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Advances in Agriculture, Food Science and Forestry

Vol. 1 (1), pp. 001-006, January, 2013
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Fragmentary regeneration of leaf lectin from cassava and veiling its fungicidal scheme

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Abstract

Various biotechnological applications in Lectins including their use in agriculture as antimicrobials and pesticides. Lectin from manioc leaf powder has been extracted, partially purified and evaluated in terms of its haemagglutination activity and fungicidal properties against Fusarium oxysporum. Ground, dried and the entire leaves of Manihot esculenta (Cacao cultivar) were extracted with distilled water and the protein extract was concentrated by precipitation in 80% saturated ammonium sulphate solution. The concentrated protein solution was partially purified on a Sepharose column and the unbound protein fraction was submitted to native and denaturing polyacrylamide gel electrophoresis. Lectin from manioc leaf powder exhibited a haemagglutination (HU) activity of 0.5 HU/100 µl and was stable over a wide temperatures range (up to 70°C). Four protein bands (of 13, 58, 61, 64 kDa) were identified in the unbound protein fraction obtained by column chromatography, and the presence of a lectin was demonstrated by the determination of an HA value of 0.5 HU/100 µl. The concentrated protein solution was not, however, active against F. oxysporum at concentrations up to 100 µg/ml.

Keywords: *Manihot esculenta,* Euphorbiaceae, manioc, fungicidal activity, haemagglutination activity, lectin.

INTRODUCTION

Lectins are proteins that bind reversibly to carbohydrates without inducing chemical modifications in the covalent structure of the glycosyl ligands. Such proteins exhibit high levels of specificity and affinity towards the sugar moieties of the glycoconjugates, but do not possess enzymatic activity (Peumans and Van Damme, 1995; Gabius et al., 2002; Loris, 2002).

The interaction between a lectin and a sugar moiety occurs at the carbohydrate-recognition domain and involves Van der Waals forces and the formation of hydrogen bonds between sugar hydroxyls and amino acid residues at the active site of the lectin (Weis and Drickamer, 1996). The cross-linkages formed between adjacent cells through binding between lectins and glycoconjugates causes cell agglutination (Peumans and Van Damme, 1995). When erythrocytes are involved in this type of interaction, the phenomenon of haemagglutination occurs (Alonso et al., 2001).

Ingestion of lectins by animals may cause anti-nutritional effects, degeneration of cell membranes and inhibition of digestive enzymes (Vasconcelos and Oliveira, 2004), and the resulting interference with the absorption of nutrients may give rise to serious physiological consequences. On the other hand, the strong sugar specificity of lectins has led to their exploitation in many biological and medicinal areas, and they have found application as fungicidal, bactericidal and insecticidal agents in agriculture (Peumans et al., 2000; Grahamstown and Van Staden, 2002), in the purification and characterisation of polysaccharides and in the glycoconjugates industry (Lima et al., 1997). Furthermore, it is used in the identification of blood groups (Lis and Sharon, 1998), as stimulators of lymphocyte mitogenesis and immunological functions (Sharon and Lis, 2004), in the agglutination of cancer cells and in studies of oncogenesis (Sharon and Lis,

2004), and in the investigations concerning the structures of proteins and carbohydrates in cells (Silva and Silva, 2000).

The use of synthetic chemicals in the control of agricultural pests is not only costly, but may also be environmentally unacceptable in terms of pollution and the induction of pathogen resistance. Application of natural insecticides and fungicides could provide a cheap and environmental friendly alternative (Macedo et al., 2000). Within this context, the leaves of manioc (*M. esculenta* Crantz) represent a rich source of lectins that could be used in the protection of crops against diseases. Currently, manioc leaves have no economical value and is normally treated as agricultural waste.

The aim of the present study was, therefore, to extract and partially purify the lectins from manioc leaf powder (MLP) in order to evaluate their haemagglutination activity (HA) and to test their fungicidal activity on *F. oxysporum*, the causal agent of a wilt disease that attacks a variety of commercial crops. The possibility of using manioc leaf lectin as a fungicidal agent could transform an abundant waste material into a valuable commodity.

