Immobilization of lectin preparations from *Moringa oleifera* seeds in inert supports for water purification

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Abstract

Humic acids are linked to the formation of carcinogenic disinfection by-products upon chlorination of drinking water. In this work the first focus was tocharacterize the affinity of protein preparations obtained from *Moringa oleifera* seeds (extract, E, fraction, 0-60F and *M.oleifera* lectin, MoL) to bind humic acids. The second focus was to select a suitable support to immobilize MoL and to assess humic acid removal from water in a packed bed column. Specific hemagglutinating activity (SHA) decreased by 94 % for both E and MoL and by 50 % for 0-60F in the presence of a commercial humic acid. Humic acid-MoL precipitation bands were observed in the diffusion gel. Both results indicate humic acid-lectin binding. Carbohydrates, potassium and calcium ions as well as pH va-lues affected the SHA of MoL. A humic acid removal of 30.4 mg/g (expressed as mass of humic acid per mass of support) was obtained in a column packed with sepharose immobilized MoL receiving a 20 mg/L of carbon humic acid solution.

Keywords: lectin, *Moringa oleifera*, immobilization, humic acid, water treatment.

1. Introduction

A lectin is a type of receptor protein of non-immune origin that interacts with carbohydrates without modifying them. These proteins recognize and bind carbohydrates with specific characteristics and have the ability to induce cell agglutination phenomenon. The presence of these proteins is detected by a hemagglutination assay performed by serial dilution of lectin and incubation with human or animal red blood cells [1]. Lectins have mainly been obtained from seeds of leguminous plants, but also from many other plant and animal tissues. They are valuable tools in biotechnological research and biomedical applications. An important application of immobilized lectins [2] is the isolation of biomolecules [3].

Moringa oleifera is a plant of the Moringaceae family and seeds have been used in water treatment [4]. Aqueous extract from *M. oleifera* seeds contains a flocculating protein that works as a clarifying agent of turbid water [5] and lectins [6; 7]. Shelled *M. oleifera* seeds have also been used for decontamination of water containing arsenic [8].

Organic compounds of biological origin are found in all surface waters. They are referred as natural organic matter (NOM) and are divided into hydrophilic (mainly carboxylic acids, carbohydrates and proteins) and hydrophobic (humic substances, HS) fractions [9]. HS are divided in two fractions, humic acids and

fulvic acids [10]. Humic acids are heterogeneous mixtures of organic compounds containing several chemical groups. 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 [11]. NOM causes odor, taste and color in water and support bacterial growth [12]; it is linked to the formation of disinfection by-products upon chlorination of drinking water. Trihalomethanes (THM) constitutes the most commonly observed of these by-products; they may originate adverse health effects in animals and humans [13]. For this reason, several strategies have been investigated for the removal of humic substance from water.

The aim of the present study was to evaluate a new process for humic acid removal from water. Important goals were to characterize the affinity of protein preparations obtained from *M. oleifera* seeds (extract, fraction and lectin) to bind a commercial humic acid, to select a suitable support to immobilize the *M. oleifera* lectin, MoL, previously called SSMoL [14] and to assess humic acid removal from water in a bed column packed with the selected support.

2. Materials and methods

2.1 Lectin isolation

M. oleifera seeds were ground to flour that was extracted with 0.15 M NaCl for 6 h at room temperature (25 °C) and a saline extract (E) was obtained. Proteins present in E were precipitated with ammonium sulphate (60 %, w/v) for 4 h at room temperature (25 °C). The fraction (0-60F) obtained was chromatographed (10 mg of protein) on a guar gel column (10 x 1.0 cm) previously equilibrated (20 mL/h flow rate) with 0.15 M NaCl. The lectin (MoL) was eluted with 1.0 M NaCl.

