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ORIGINAL PAPER

Interaction of *Moringa oleifera* seed lectin with humic acid

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The aim of this work was to characterise the affinity of protein preparations from *Moringa oleifera* seeds, specifically extract (seeds homogenised with 0.15 M NaCl), fraction (extract precipitated with 390 mg mL^{-1} of ammonium sulphate) and *cMoL* (coagulant *M. oleifera* lectin) to bind humic acids using a haemagglutinating activity assay with rabbit erythrocytes and a radial diffusion assay in agarose gel. Specific haemagglutinating activity (SHA) decreased by 94 % for the extract and *cMoL* and by 50 % for the fraction in the presence of humic acid. Precipitation bands were observed in the diffusion gel. Both results suggested humic acid–*cMoL* binding. Carbohydrates, potassium, and calcium ions and pH affected the SHA of *cMoL*. As an example of application, *cMoL* was immobilised on a column packed with sepharose receiving 20 mg mL^{-1} of carbon humic acid solution, 30 mg of humic acid per gram of support was removed. This result suggested that protein preparations might be used in water treatment to remove humic acids.

Keywords: humic acid, lectin, *Moringa oleifera*, protein preparations, water treatment

Introduction

Lectin is a type of receptor protein of non-immune origin that interacts with carbohydrates without any modifying effect. These proteins recognise and bind carbohydrates with specific characteristics and have the ability to promote cell agglutination. The presence of these proteins is detected by a haemagglutination assay performed by the serial dilution of lectin and incubation with human or animal red blood cells (Correia et al., 2008).

A broad application for plant lectins is based on their ability to discriminate between different sugars (Pedroso et al., 2008). Most lectins recognise *N*-acetylneuraminic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, mannose, and fucose (Peumans & Van Damme, 1995). The key interactions between lectins and carbohydrates occur via hydrogen bonds between the sugar hydroxyl groups and the groups in the main-chain and side-chain of a protein, hydrophobic bonds, metal co-ordination, water-mediated contacts, van der Waals packing of the hydrophobic sugar ring face against an aromatic residue, and hydrophobic interactions (Elgavish & Shaanan, 1997; Del Sol et al., 2007).

Lectins have mainly been obtained from the seeds of leguminous plants but also from many other plant and animal tissues (Santos et al., 2005, 2009; Rameshwaram & Nadimpalli, 2008; Wong & Ng, 2006). They are valuable tools in biotechnological research (Fraguas et al., 2004) and biomedical applications (Kelly et al., 2005; Favacho et al., 2007). An important application of immobilised lectins reported in the literature (Aniulyte et al., 2006) is the isolation of biomolecules (Kelly et al., 2005) i.e. plasma glycoproteins (lecithin-cholesterol acytransferase) (Lima

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et al., 1997), polysaccharides (Franco-Fraguas et al., 2003), trypsin inhibitor (Paiva et al., 2003), and enzymes (peroxidases) (Fraguas et al., 2004). Studies in the biomedical field reported the isolation of lectins with mitogenic activity on human lymphocytes (Maciel et al., 2004), antifungal activity (Yan et al., 2005), and activity against the proliferation of human cancer cell lines (Kaur et al., 2005). Purified lectins are used in clinical analyses for blood-type classification; some glycolipids and glycoproteins present on individual red blood cells can be identified by lectins.

Moringa oleifera is a plant belonging to the Moringaceae family and the seeds have been used in water treatment (Okuda et al., 1999; Ghebremichael et al., 1995). The aqueous extract obtained from *M. oleifera* seeds contains a flocculating protein that acts as a clarifying agent on turbid water (Pritchard et al., 2010) and lectin with coagulant properties was purified from the seeds (Santos et al., 2009). Shelled *M. oleifera* seeds have also been used for the decontamination of water polluted with arsenic (Kumari et al., 2006). In addition, antioxidant activity and antimicrobial properties of *M. oleifera* seeds have been reported in the literature (Santos et al., 2005; Ghebremichael et al., 2005).