MATERIALS AND METHODS

Preparation of MLP

Mature aerial parts of manioc (Cacao cultivar) were collected from 12 month old plants grown in Fazenda Rio Grande, Lavras, MG, Brasil. The whole leaves and petioles were washed with tap water and dehydrated in a fan-assisted oven at 30 to 35°C for 24 h, following which the petioles were removed and the leaves were dried further for 24 h. Dried leaves were ground to a powder (MLP) using a Willy type mill (Tecnal, Piracicaba, Brasil).

Extraction and concentration of protein

A 25 g portion of MLP was extracted with distilled water (1:20; w/v) under constant stirring at room temperature for 15 min. The suspension was filtered through muslin cloth and centrifuged at 8000 x *g* for 15 min at 7°C. The supernatant was removed and the residue submitted to two successive extractions under the conditions as described. The crude MLP extracts were combined, cooled in an ice bath and ammonium sulphate was added under constant stirring, and pH control until 80% saturation was achieved (Cooper, 1942; Pereira et al., 2008). The mixture was maintained in the refrigerator for 24 h and subsequently centrifuged at 8000 x *g* for 15 min at 7°C. The residue was resuspended in 0.15 M NaCl solution and dialysed against the same solution. All procedures were carried out in triplicate, and the concentrated protein solution was submitted to further purification.

Assessment of the protein concentration, haemagglutination activity and thermostability

The protein concentration, HA and thermostability of the crude MLP extract and of the concentrated protein solution were estimated according to standard procedures. All assays were performed in triplicate. Total protein content was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as standard. The HA assay was performed according to Calderón de la Barca et al. (1985). Briefly, aliquots (100 µL) of buffered saline solution were added to each well of a 96-well microtiter plate, and a 100 µl of the sample was transferred to the first well. Serial dilutions (base 2) were prepared by transferring a 100 µl aliquot of a more concentrated sample to an adjacent well containing diluent alone, it was mixed and dilution procedure continued. An aliquot (100 µl) of 2% suspension of erythrocytes prepared from human blood (type A, Rh +) was added to each well and the microtiter plate was maintained at room temperature. HA was determined visually at 60 and 90 min, and the results were expressed in haemagglutination units (HU), defined as the reciprocal of the maximum dilution presenting visible agglutination of red blood cells (that is, if the 1/4 titre represented the maximum dilution exhibiting agglutination and the volume of sample assayed was 100 µl, then the HA value would be 4 HU/100 µl). Negative control samples consisted of buffered saline solution and erythrocyte suspension only.

The thermostability of MLP lectin was assessed by submitting aliquots (1 ml) of the concentrated protein solution to temperatures in the range 40 to 90°C for 30 min, cooled in an ice bath and the HA and protein concentration of the samples was determined.

Partial purification of MLP lectin

Chromatographic procedures were conducted in a refrigerator at 4°C. A glass column (40 x 2 cm) was packed with Sepharose CL-4B resin (GE Healthcare, Chalfont St. Giles, UK) according to the manufacturer's instructions and equilibrated with 0.15 M NaCl until the A_{280} of the effluent was < 0.02. Subsequently, a 30 ml sample of the concentrated protein solution (containing 5.84 mg of protein) was loaded onto the column and eluted with 0.15 M NaCl at a constant flow rate of 1 ml/min with the aid of a peristaltic pump (Amersham Pharmacia Biotech, Piscataway, EUA). Unbound proteins fractions (2.5 ml) were collected, monitored at 280 nm, and those showing absorption at this wavelength were combined. The protein content was determined and, following lyophilisation, an aliquot was submitted to polyacrylamide gel electrophoreses (PAGE) and sodium dodecyl sulphate (SDS)-PAGE. The column was finally eluted with 0.15 M NaCl containing 0.1 M galactose in order to elute any proteins that remained bound to the Sepharose.

