °C) and HA was measured with $MgCl_2$, $CaCl_2$ and KCl (5 mM). For all experiments, HA was measured as described in Section 2.3 using 50 μL of a 2.5% (v/v) suspension of rabbit glutaraldehyde treated erythrocytes.

2.6. Hemagglutinating activity inhibition

The lectin inhibitory assays using extracts and cMoL were evaluated with solutions of carbohydrates D(−)-fructose, D(+)-raffinose, D(+)-glucose and D(+)-mannose. cMoL was also assayed with solutions of D(+)-lactose, (+)-arabinose, trehalose, L(+)-rhamnose and galactose. E and cMoL were assayed with 0.5 mg/mL glycoprotein solutions (fetuin, asialofetuin, ovalbumin, casein and azocasein). The inhibitory assays were similar to the hemagglutinating assay with exception of an incubation step (room temperature, 15 min) before erythrocyte addition.

2.7. Protein evaluation

The protein was estimated according to the Lowry et al. method [27] and by absorbance at 280 nm.

2.8. Lectin characterization

cMoL molecular weight was estimated by sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE) as described by Laemmli [28]. Gels were stained either for protein with Coomassie Brilliant Blue [28] or for carbohydrate using Schiff reagent (Sigma) according to Pharmacia Fine Chemicals (1980). The native molecular weight was determined by Sephacryl S-300 gel filtration column (16 mm × 60 cm) with 0.5 M NaCl (20 mL/h flow rate) using molecular weight markers (bovine serum albumin: 66 kDa, fetuin: 64 kDa, ovalbumin: 44 kDa and ovoinhibitor: 28 kDa) using Äkta FPLC system (Amersham Pharmacia Biotech, Sweden).

2.9. Coagulation activity assay

The coagulation activity assay was based on the method described by Ghebremichael et al. [24]. Initially, the water sample was treated with kaolin clay (10 g) to 1 L tap water, stirred for 30 min and allowed to settle for 24 h to achieve complete hydration. Desired turbidity was obtained by dilution. An aliquot (100 μL) of samples (1 mg/mL, E3, seed 0-60F and cMoL) and positive control (aluminium sulphate 5%) were added to high turbidity clay suspension (900 μL, 250–300 NTU, Nephelometric Turbidity Units) in 1 mL cuvette and homogenized instantly. Dilution (1:2) of clay suspension was done to obtain low turbidity (125–150 NTU) and samples were assayed. Samples were allowed to settle for 1 h and absorbance was measured at 500 nm using a UV–Visible spectrophotometer FEMTO 700 S. In order to reduce background effect, a sample volume of 800 μL from the top was transferred to the cuvette for absorbance measurements every 5 min up to 60 min and subsequently to each 10 min up to 140 min. Reduction in absorbance relative to negative control defines coagulation activity. The assays were performed three times and under different temperatures (15, 30, 45 and 60 °C) or 5 mM solutions of ions (Ca^{+2}, Mg^{+2} and Mn^{+2}).

2.10. Statistics analysis

Values of experimental results shown in tables and figures are the mean of at least three determinations (± standard deviation). Linear regression equations were established using the Origin version 6.0 program (Microcal, Northampton, MA, USA).

3. Results

HA was detected in all extracts obtained from *M. oleifera* tissues. The highest specific activities, however, were detected in extracts from steam bark, flowers and inflorescence rachis (Table 1). The differences in carbohydrate inhibition of lectin preparations from distinct tissues of the plant may indicate the presence of different lectins.

When 0-60F (Specific HA, SHA: 371) of *M. oleifera* seeds was chromatographed on guar gel affinity column (Fig. 1(A)) HA was detected in unabsorbed (SHA: 35) and 1.0 M NaCl eluted fractions (SHA: 864). The 1.0 M NaCl eluted fractions (cMoL) retained 59% of chromatographed lectin activity and the obtained purification factor was 2.3 (Table 2).

cMoL showed HA to rabbit (titer: 256^{-1}) and A, B, O and AB human fresh erythrocytes (titer: 64^{-1}, 128^{-1}, 16^{-1} and 256^{-1}, respectively) like the seed lectin from *Crotalaria pallida* [8]. cMoL is a highly stable protein, active at pH range 4.0–9.0 and resistant up to 7 h at 100 °C. The presence of ions Mg^{2+}, Ca^{2+} and K^+ increased cMoL HA.

Inhibition assays revealed that HA of extracts was partially inhibited with carbohydrates and glycoproteins (Table 3); the latter macromolecules were more effective inhibitors to cMoL than carbohydrates. cMoL HA was strongly inhibited by ovalbumin; azocasein and asialofetuin completely abolished HA activity (Table 3) indicating complex sugar specificity.

