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Isolation and antiproliferative activity of *Lotus corniculatus* lectin towards human tumour cell lines

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ABSTRACT

The objective of the study was to investigate the anti cancer activity of a lectin isolated from *Lotus corniculatus* seeds. A tetrameric 70 kDa galactose specific lectin was purified using two step simple purification protocol which involved affinity chromatography on AF-BlueHC650M and gel filtration on Sephadex G-100. The lectin was adsorbed on AF-BlueHC650M and desorbed using 1 M NaCl in the starting buffer. Gel filtration on Sephadex G-100 yielded a major peak absorbance that gave two bands of 15 kDa and 20 kDa in SDS PAGE. Hemagglutination activity was completely preserved, when the temperature was in the range of 20–60 °C. However, drastic reduction in activity occurred at temperatures above 60 °C. Full hemagglutination activity was retained at ambient pH 4–12. Thereafter no activity was observed above pH 13. Hemaglutination of the lectin was inhibited by D-galactose. The lectin showed a strong antiproliferative activity towards human leukemic (THP-1) cancer cells followed by lung cancer (HOP62) cells and HCT116 with an IC_{50} of 39 μg/ml and 50 μg/ml and 60 μg/ml respectively. Flow cytometry analysis showed an increase in the percentage of cells in sub G0G1 phase confirming that *Lotus corniculatus* lectin induced apoptosis. Morphological observations showed that *Lotus corniculatus* lectin (LCL) treated THP-1 cells displayed apparent apoptosis characteristics such as nuclear fragmentation, appearance of membrane enclosed apoptotic bodies and DNA fragmentation. *Lotus corniculatus* lectin (LCL) effectively inhibits the cell migration in a dose dependent manner as indicated by the wound healing assay.

Introduction

Lectins are heterogeneous group of proteins with at least one non catalytic domain that selectively recognises and specifically binds to free sugars present on glycoproteins and glycolipids without altering the structure of the carbohydrates (Lannoo and Van Damme, 2010). Lectins are widely distributed in nature, mainly in the plant kingdom, even though they also occur in other organisms, such as animals and microorganisms (Pustai 1991; Drikarmer and Taylor 1993; Gabius 1997; Wong et al., 2010). Plant lectins have been established to possess remarkable anticancer properties *in vivo*, *in vitro* and in human case studies and have been successfully adopted for alternative cancer therapy (De Mejia and Prisecaru, 2005; Liu et al., 2010; Pusztai et al., 2008). Legume lectins are one of the most comprehensively studied plant lectins for their molecular basis of the protein-carbohydrate interactions for several decades (Damodaran et al., 2008). In recent years, the main interest in this lectin family lay in their prospective application as antitumor agents that could bind specific cancer cell surface glycoconjugates (Ueno et al., 2000) *e.g.* a typical legume lectin with specificity towards sialic acid purified from *Phasoleus coccinus* L.(Phasoleus Multiflorus wild) seeds possess a remarkable antiproliferative activity.

Lotus corniculatus commonly known as Bird's foot trefoil belongs to a genus that contains many dozen of species distributes worldwide *e.g. Lotus aboriginus* (Rosy Bird's foot trefoil), *Lotus angustissimus* (Slender Bird's foot trefoil) and *Lotus argophyllus* (Silver Bird's foot trefoil). *Lotus corniculatus* is a species of the leguminosae family. *Lotus corniculatus* can fix nitrogen through the root nodules making it useful as cover crop. It has been studied for its flavonoid content (Jay and Ibrahim 1986; Sarelli et al., 2003; Reynaud and Lassignol, 2005). *Lotus corniculatus* is known for its medicinal values. The flowers are antispasmodic and sedative (Chiej 1984). The root is carminative and febrifuge (Duke and Ayensu 1985). Although *Lotus corniculatus* belongs to legume family, none of the Lotus species have been investigated for the lectins.

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We therefore isolated and purified a lectin from *Lotus corniculatus* seeds with two simple chromatographic steps. Some physical and chemical properties such as thermo stability, pH stability and carbohydrate specificity of the lectin have been studied. Biological activities of the *Lotus corniculatus* lectin were also studied. The lectin possessed antiproliferative effects on some tumour cell lines by inducing apoptosis and also possesses anti metastatic effects.

Materials and methods

Purification of Lotus corniculatus lectin

The pods of *Lotus corniculatus* were obtained from the surroundings of Kashmir University and authenticated at centre of plant taxonomy (COPT), Department of Botany, Kashmir University, India. The seeds (60 g) were crushed and powdered in liquid nitrogen, dissolved in 300 ml of 10 mM Tris–HCl buffer (pH 7.6), followed by centrifugation at 3000 × *g*, at 4 °C, for 30 min. Ammonium sulphate was added to the supernatant to 40–60% saturation. The precipitate was resuspended in 10 mM Tris–HCl buffer and dialysed extensively overnight at 4 °C. The sample was then adjusted to 10 mM Tris–HCl (pH 7.6) by adding Tris–HCl buffer (1.5 M, pH 7.6). The sample was then applied to AF-BlueHC650M (Toyopearl) column (18 cm × 5 cm) that had been equilibrated with 10 mM Tris–HCl buffer (pH 7.6). Unabsorbed proteins were eluted with the starting buffer. The column was washed with 1 M NaCl in10 mM Tris–HCl buffer to elute the bound protein. The active fractions were pooled, dialysed extensively against double distilled water and concentrated by Ultra filtration using 10 kDa cut-off membrane. The purified lectin was dissolved in sterile distilled water (16 mg/ml). The solution was subjected to Gel filtration on Sephadex G-100 column. A major absorbance peak that contained purified LCL was obtained.