Gel electrophoresis

Native PAGE was performed according to the method of Laemmli (1970) and Robyt and White (1987) with a stacking gel of 5% polyacrylamide in 0.6173 M Tris/HCl buffer (pH 6.8) and a separation gel of 12.5% polyacrylamide in 0.3777 M Tris/HCl buffer (pH 8.9). The lyophilised unbound protein fraction was prepared in 0.6173 M Tris/HCl buffer (pH 6.8) containing 20% glycerol. Electrophoresis was carried out at 60 volts for 5 h using an Amersham Pharmacia Biotech model EPS 1001 vertical electrophoresis system. Protein bands were visualised by staining the gel with Coomassie Blue R–250.

SDS-PAGE was performed according to Laemmli (1970). The stacking gel consisted of 6% polyacrylamide in 0.5 M Tris/HCl buffer (pH 6.8) containing 0.4% SDS, concentrated *N,N,N',N'*-Tetramethylethylenediamine (TEMED) and 10% ammonium persulphate, while the separation gel comprised 12.5% polyacrylamide in 1.5 M Tris/HCl buffer (pH 8.8) containing 0.4% SDS, concentrated TEMED and 10% ammonium persulphate. The lyophilised unbound protein fraction and molecular markers (BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soy-bean trypsin inhibitor and lactoalbumin) were prepared in 62.5 M Tris/HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS and 5% 2-mercaptoethanol.

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Table 1. Total protein content and haemagglutination activity of the manioc leaf powder (MLP) samples.

Sample	Total protein (mg)	Haemagglutination activity (HU /mg protein)
Crude MLP extract [a]	41.58 ± 5.42	2164.0 ± 386.0
Concentrated protein solution [b]	24.05 ± 3.21	2856.0 ± 430.0

[a] Obtained by extraction of 25 g of MLP with distilled water. [b] Obtained by precipitation of the crude MLP extract with 80% saturated ammonium sulphate solution. Values shown represent the mean ± standard deviation.

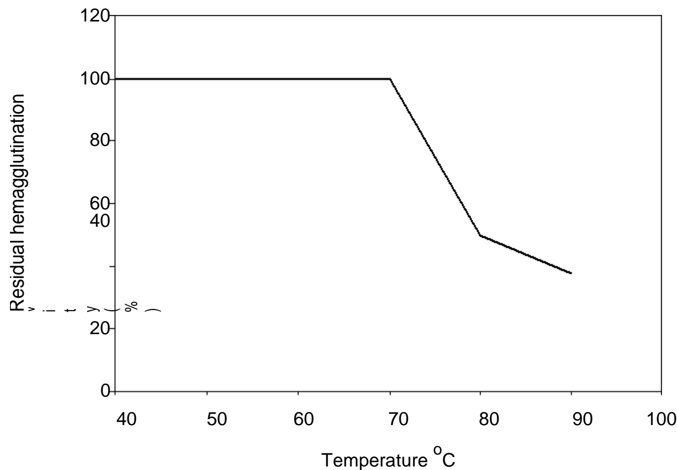

Figure 1. Effect of temperature on the haemagglutination activity of the concentrated protein solution obtained from manioc leaf powder. Incubations were carried at 40, 60, 70, 80 and 90ºC for 30 min.

Moreover, the running conditions and visualisation of bands had been described.

Fungicidal activity

Assays were conducted in the form of a random design comprising 5 treatments with 4 repetitions each. Lyophilised protein material was redissolved in distilled water at concentrations of 1, 10, 50 and 100 µg/ml and was added to molten (45 - 50°C) potato dextrose agar (PDA) medium. Aliquots (25 ml) of the medium were poured into Petri dishes (9 cm diameter) and left to solidify. Negative control plates were prepared with PDA medium alone. Cultures of *F. oxysporum* were obtained from the culture bank of the Departmento de Fitopatogia, Universidade Federal de Lavras. Disks (9 mm) of 6 day-old fungal mycelia, previously cultivated in PDA medium, were placed in the centre of Petri dishes containing the analyte and the dishes sealed with plastic film. Incubation was carried out under biological oxygen demand at 25°C. After incubation for 7 days, the diameters of the growing mycelia were measured (cm) and compared with the control culture, and the results were submitted to analysis of variance.