Under denatured and reduced conditions a main polypeptide band of 26.5 kDa was revealed (Fig. 2(A)); PAGE confirmed cMoL as a basic protein (Fig. 2(B)) and no staining was detected with Schiff's reagent. Native cMoL Sephacryl S-300 gel filtration chromatography revealed a single peak of 30 kDa (Fig. 1(B)).

Flour of *M. oleifera* seeds has been broadly used in developing countries as a natural coagulant to treat water for human consumption. Seed extract, 0-60F and cMoL showed coagulant

Table 1
Protein concentrations and specific hemagglutinating activities of tissue extracts from *Moringa oleifera*.

Tissue extract[a]	Protein concentration (mg/L)	SHA[b] (HA/protein concentration)
Flowers	0.9	1185
Inflorescence rachis	0.4	680
Seeds	4.9	208
Leaf tissue	1.0	8
Fundamental tissue of steam	0.4	84
Steam bark	1.7	2444

[a] All tissues were colleted from a single plant.
[b] SHA, specific hemagglutinating activity. Samples were assayed with glutaraldehyde treated rabbit erythrocytes.
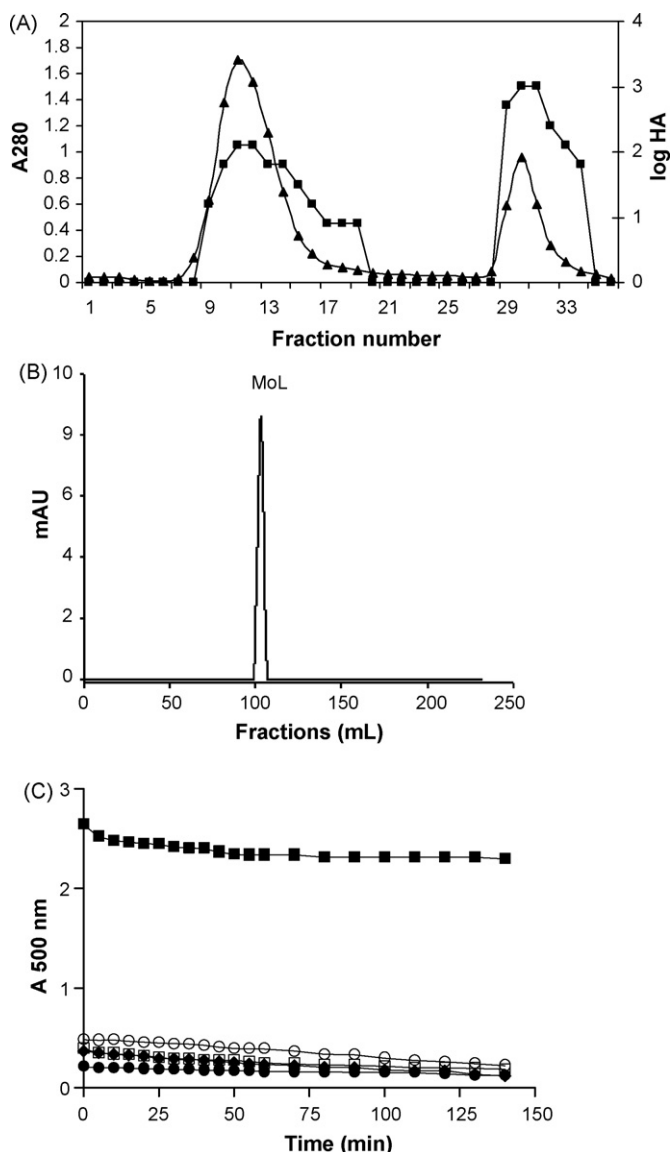
Fig. 1. (A) cMoL (10 mg of 0-60F protein) purification on a guar gel affinity column (10.0 cm × 1.0 cm). More than 2 mg of cMoL were obtained. At the point indicated (arrow) elution buffer was changed to 1.0 M NaCl. Absorbance at 280 nm (■) and log of HA (▲) are represented. (B) cMoL native molecular weight determination by gel filtration chromatography on a Sephacryl S-300 column. A peak of 30 kDa is represented. Molecular weight markers used were: bovine serum albumin, 66 kDa; fetuin, 64 kDa; ovalbumin, 44 kDa; ovoinhibitor, 28 kDa. (C) Coagulation activities of cMoL and lectin preparations with kaolin clay (10 g/L). cMoL (1 mg/mL, □), extract (1 mg/mL, ●), 0-60F (1 mg/mL, ◆), aluminium sulphate (5% p/v, ○) and negative control (■). The values represent the mean of three assays (± standard deviation): significant differences between groups were determined at $\rho < 0.05$.