2.2 Hemagglutinating activity and inhibition hemagglutinating activity assays

The hemagglutinating activity assay (HA) was performed in microtiter plates [15]. The inhibition assay (IHA) followed the same protocol as HA with the exception of an incubation step with inhibiting substance for 15 min at room temperature (25 ºC), before addition of erythrocyte suspension. The following substances were tested: i) humic acid (Sigma Aldrich 53680), a solution of 10 mg/L of carbon prepared in 0.1 M NaOH; ii) carbohydrates, aqueous solutions of D(+)-glucose, D(+)-galactose, L(+)-arabinose and, D(-)-galacturonic acid (0.2 M, 0.1 M, 0.05 M, 0.025 M and 0.0125 M); iii) glycoprotein, solution of azocasein 5 mg/L; iv) halogenated organic compounds, aqueous solutions of dichloroacetic acid (5.6 µg/L) and trichloroacetic acid (56 µg/L), as well as chloroform (56 µg/L).

2.3 Effect of pH values and concentration of calcium and potassium on lectin hemagglutinating activity

The effect of pH in an interval of 7.5 to 10 on lectin HA was assessed in the presence of humic acid according to the procedure previously described to HA. The pH was adjusted with phosphate buffer. The effect of potassium and calcium in the lectin HA was tested in the presence of the humic acid. A humic acid solution of 100 mg/L of carbon was diluted (1:10) with $CaCl_2$ or KCl_2 solutions, 5 and 10 mM, prepared in 10 mM sodium phosphate buffer. Before the addition of erythrocyte suspension, lectin was incubated with the humic acid in the presence of K^+ and Ca^{2+} for 15 min at room temperature (25 ºC).

2.4 Protein evaluation

The protein was estimated according to Lowry *et al.* [16]. A calibration curve was prepared using bovine serum albumin (BSA) as standard in a range between 0 µg and 400 µg.

2.5 Single radial diffusion assay

A diffusion assay was carried out in agarose gel formed in a Petri dish. The gel (1%, w/v) was prepared in 0.15 M NaCl. A humic acid solution (30 μL), with a concentration of 100 mg/L and 200 mg/L of carbon, was placed in a central well; peripheral wells were occupied with 15 μL (1 mg/mL) of E, 0-60F and MoL. Assay was also carried out with azocasein (0.5 mg/mL) incubated with each protein preparation for 15 min at room temperature (25 ºC). Diffusion experiments were performed in a humid chamber at 4 °C for 48 h. Gels were exhaustively washed with 0.15 M NaCl and stained for 2 h with 0.1% (w/v) Coomassie Brilliant Blue, prepared in a mixture of ethanol 45 % (v/v) and acetic acid 10 % (v/v).

2.6 Humic acid characterization

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

2.7 Total organic carbon

Total organic carbon (TOC) was measured spectrophotometrically at 600 nm using the Method 10129 from Hach Lange GmbH (0.0 mg/L to 20.0 mg/L of carbon). Organic carbon was oxidized with persulphate in the presence of acidic conditions and the carbon dioxide formed was captured by and indicator solution that changes color proportionally to the amount of organic carbon originally present in the sample. Results were expressed in mg/L of carbon.

2.8 MoL immobilization in silica, clay and cellulose

Silica previously treated with (3-aminopropyl) triethoxysilane (APT-silane), 6 g, was added to 120 mL of APT-silane solution 10 % (p/v) in distilled water (pH 3.0) and mixture was agitated (2 h, 75 ºC). APT-silane solution was removed by centrifugation and distilled water washing (5 times). Silica was dried at 110 ºC during 17 h. Pretreated silica, clay and cellulose were activated with 2.5 % (v/v) glutaraldehyde solution in 5 mM sodium phosphate buffer (pH 7.0) and incubated during 1 h at room temperature; glutaraldehyde was removed and supports were washed with the buffer. MoL immobilization (1.9 mg/L) was performed with 50 mg of activated supports mixed with 1.5 mL of 5 mM sodium phosphate buffer (pH 7.0) for 17 h at 4 ºC. Supports were washed with the buffer (4 times) and 2 M urea (once). Supernatant was removed, volume was measured and protein was determined by Lowry.