Organic compounds of biological origin are found in all surface waters. They are referred to as natural organic matter (NOM) and are contained in hydrophilic as well as hydrophobic fractions. The hydrophilic fraction mainly includes carboxylic acids, carbohydrates and proteins, while the hydrophobic fraction includes the so-called humic substances (HS) (Matilainen et al., 2006). HS can be found in two fractions, humic acids and fulvic acids (Moriguchi et al., 2005). Humic acids are heterogeneous mixtures of organic compounds containing several chemical groups and have been characterised by several methods. Fourier-transformed infrared spectroscopy (FTIR) suggests that humic acids contain aliphatics, carbohydrates, aromatics, amides, carboxylic and phenolic groups (Shirshova et al., 2006). Characterisation by infrared (IR) and cross-polarisation (CP) with magic-angle spinning (MAS) ^{13}C nuclear magnetic resonance (NMR) spectroscopy indicates that humic acids have aliphatic and aromatic fractions and carboxylate groups (Shin et al., 1999; Xu et al., 2006). The molecular sizes of humic acids are reported to range from several hundred to several hundred thousand daltons and the chemical structures show no repetitive pattern (Shin et al., 1999).

NOM is responsible for odour, taste and colour in water and it supports bacterial growth (Yan et al., 2006). It is associated with the formation of disinfection by-products upon chlorination of drinking water, the most commonly observed of which are trihalomethanes (THMs). They may cause adverse health effects in animals and humans (Eish & Wells, 2006). For this reason, several strategies for the removal of humic substances from water have been investigated, such as coagulation/flocculation using iron(III) chloride or aluminium sulphate, adsorption (Le Clech et al., 2006; Moriguchi et al., 2005), activated carbon filtration (Matilainen et al., 2006), and photocatalytic degradation on Ti-modified silica (Moriguchi et al., 2005).

The aim of this work is to characterise the affinity of protein preparations obtained from *M. oleifera* seeds (extract, fraction and *cMoL*) to bind commercial humic acid. As an example of the application, a suitable support to immobilise *cMoL* was chosen, and humic acid removal from water was evaluated in a column packed with the selected support.

Experimental

The extract, fraction, and lectin from *M. oleifera* seeds were prepared in accordance with Santos et al. (2009). Seeds were ground to flour that was extracted with 0.15 M NaCl at room temperature (r.t., 25 °C) for 6 h and a saline extract was obtained. The proteins present in the extract were precipitated with ammonium sulphate 390 mg mL^{-1} at r.t. for 4 h and a protein fraction was obtained. The fraction was isolated (10 mg of protein) on a guar gel column (10 cm × 1.0 cm) previously equilibrated (20 mL h^{-1} flow rate) with 0.15 M NaCl. The lectin, named *cMoL* (coagulant *M. oleifera* lectin), was eluted with 1.0 M NaCl. The extract, fraction, and *cMoL* were used in the experiments described below.

The haemagglutination assay (HA) was performed in microtiter plates in accordance with Correia & Coelho (1995). The extract, fraction, and *cMoL* (50 μL) underwent two-fold serial dilution with 0.15 M NaCl before the addition of 50 μL suspension of rabbit erythrocytes previously treated with glutaraldehyde $\varphi_r = 2.5$ %. After an incubation period of 45 minutes, the titer was expressed as the highest dilution resulting in haemagglutination. Assays were performed with the extract (2485 mg L^{-1}), fraction (219 mg L^{-1}), and *cMoL* (129 mg L^{-1}).