Table 2
Summary of cMoL purification.

Preparation	Total protein (mg)	HA	Total HA	SHA	Yield[a] (%)	Purification (folds)
0-60F	10.0	2048	3710	371	100	1.0
cMoL	2.5	256	2201	864	59	2.3

[a] Percentage of total activity recovered. Hemagglutinating activity (HA) assays and SHA, specific HA were performed with rabbit erythrocytes. More than 2 mg of cMoL were obtained from 10 g of seed powder when a 10% (w/v) extract was submitted to 60% ammonium sulphate fractionation followed by guar gel affinity chromatography.

Table 3
Inhibition hemagglutinating activity of tissue extracts and cMoL by carbohydrates and glycoproteins.

Inhibitor	E1	E2	E3	E4	E5	E6	cMoL
D(−)-Fructose	148.1	N	N	N	N	305.5	N
D(+)-Raffinose	592.5	NS	76.8	N	10.5	N	320
D(+)-Glucose	148.1	167.5	153.6	N	N	N	80
D(+)-Mannose	N	335	153.6	N	N	N	N
D(+)-Lactose	ND	ND	ND	ND	ND	ND	160
D(+)-Arabinose	ND	ND	ND	ND	ND	ND	320
Trehalose	ND	ND	ND	ND	ND	ND	160
L(+)-Rhamnose	ND	ND	ND	ND	ND	ND	160
Galactose	ND	ND	ND	ND	ND	ND	80
Asialofetuin	ND	ND	ND	ND	ND	ND	0
Fetuin	0	41.9	38.4	4.1	0	152.7	N
Ovalbumin	N	335	76.8	4.1	0	611	10
Casein	296.2	0	38.4	2.0	0	19	80
Azocasein	0	0	9.6	2.0	0	2.4	0

Flowers (E1), inflorescence rachis (E2), seeds (E3), leaf tissue (E4), fundamental tissue of steam (E5), steam bark (E6). Specific hemagglutinating activity (SHA) of E were 1185 (E1), 2680 (E2), 307 (E3), 8.16 (E4), 84.21 (E5) and 2444 (E6). SHA of cMoL was 640. Assays were performed with rabbit erythrocytes. Carbohydrates (0.2 M) and glycoproteins (0.5 mg/mL) were used. N indicates that no inhibition of HA was detected and ND indicates that the assays were not determinate.

[29]. Some lectins were found to be toxic or anti-nutritious for man and animals. In general, nausea, bloating, vomiting and diarrhoea characterize the oral acute toxicity of lectins on humans [30]. HA was detected in all extracts obtained from *M. oleifera* tissues; these findings make this plant an important model for study of different molecular forms of lectins and their correspondent functions. The same has been observed for *Bauhinia monandra* extracts [31] and lectins were purified from different tissues of *Dolichos lablab* [32]. The presence of lectins in different parts of *M. oleifera* that are consumed by the population requires a nutritional assessment of these tissues.

The presence of lectin is mainly detected through the hemagglutinating activity assay which is performed with a serial dilution of the lectin before an incubation step with human or other animal erythrocytes [6]. The lectin interaction with carbohydrates are performed through a combination of hydrogen bonds between sugar hydroxyl groups and protein main-chain, as well as side-chain groups, water-mediated contacts, van der Waals packing of the hydrophobic sugar ring face against an aromatic residue, and hydrophobic interactions [33].