2.9 Lectin immobilization in sepharose and agarose

Cyanogen bromide-activated sepharose 4B (0.25 g) and cyanogen bromide-activated agarose (0.25 g) were used for MoL immobilization [17]. Supports were washed with 0.5 M NaCl, pH 2.5, followed by 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.2. Incubation (24 h, 4 C) was performed with MoL (0.6 mg/L). After filtration and washing with $NaHCO_3$ solution, ethanolamine was added to a final concentration of 1 M.

2.10 Removal of humic acid from water in a packed bed column

A humic acid solution of 20 mg/L of carbon was applied to 1 mL column (0.68 x 4 cm) containing MoL immobilized in sepharose at a flow rate of 12 mL/h. The assay was carried out at room temperature and 5 mL samples were collected at 20 min intervals. TOC and protein were determined.

3. Results and discussion

The HA were normalized by the amount of protein used in each assay (specific hemagglutinating activity, SHA), detected in E (SHA: 825.1), 0-60F (SHA: 9351.6) and MoL (SHA: 3969.0) obtained from *M. oleifera*

seeds (figure 1A); in the presence of humic acid, SHA decreased by 94 % for both E (SHA: 51.5) and MoL (SHA: 248.0) and by 50 % for 0-60F (SHA: 4675.8).

Carbohydrates are present in surface water originating from the degradation of organic compounds namely, lignin, cellulose, hemicellulose and proteins [18]. Inhibition hemagglutinating activity assays were carried out in the presence of carbohydrates to assess their potential to interfere with humic acid binding to the MoL. D(+)-Galactose (0.2 M) and L(+)-arabinose (0.0125 M) reduced MoL 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 MoL. Reduction in the percentage of SHA in the presence of humic acid was slightly higher than 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 [19] competition constitutes a minor problem for a future application of lectins to water treatment. D(+)-Glucose and D(-)-galacturonic acid had no effect on MoL HA at all tested concentrations.

Haloacetic acids and trihalomethanes are disinfection by-products formed in the reaction of chlorine with natural organic matter present in water and have adverse environmental and health effects [13]. Trichloroacetic acid, dichloroacetic acid, and chloroform had no effect in MoL activity under assayed concentrations.

The SHA of the MoL assessed in the presence of the humic acid decreased by 50 % with an increase in pH from 7.5 to 8.0. A further increase in pH in the range of 8.0 to 10 had no effect on MoL SHA. The later observation can be explained by assuming that pH increases the solubility of humic acid and thus its capability to compete with MoL for the carbohydrates on the surface of the erythrocytes.

The effect of mono- and divalent-cation in the SHA of MoL in the presence of humic acid is depicted in Figure 1B. The experimental results showed that K^+ (5 mM and 10 mM KCl) enhanced the interaction MoL-humic acid, since SHA was lower than the one determined in absence of potassium and magnesium, 124 and 248, respectively. An opposite result was obtained with Ca^{2+} (5 mM and 10 mM $CaCl_2$) that increased SHA to 992; calcium may decrease the availability of humic acid to compete with MoL for carbohydrates on erythrocyte's surface. Zhou *et al*. [20] suggested that calcium is able to form a metal bridge or salt linkage among carboxyl groups of humic acids which result in formation of macromolecules and might have a lower ability to bind carbohydrates than single humic acid molecules.