Assay of hemagglutination activity

In a 96-well microtitre U-plate, a serial two fold dilution of the test sample (50 μl) in phosphate buffer saline (PBS) (pH 7.2) was performed. A 2% rabbit red blood cell suspension (50 μl) in PBS was added to the sample. The mixture was incubated at room temperature until the red blood cells in the blank (without protein sample) had fully sedimented and appeared as a red spot at the bottom of the well. Presence of agglutinated red blood cells in the wells indicated hemagglutinating activity. One hemagglutination unit is the reciprocal of the highest dilution of the lectin sample inducing hemagglutination. Specific activity is the number of hemagglutination units per mg protein (Yagi et al., 2002).

Carbohydrate binding specificity

The carbohydrate specificity was investigated by observing the inhibition of the lectin induced hemagglutination by various sugars namely D-glucose, D-galactose, D-mannose, fructose, lactose, maltose, sucrose, ribose, xylose, manitol and sugar derivatives like N-acetyl galactosamine and N-acetyl glucosamine. The inhibition assay was performed in 96-well plate. Different dilutions of the above sugars (final volume 20 μl) were added to the wells in which agglutination was performed. To each dilution, 20 μl of purified lectin was added. The mixture was incubated at room temperature for 1 h after which 80 μl of 2% suspension of erythrocytes was added to each well. The minimum concentrations of each sugar capable of fully inhibiting agglutination after 1 h at room temperature were noted.

Molecular mass determination

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 8% separating gel and a 5% stacking gel was performed. After electrophoresis, the gel was stained with Coomassie brilliant blue and destained with 10% acetic acid overnight (Laemmli and Favre, 1973).

Gel permeation chromatography was carried out using Sephadex G-100 column calibrated with molecular mass standard.

Protein concentration determination

The protein concentration was determined by the method of (Lowry et al., 1951) using BSA as the standard protein.

Effect of temperature, pH on lectin-induced hemagglutination

Thermal stability of LCL was monitored in the range of 10–100 °C by incubating the lectin for 60 min at the respective temperatures, followed by cooling on ice and determination of agglutination activity under standard conditions.

The pH dependence of the lectin was determined by incubating 50 μg of LCL with buffers in different pH:HCl: pH 0–1, glycine/HCl (pH 2–3), 0.05 sodium acetate/acetic acid (pH 4–5), potassium phosphate (pH 6–7), Tris–HCl (pH 8–9) and glycine-NaOH (pH 10–11), pH 11–12 $NaHCO_3$ and pH 13–14, NaOH, for 5 h at 25 °C and pH was adjusted to 7.2 just prior to hemagglutination assay.

Tumour cell lines and culture conditions

Human leukemic cell line (adherent Type) (THP-1), lung cancer cell line (HOP62) and colon cancer cell line (HCT116) were obtained from American Type Culture Collection. All the cell lines were cultured in RPMI 1640 (Sigma) medium which contained FCS 10% (Sigma), 100 U/ml of pencillin (Sigma) and 100 μg/ml of streptomycin (Sigma). Cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The cells were fed every 2–3 days, and at confluency they were harvested with 0.05% (w/v) trypsin–EDTA (Sigma) and sub-cultured in identical medium.

Sulphorhodamine B (SRB) assay

Human leukemic (Adherent type) (THP-1), Lung cancer (HOP62), and colon (HCT116) cancer cells from American Type Culture Collection were adjusted to a cell density of 5×10^4 cells/ml in RPMI medium. The cells (100 ml) were seeded onto the wells of a 96-well plate and incubated overnight. Different concentrations of *Lotus corniculatus* lectin (100 ml, final concentrations at 30–100 μg/ml) were added to the wells followed by incubation for 48 h. The plates was taken out from the incubator after 48 h of adding the sample. To stop the reaction, 50 μl of chilled 50% TCA (trichloroacetic acid) to each well of the plate was added, making final concentration to 10%. The plate was incubated at 4 °C for 1 h to fix the cells attached to bottom of the wells. The plate was washed 5–6 times with distilled water. Plate was air-dried. 100 μl of SRB dye (0.4% in 1% acetic acid) was added to each well of the plate and the plate left at room temperature for 30 min. The plate was washed with 1% acetic acid after 30 min. The plate was again air-dried. 100 μl of tris buffer (10.5 M) was added to each well. The plate was shaken gently for 10–15 min on a mechanical shaker. The optical density was recorded with ELISA reader at 540 nm.