RESULTS AND DISCUSSION

Extraction and concentration of MLP lectin

Initially, purification of lectins followed the scheme used for proteins in general without exploiting their special characteristics. The methods used included precipitation by salts, acids and organic solvents. Progress in this area was achieved by the introduction of preparative chromatographic methods with ion exchangers, gel filtration (size exclusion chromatography) media and, above all, affinity adsorbents (Rüdiger and Gabius, 2001).

In this work, the partial purification of the lectin, we used ammonium sulphate as precipitating agent.

The amounts of protein present in the crude MLP extract and in the concentrated protein solution derived therefrom are shown in Table 1. Approximately 58% of the proteins present in the crude MLP extract could be recovered following precipitation in 80% ammonium sulphate and dialysis against 0.15 M NaCl solution. The recovery efficiency obtained in the present study was much greater than that reported previously (11%; Pereira et al., 2008). The haemagglutination activity (expressed in HU/mg protein) of the concentrated protein solution was approximately 30% higher than that of the crude MLP extract (Table 1).

Thermostability of MLP lectin

The HA of the concentrated protein solution was stable at temperatures up to 70°C (Figure 1), but was diminished by 50 and 62.5%, respectively, at 80 and 90°C. Stability over a wide temperature range is typical of plant lectins and can be attributed to their role in plant defence mechanisms against environmental stress (Peumans and Van Damme, 1995).

Partial purification of MLP lectin

Column chromatography of the concentrated protein solution over Sepharose CL-4B resin eluted with 0.15 M NaCl gave rise to a single 280 nm peak corresponding to the unbound protein fraction (fractions 24 to 51; Figure 2). The total protein content of these combined fractions was 1.75 mg, giving a recovery efficiency of 29.91%, and the HA was 0.5 HU/100 µl.

Normally, elution of proteins bound to a stationary phase can be achieved by introducing a soluble ligand that competes with the immobilised ligand to which the target protein is attached (Collins et al., 1997). In the case of lectins, the linkage with a soluble ligand (e.g. galactose) could occur either through the carbohydrate

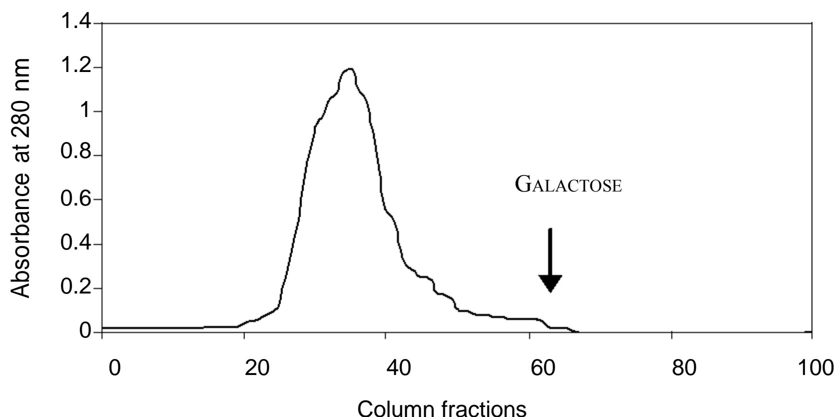

Figure 2. Chromatographic profile of the concentrated protein solution derived from manioc leaf powder. Column chromatography was carried out using a Sepharose CL-4B glass column (20 x 2 cm i.d.; approximate volume 63 ml). Non-bound proteins were eluted with 0.15 M NaCl at a flow rate of 1 ml/min, whilst matrix-bound proteins were eluted with 0.15 M NaCl and 0.1 M galactose (initiation of elution with galactose arrowed).