Guar gel affinity chromatography efficiently purified seed cMoL, with good recoveries (59%). This new and simple protocol purified a lectin different from others already published, like the water soluble *M. oleifera* lectin, WSMoL, previously described in seeds [34]. cMoL is a basic protein with 30 kDa, active at pH range 4.0–9.0, thermostable at 100 °C during 7 h and HA was completely inhibited by azocasein and asialofetuin. WSMoL is an acidic glycoprotein and was mainly active with rabbit cells at pH 4.5; HA was abolished by heat treatment, pH 7.0, fructose and porcine thyroglobulin [34]. Katre et al. [35] isolated a hemagglutinin from
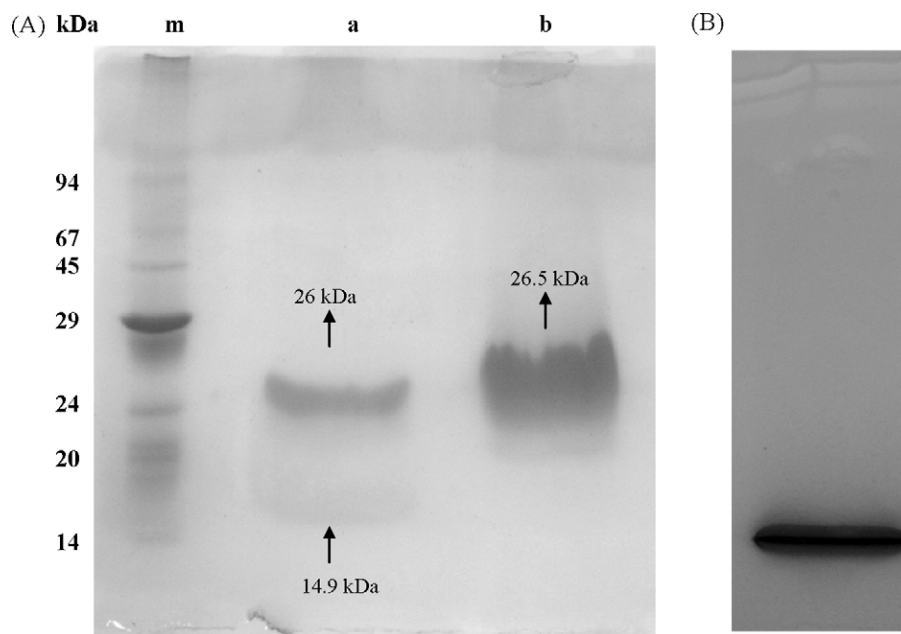
activities (reduction in absorbance at 500 nm of approximately 92% in relation to negative control) similar to the positive control aluminium sulphate on water with high (250–300 NTU) and low (125–150 NTU) turbidity of kaolin clay suspension (Fig. 1(C)). Significant differences in coagulant activity were not observed at the temperatures the analysis was done. No coagulant activity was detected in seed extract with all tested ions, i.e. coagulation was also absent to 0-60F or cMoL with Ca^{+2} and Mg^{+2}. However, Mn^{+2} with 0-60F and cMoL decreased water turbidity similarly to sulphate.

4. Discussion

Tissues from *M. oleifera* such as leaves and flowers are eaten as nutritious vegetables and fried seeds have also been consumed

Fig. 2. SDS-PAGE (A) of lectin (100 μg) treated (a) and untreated (b) with β-mercapthoethanol and PAGE showing purified native and basic cMoL (B). Molecular weight markers (m): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactoalbumin (14 kDa); the gels were stained with Coomassie Brilliant Blue (A) and starch black (B).

seeds of *M. oleifera*, a homodimer with molecular mass of 14 kDa and subunits (7.1 kDa) linked by disulfide bond(s).

cMoL is also distinct from other coagulant proteins previously reported in *M. oleifera* seeds. Nadabgengesere et al. [13] described dimeric cationic proteins with molecular mass of 12–14 kDa and isoelectric point (p*I*) between 10 and 11; Ghebremichael et al. [24] studied a cationic protein with p*I* greater than 9.6 and molecular mass less than 6.5 kDa that showed coagulation efficiencies of heat-treated samples slightly higher than raw samples. Heat treatment promotes the molecular vibration and this can be responsible for better coagulation efficiency. Gassenschimdt et al. [36] purified a flocculating cationic protein with p*I* greater than 9.6 and molecular mass less than 6.5 kDa. Furthermore, Okuda et al. [37] founded a coagulant compound in saline extract of *M. oleifera* seeds that was neither protein, nor polysaccharide or lipid.

Coagulant activity of high-molecular cationic polyacrylamide derivatives has been explained by the bridge formation model; coagulation of negatively charged particles is a result of binding by Coulomb forces of positively charged particles and neutralization of part of the surface charge. Reduced electrostatic repulsion leads to the agglomeration and formation of flocs by bridges between negatively charged particles [36]. This mechanism may explain cMoL coagulant properties when positively charged proteins bind to parts of surface from negatively charged particles, flocs are formed and coagulation is observed.

M. oleifera may provide an interesting model for structural comparison of lectins since all extracts from distinct *M. oleifera* tissues revealed HA and others different lectins are present in the seeds.

In this paper a new thermoresistant coagulant lectin (cMoL) was purified from seed flour using a simple technique. *M. oleifera* coagulant proteins, cMoL and WSMoL, could contribute to unravel the function of products from a unique gene or genes in the plant.

Extracts, 0-60F and cMoL showed water coagulant properties in the same proportions. Similar results were found with aluminium sulphate, the most common synthetic coagulant used in water treatment all over the world. In conclusion, *M. oleifera* seed preparations and cMoL can be applied to water treatment for

human consumption. The production of filters containing cMoL is already being developed in our laboratories.

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