The haemagglutination inhibition assay followed the same protocol as the haemagglutination assay with the omission of the incubation step with the inhibiting substance at r.t. for 15 min, before addition of the erythrocytes suspension. The following substances were tested: *i*) humic acid (Sigma–Aldrich, Australia, a solution of 10 mg L^{-1} of carbon prepared in 0.1 M NaOH; *ii*) carbohydrates, aqueous solutions of D-(+)-glucose, D-(+)-galactose, L-(+)-arabinose, and D-(−)-galacturonic acid with concentrations of 0.2 M, 0.1 M, 0.05 M, 0.025 M, and 0.0125 M; *iii*) a solution of azocasein 5 mg L^{-1}; *iv*) halogenated organic compounds, aqueous solutions of dichloroacetic acid (5.6 g L^{-1}), trichloroacetic acid (56 g L^{-1}), and chloroform (56 g L^{-1}).

The effect of pH in an interval from 7.5 to 10 on

cMoL haemagglutinating activity was assessed in the presence of humic acid (10 mg L^{-1} of carbon) following the procedure previously described (Correia & Coelho, 1995). pH was adjusted with phosphate buffer.

The effect of potassium and calcium in the *cMoL* haemagglutinating activity was tested in the presence of humic acid. A humic acid solution (100 mg L^{-1} of carbon) was diluted ($\varphi_r = 1 : 10$) with 5 mM $CaCl_2$ or 10 mM KCl solutions prepared in 10 mM sodium phosphate buffer. Before addition of the erythrocytes suspension, *cMoL* was incubated with the humic acid in the presence of K^+ and Ca^{2+} at r.t. for 15 min.

A diffusion assay was carried out in agarose gel in a Petri dish. The gel of a concentration of 10 mg mL^{-1} was prepared in 0.15 M NaCl. A humic acid solution (30 μL) of a concentration of 100 mg mL^{-1} and 200 mg mL^{-1} of carbon, was placed in a central well, while peripheral wells were filled with 15 μL (1000 mg mL^{-1}) of *M. oleifera* seed extract, fraction, and *cMoL*. This assay was also carried out in the presence of azocasein (500 mg mL^{-1}) which was incubated with each protein preparation at r.t. for 15 min. Diffusion experiments were performed in a humid chamber at 4 ℃ for 48 h. Subsequently, the gels were thoroughly washed with 0.15 M NaCl and stained for 2 h with 1 mg mL^{-1} Coomassie Brilliant Blue, prepared in a mixture of ethanol $\varphi_r = 45$ vol. % and acetic acid $\varphi_r = 10$ vol. %.

cMoL was immobilised on silica, clay and cellulose. The silica was previously treated with (3-aminopropyl)-triethoxysilane (APT-silane); 6 g of this support was added to 120 mL of APT-silane solution 100 mg mL^{-1} prepared in distilled water (pH 3.0) and the mixture was agitated at 75 ℃ for 2 h. The APT-silane solution was centrifuged, the supernatant discarded and the silica washed with distilled water (5 times). Then the silica was dried at 110 ℃ for 17 h. Pre-treated silica, clay and cellulose were activated with $\varphi_r = 2.5$ vol. % glutaraldehyde solution prepared in the 5 mM sodium phosphate buffer (pH 7.0) and incubated at r.t. for 1 h; glutaraldehyde was removed and the supports washed with the same buffer. *cMoL* immobilisation (1.9 mg L^{-1}) was performed with 50 mg of the activated supports mixed with 1.5 mL of 5 mM sodium phosphate buffer (pH 7.0) at 4 ℃ for 17 h. The supports were washed with the same buffer (4 times) and with 2 M urea (once). The supernatant was removed, the volume measured and the protein determined by the Lowry method (Lowry et al., 1951).

Cyanogen bromide–activated sepharose 4B (0.25 g) and cyanogen bromide–activated agarose (0.25 g) were used for *cMoL* immobilisation in accordance with Paiva et al. (2003). The supports were washed with 0.5 M NaCl, pH 2.5, followed by 0.1 M $NaHCO_3$ in 0.5 M NaCl, pH 8.2. Incubation (4 ℃, 24 h) was performed with *cMoL* (0.6 mg L^{-1}). After filtration and washing with $NaHCO_3$ solution, ethanolamine was added up to a final concentration of 1 M.