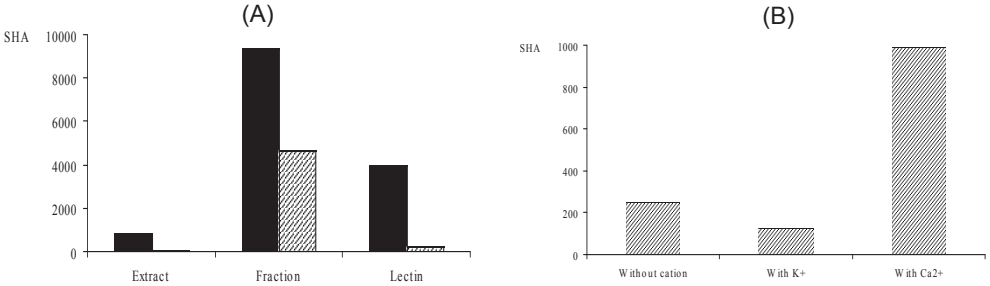

Figure 1. Specific hemmaglutinating activity (SHA) of E, 0-60F and MoL without (black bars) and with (black and white bars) humic acid (A) and MoL SHA plus humic acid (B) without and with cations (10 mM $CaCl_2$ or 5 mM and 10 mM KCl_2). SHA was determined dividing HA by protein concentration. Values represent the mean of three assays (± standard deviation): significant differences between groups were determined at ñ < 0.05.

The single radial diffusion gel showed precipitation bands indicating that E, 0-60F and MoL did bind to humic acid (100 mg/L and 200 mg/L of carbon); results are in agreement with those obtained in HA

(Figure 2). Precipitation bands were also observed with azocasein, a glycoprotein that completely inhibited MoL HA. These results suggest that affinity of humic acid functional groups might not be related to MoL azocasein binding sites, which are occupied by this glycoprotein.

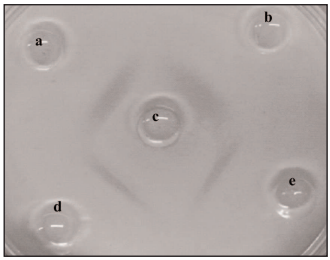

Figure 2. Precipitation bands observed in agarose gel corresponding to interaction among humic acid (c) and E (a), 0-60F (b), as well as MoL (d, e).

Immobilization of lectins in a matrix generates a new surface from the combination of physical and chemical properties of lectin and carrier. In ideal cases, surfaces with hydrophilic character (free of hydrophobic binding sites) are considered optimal to minimize non-specific interactions with protein samples and also to maximize stability. Another consideration is the type of linkers to be used to attach lectins to surface of stationary phase. Good matrices allow immobilized lectins to act in a similar way to that in nature, to recognize and subsequently to bind biomolecules of interest without steric hindrance [21]. Most reactive groups used for immobilizing proteins (glutaraldehyde, cyanogen bromide, etc.) are able to yield very stable enzyme-support bonds under mild immobilization conditions, such as neutral pH values [22]. The support with the highest amount of MoL immobilized was sepharose, 2.4 mg/g, followed by agarose, 0.2 mg/g, expressed as mass of protein per mass of support. MoL was not immobilized in silica, clay or cellulose. Figure 3 presents the breakthrough curve of a humic acid in solution (20 mg/L of carbon) obtained in a column containing immobilized MoL on sepharose. The considerable removal of humic acid (30.4 mg/g, expressed as mass of humic acid per mass of support) in the column indicates that the proposed process might be an interesting alternative to the existing ones.

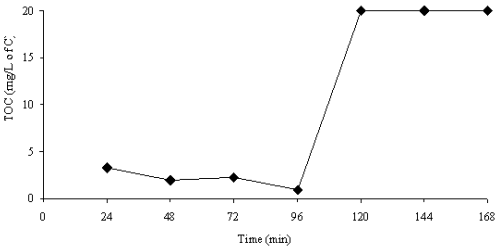

Figure 3. Removal of humic acid by *M. oleifera* lectin immobilized in sepharose.

In this context, immobilized lectin from *M. oleifera* seeds can be used for humic acid removal in water treatment and the immobilization assays with the other preparations (extract and fraction) are being conducted in our laboratory for the production of a filter to be used in a larger scale.

4. Conclusions

With this study, we can conclude that *M. oleifera* lectin preparations showed affinity with humic acid. The immobilization stage can be limiting and new methodologies will be tested. The assays with lectin immobilized in a packed bed column removed humic acid (30.4 mg/g, expressed as mass of humic acid per mass of support). This feature could be explored in the field of water treatment with perspectives to produce a filter for water purification with capacity to remove humic acids.

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