receptor site (specific linkage) or through ionic, hydrophobic and hydrogen interactions (non-specific linkage). In the present study, the Sepharose column was eluted with 0.15 M NaCl containing 0.1 M galactose in an attempt to elute the bound protein fraction, but none of the eluted fractions showed absorption at 280 nm as shown in Figure 2. There are two possible explanations for the interaction between the MLP lectin and the stationary matrix and the absence of lectin in the eluted bound fraction. The first hypothesis is that the carbohydrate receptor site of the MLP lectin is not specific for the galactose ligand such that 0.15 M NaCl was sufficient to break the weak non-specific interactions, thus displacing the bound lectin from the stationary phase. The second proposition is based on the fact that the agarose gel of the stationary phase is highly cross-linked forming pores of different sizes such that the proteins were separated according to molecular weight.

Column chromatography of the concentrated protein solution over Sepharose CL-4B resin eluted with 0.15 M NaCl was found only at the peak of lectin activity, but it was found heterogeneous on native electrophoresis. It is possible since plants contain families of lectin genes that may have altered patterns of expression in a tissue or in different tissues of the same plant. Common lectin gene products may also differ during posttranslational modification (Etzler, 1985). However, incomplete separation of proteins may have occurred.

Gel electrophoresis

When the unbound protein fraction obtained from the Sepharose column was submitted to native PAGE (Figure 3A), four bands were stained with Coomassie Blue indicating the presence of four separate proteins. Under denaturing conditions, the SDS-PAGE showed separate bands at 64, 61, 58 and 13 kDa (Figure 3B) confirming the presence of four distinct proteins. It is not possible at this stage to determine, which of these bands correspond to MLP lectin since plant lectins present molecular weights that vary over a wide range, as exemplified by those isolated from *Urtica dióica* (8.5 kDa; Broekaert et al., 1989) and *Phaseolus lunatus* (265 kDa; Moreira et al., 1991).

Fungicidal activity

With the insecticidal action of plant lectins, the spectrum of external functions is certainly not completely covered yet. Binding to cell wall constituents in fungi can interfere with their growth. Fungal cell walls contain chitin, the β1 → 4 linked polymer of GlcNAc. It is, therefore, likely that a fungicidal action is exerted by GlcNAc-binding lectins (Sharon and Goldstein, 1998).

MLP lectin showed no inhibitory activity against *F. oxysporum* as demonstrated by the growth of mycelia when cultured on medium containing variable concentrations (0 to 100 µg/ml) of protein solution (Figure 4). It has been shown previously that some plant lectins do exhibit fungicidal activity against *F. oxysporum*, e.g. lectins isolated from *Talisia esculenta* (Freire et al., 2002) and *Pouteria torta* (Boleti et al., 2007) were both active at a concentration of 280 µg/ml.