A humic acid solution of 20 mg L^{-1} of carbon was applied at a flow rate of 12 mL h^{-1} onto a 1 mL packed bed column (0.68 cm × 4 cm) containing *cMoL* immobilised on cyanogen bromide–activated sepharose 4B (lectin–sepharose). The assay was carried out at r.t. and 5 mL samples were collected at 20 min intervals. Total organic carbon and protein were determined.

The protein was estimated in accordance with Lowry et al. (1951). A calibration curve was prepared using bovine serum albumin (BSA) as the standard in a range between 0 μg mL^{-1} and 400 μg mL^{-1}.

The elemental composition of the humic acid was determined on an elemental analyser (Carlo Erba EA 1108, Italy). The elemental composition on a mass basis was 48.36 % of C, 26.91 % of O, 4.24 % of H, 0.78 % of N, and 0.78 % of S.

Total organic carbon (TOC) was measured spectrophotometrically at 600 nm using Method 10129 adopted from Hach Lange GmbH (Germany) (0.0 mg L^{-1} to 20.0 mg L^{-1} of C). Organic carbon was oxidised with persulphate under acidic conditions and the carbon dioxide formed trapped in an indicator solution which changed colour proportionally to the amount of organic carbon originally present in the sample. The results are expressed in mg L^{-1} of C.

The values of experimental results in the tables and figures are the mean of at least three determinations (± standard deviation, SD) and the mean value obtained from the three replicates was computed.

Results and discussion

The specific haemagglutinating activity (SHA) was calculated by dividing haemagglutinating activity (HA) with protein concentration (mg mL^{-1}). Haemagglutinating activity was determined in the extract (SHA: 824.1), fraction (SHA: 9351.6), and *cMoL* (SHA: 3969.0) obtained from *M. oleifera* seeds as depicted in Fig. 1A; in the presence of humic acid specific haemagglutinating activity (SHA) decreased by 94 % for both the extract (SHA: 51.5) and *cMoL* (SHA: 248.0) and by 50 % for the fraction (SHA: 4675.8).

Carbohydrates present in surface water originate from the degradation of organic compounds such as lignin, cellulose, hemicellulose, and proteins (Sánchez-Monedero et al., 1999). Haemagglutination inhibition assays were carried out in the presence of D-(+)-galactose, L-(+)-arabinose, D-(+)-glucose, and D-(−)-galacturonic acid to assess their potential to interfere with humic acid binding to *cMoL*. D-(+)-Galactose (0.2 M) and L-(+)-arabinose (0.0125 M) reduced *cMoL's* SHA from 3969 to 496 (87.5 %). This result might be explained by the fact that D-(+)-galactose and L-(+)-arabinose compete with the carbohydrates on the surface of the erythrocytes to bind the *cMoL*. Reduction in the percentage of SHA in the presence of humic acid was slightly higher than

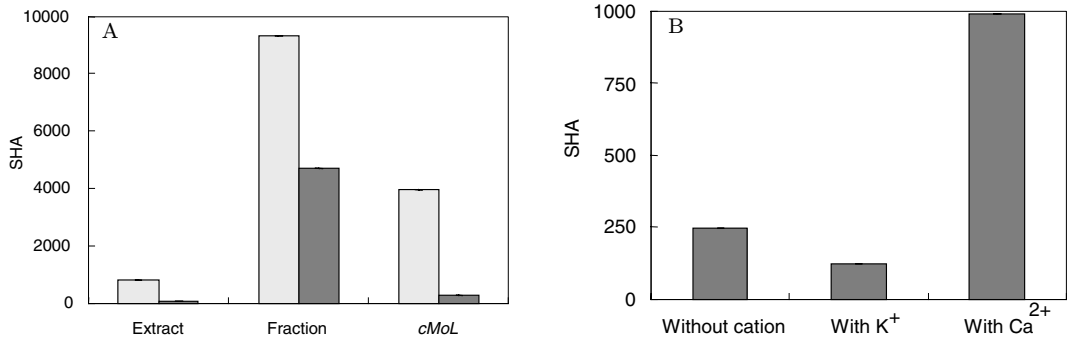

Fig. 1. A: Comparison of specific haemagglutinating activity (SHA) of *M. oleifera* extract (2485 mg L^{-1}), fraction (219 mg L^{-1}), and *cMoL* (129 mg L^{-1}) (grey bars) with SHA obtained in the presence of humic acid 10 mg L^{-1} of carbon (dark grey bars). B: Comparison of SHA of *cMoL* in the presence of humic acid 10 mg L^{-1} of carbon with SHA obtained in the presence of humic acid 10 mg L^{-1} of carbon + cations (10 mM $CaCl_2$ or 5 mM, and 10 mM KCl). SHA was determined by dividing HA with protein concentration. Values represent the mean of three assays.

that obtained with the aforementioned carbohydrates, 94 % and 87.5 %, respectively. Nevertheless, considering that about 75 % of the dissolved organic carbon in rivers consists of humic substances (Barreto et al., 2003), competition constitutes a minor problem for the future application of lectins to water treatment. D-(+)-Glucose and D-(−)-galacturonic acid had no effect on *cMoL* HA at all the concentrations tested.

Haloacetic acids and trihalomethanes are disinfection by-products formed by the reaction of chlorine with natural organic matter present in water and have adverse environmental and health effects (Scott et al., 2000; Eish & Wells, 2006). The affinity of *cMoL* to bind these compounds was also tested. The experimental results have shown that trichloroacetic acid, dicholoroacetic acid, and chloroform had no effect on the *cMoL*'s activity under the concentrations tested.

The SHA of *cMoL* assessed in the presence of humic acid decreased by 50 % with an increase in pH from 7.5 to 8.0. A further increase in pH with an increment of 0.5 in the range from 8.0 to 10 had no effect on *cMoL*'s SHA. The latter observation can be explained by the assumption that pH increases the solubility of humic acid and thus its ability to compete with *cMoL* for the carbohydrates on the surface of the erythrocytes.

The effect of mono- and divalent cations in the specific haemagglutinating activity of *cMoL* in the presence of humic acid is depicted in Fig. 1B. The experimental results showed that K^+ (5 mM and 10 mM of KCl) enhanced the *cMoL*–humic acid interaction, since the SHA was lower (124) than the SHA determined in the absence of potassium and magnesium, (248). A higher result was obtained with Ca^{2+} (5 mM and 10 mM $CaCl_2$) which increased the SHA up to 992. A possible explanation is that calcium decreases the availability of humic acid to compete with *cMoL* for the carbohydrates on the erythrocyte's surface. The study by Zhou et al. (2005) suggested that calcium was able to form a metal bridge or salt linkage between the carboxyl groups of humic acids which results in the formation of macromolecules which might have a lower ability to bind carbohydrates than humic acid molecules alone.

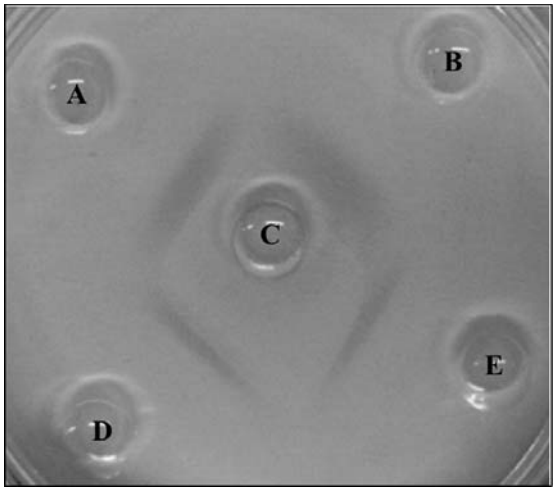

Fig. 2. Precipitation bands observed in agarose gel corresponding to the interaction between humic acid 100 mg L^{-1} of carbon (C) and extract (A), fraction (B), as well as *cMoL* (D and E), 1000 mg L^{-1}.