Conclusions

This study indicated that Leaf lectin from Manihot

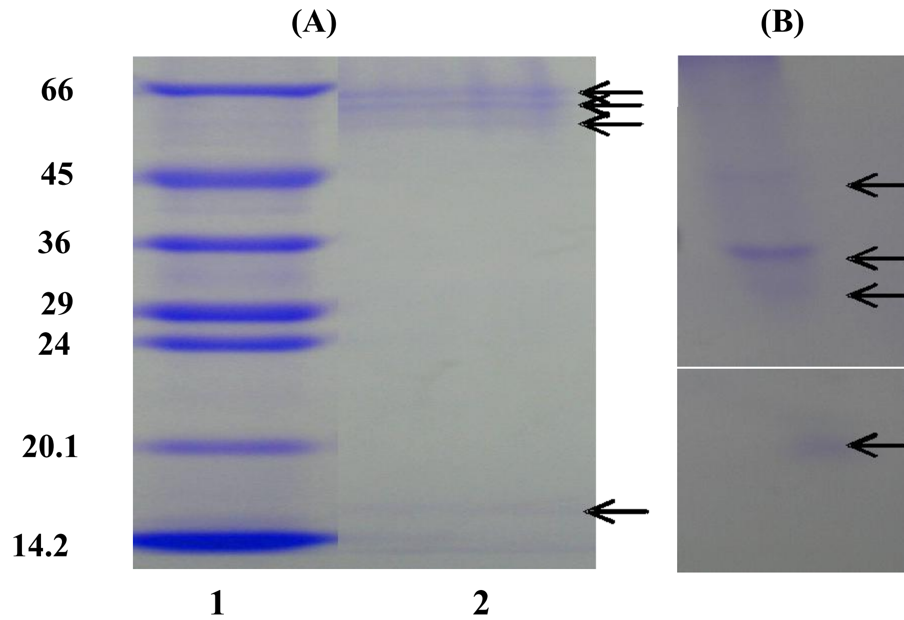

Figure 3. Gel electrophoresis of the unbound protein fraction obtained via column chromatography over Sepharose CL-4B of a manioc leaf powder solution. (A) Native PAGE: Bands corresponding to the unbound protein pool; (B) SDS- PAGE : Lane 1 - Bands corresponding to the molecular markers BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soy-bean trypsin inhibitor (20.1 kDa) and lactoalbumin - 14.2 kDa; Lane 2 - Bands corresponding to the unbound protein pool.

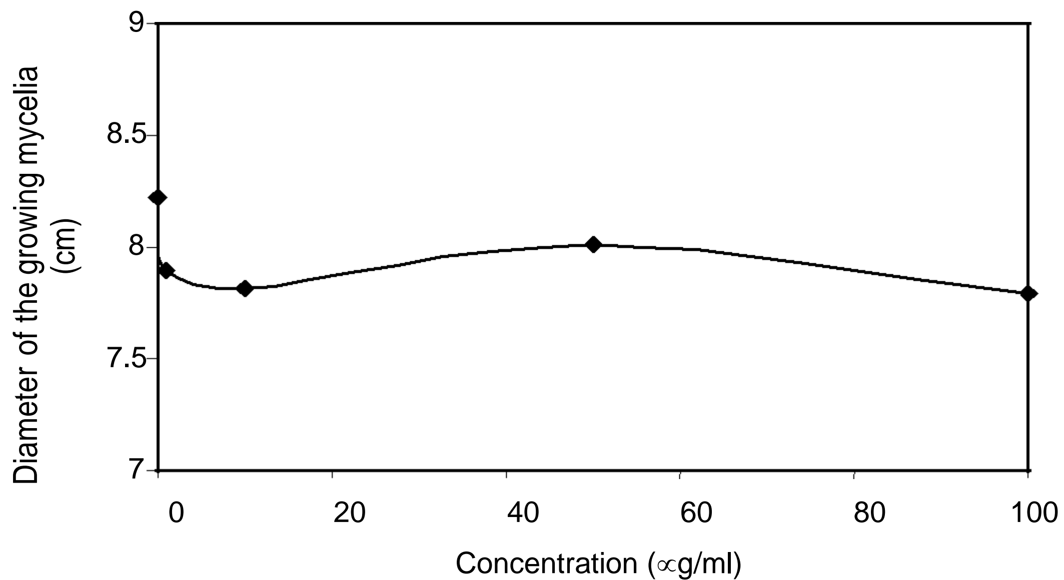

Figure 4. Effect of the concentrated protein solution derived from manioc leaf powder on the growth of mycelia of *F. oxysporum* after 7 days incubation at 25°C under biological oxygen demand.

esculenta has four protein bands (13, 58, 61, 64 kDa), which were identified in the unbound protein fraction. This fraction exhibited a haemagglutination activity of 0.5 HU/100 µl indicating the presence of lectin, although it was not possible to associate the activity with a specific band. MLP-lectin was stable over a wide temperature

range (up to 70°C), but was not active against *F. oxysporum* in concentrations up to 100 µg/ml.

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