The single radial diffusion gel showed precipitation bands indicating that the extract, fraction and, *cMoL* bound to humic acid present at a concentration of 100 mg L^{-1}. Similar results were obtained for a humic acid concentration of 200 mg L^{-1} of carbon. These results are in agreement with those obtained in the haemagglutinating activity assay (Fig. 2). Precipitation bands were also observed in an experiment carried out in the presence of azocasein, a protein that totally inhibited *cMoL*'s haemagglutinating activity (Santos et al., 2009). These results suggest that the affinity of humic acid functional groups might not be related to *cMoL* azocasein binding sites, which are occupied by this protein.

Immobilisation of lectins in a matrix generates a new surface by combining the physical and chemical

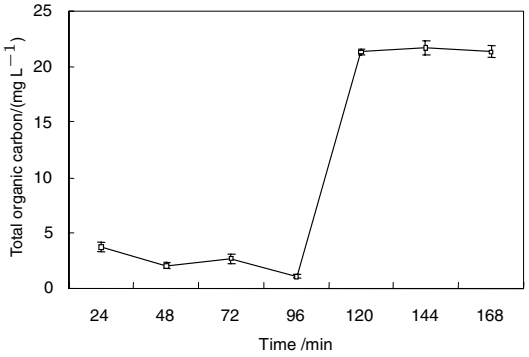

Fig. 3. Removal of humic acid 20 mg L^{-1} of carbon by *M. oleifera* lectin 0.6 mg L^{-1} (*cMoL*) immobilised on cyanogen bromide–activated sepharose 4B.

properties of lectin and a carrier. In ideal cases, surfaces of a hydrophilic character (free from hydrophobic binding sites) are considered optimal for minimising non-specific interactions with the protein samples and also for maximising stability. Another consideration is the type of linkers to be used to attach lectins to the surface of the stationary phase. Good matrices allow immobilised lectins to act in a way similar to the way they act in nature (i.e. to recognise and subsequently to bind biomolecules of interest without steric hindrance) (Monzo et al., 2007). Most reactive groups used for immobilising proteins (glutaraldehyde, cyanogen bromide, etc.) are able to yield very stable enzyme-support bonds under mild immobilisation conditions (e.g., neutral pH) (Grazu et al., 2006). The support that immobilised the highest amount of *cMoL* was cyanogen bromide–activated sepharose 4B, 2.4 mg g^{-1}, followed by cyanogen bromide-activated agarose, 0.2 mg g^{-1}, expressed as mass of protein per mass of support. *cMoL* was not immobilised on the other supports tested, specifically, silica, clay and cellulose. Fig. 3 presents the breakthrough curve of a humic acid solution (20 mg L^{-1} of C) obtained in a packed column containing *cMoL* immobilised on sepharose. The amount of humic acid removed was 30 mg g^{-1} (expressed as mass of humic acid per mass of support) and this result suggested that protein preparations might be used in water treatment to remove humic acids.

Conclusions

The basic question addressed in the present work is whether protein preparations, specifically, the extract, fraction and *cMoL* from *M. oleifera* seeds, might bind humic acids present in aqueous solutions. From the results of this study we can conclude that *M. oleifera* lectin preparations showed an affinity towards humic acid, based on experiments of inhibition of the *cMoL* haemagglutinating activity and the single radial diffusion assay with humic acid and protein preparations. The immobilisation stage can be limiting and new methodologies will be tested. In the assays, *cMoL* immobilised on a packed bed column removed humic acid (30 mg g^{-1}, expressed as mass of humic acid per mass of support). This feature could be explored in water treatment to remove humic acids, because it presents a new separation and purification process.

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