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Pea lectin inhibits growth of Ehrlich ascites carcinoma cells by inducing apoptosis and G_2/M cell cycle arrest *in vivo* in mice

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ABSTRACT

Pea (*Pisum sativum* L.) lectin is known to have interesting pharmacological activities and of great interest on biomedical research. In the current research pea lectin was purified followed by ion exchange chromatography on DEAE column and affinity chromatography on glucose-sepharose column. The lectin shown 11.7–84% inhibitory effect against Ehrlich ascites carcinoma (EAC) cells at the concentration range of 8–120 μg/ml in RPMI 1640 medium as determined by MTT assay. Pea lectin was also shown 63% and 44% growth inhibition against EAC cells *in vivo* in mice when administered 2.8 mg/kg/day and 1.4 mg/kg/day (i.p.) respectively for five consequent days. When Pea lectin injected into the EAC bearing mice for 10 days its significantly increased the hemoglobin and RBC with the decreased of WBC levels toward the normal. Apoptotic cell morphological change of the treated EAC cells of mice was determined by fluorescence and optical microscope. Interestingly, cell growth inhibition of the lectin was significantly reduced in the presence of caspase inhibitors. Treatment with the lectin caused the cell cycle arrest at G_2/M phase of EAC cells which was determined by flow cytometry. The expression of apoptosis-related genes, *Bcl-2*, *Bcl-X* and *Bax* was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR). Intensive increase of *Bax* gene expression and totally despaired of *Bcl-2* and *Bcl-X* gene expression were observed in the cells treated with Pea lectin for five consecutive days.

Introduction

Lectins are proteins or glycoproteins from non-immune origin that specifically recognize cell surface molecules with at least two binding sites to carbohydrates. They are found in all kinds of organisms, including animals, plants, fungi, bacteria and viruses. Plant lectins represent a unique group of proteins with potent biological activities, such as agglutination, toxicity, anti-proliferation of cancer cells as well as having anti-fungal and anti-bacterial activities (Kabir et al. 2011a,b, 2012; Liu et al. 2010; Sitohy et al. 2007). They can specifically recognize and bind to various sugar structures, and thus trigger several important cellular processes (Sharon and Lis 1989; Sharon 2007). Several plant lectins have been shown to have antitumor activity and induce apoptosis in a series of tumor cell lines (Kabir et al. 2011a,b, 2012; Kim et al. 2000; Liu et al. 2010; De Mejía and Prisecaru 2005).

The lectin from *Pisum sativum* L. is commonly known as Pea lectin. The amino acid sequence and the crystal structure of the lectin were determined (Einspahr et al. 1986). Pea lectin highly agglutinated human, rabbit and rat erythrocytes but weekly agglutinated chicken erythrocytes. It was also reported that Pea lectin inhibited the growth of *Aspergillus flavus*, *Trichoderma viride* and *Fusarium oxysporum* (Sitohy et al. 2007). The lectin also inhibited the migration of cardiac mesenchymal cells (Sumida et al. 1997). *In vitro* binding assays, *Pisum sativum* agglutinin showed high affinity for Ehrlich ascites tumor (Kojima and Jay 1986). In the current article we have focused on the Pea lectin induced apoptosis in Ehrlich ascites carcinoma (EAC) cells and assessed its effect on the expression of different apoptosis-related genes.

Materials and methods

Chemicals and reagents

Sepharose-4B, Hoechst 33342, propidium iodide and RPMI 1640 medium were purchased from Sigma (USA). Fetal calf

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serum and penicillin-streptomycin from Invitrogen (USA), z-DEVD-fmk and z-IETD-fmk from Biovision (USA). Trypan blue and all other chemicals/reagents were of analytical grade. The *Pisum sativum* seeds were collected from the local market.

Purification of Pea lectin

50 g of *Pisum sativum* seeds were homogenized in distilled water (DW) and then centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was dried by freeze dryer and stored at 4 °C. Freeze dried powder was dissolved in 10 mM of Tris–HCl buffer pH 7.8 and dialyzed against the same buffer and centrifuged at 10,000 rpm for 10 min. The supernatant was then applied on DEAE cellulose column previously equilibrated with 10 mM of Tris–HCl buffer pH 7.8. Unbound fraction was collected and pH was adjusted to 8.2 by 1 M Tris–HCl buffer, pH 8.4. Then the fraction was added to 20 ml of glucose–sepharose gel (glucose linked to epoxy-activated sepharose-4B) previously equilibrated with 10 mM of Tris–HCl buffer pH 8.2. NaCl and $CaCl_2$ salts solutions were added to the gel to the final concentration of 0.15 M and 2 mM respectively and kept overnight at 4 °C with shaking for effective binding of lectins. A column was prepared with the gel and the unbound proteins were washed out by 10 mM of Tris–HCl buffer saline (TBS) pH 8.2 containing 1 mM of $CaCl_2$. Lectin was eluted from the column by TBS containing 20 mM of EDTA or 1 M urea. The eluted fraction was dialyzed against 10 mM of Tris–HCl buffer containing 1 mM of $CaCl_2$ and the homogenity was checked by using SDS-PAGE in 15% polyacrylamide gel as described by Laemmli (1970). Bovine serum albumin (Mr. 67 kDa), Ovalbumin (Mr. 45 kDa), Carbonic anhydrase (Mr. 29 kDa), Trypsin inhibitor (Mr. 20 kDa) and Lysozyme (Mr. 14.6 kDa) were used as marker proteins. Protein concentration was determined by the Lowry's methods using BSA as the standard protein (Lowry et al. 1951).

Hemmaglutination activity

The hemagglutination assay of Pea lectin was performed in 96-well microtiter U-bottomed plates as described by Kabir et al. (2012). Hemagglutinating inhibition was studied by adding a serially dilution of the following sugars (D-mannose, D-glucose, D-galactose, L-arabinose, D-melibiose, D-maltose, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetyl-D-glucosamine and methyl-α-D-galactopyranoside) at the final concentration of 200 mM.

Determination of temperature and pH stability

Pea lectin (0.5 mg/ml in Tris–HCl buffer saline, pH 7.8) was heated in a water bath for 30 min at different temperatures from 40 to 80 °C and cooled to room temperature (30 °C) to examine the thermo stability. Then 50 μl of Pea lectin was serially diluted with an equal amount of hemagglutination buffer, pH 7.8 and the hemagglutination titer was performed as described by Kabir et al. (2012). The non-heated lectin sample was used as a control, which denoted 100% activity. The pH stability of Pea lectin was examined by incubating the protein solutions (0.5 mg/ml) against 0.1 M sodium acetate (pH 3.0, 4.0, 5.0 and 6.0), 0.1 M phosphate buffer (pH 7.0), 0.1 M Tris–HCl (pH 8.2) and 0.1 M glycin–NaOH (pH 10.0 and 11.8) for 8 h at room temperature. After 8 h the lectin solutions were dialyzed against 10 mM Tris–HCl buffer, pH 7.8 containing 0.9% NaCl for 12 h.

Treatment with urea and divalent cation

Pea lectin (1 mg/ml) in 0.1 M Tris–HCl buffer saline was incubated at 20 °C with 2 and 4 M of Urea for 2 h. Lectin in the same buffer without denaturants was used as a control and its activity was considered as 100%. Pea lectin (1 mg/ml) was incubated with 20 mM of EDTA for 4 h at 30 °C and then dialyzed against Tris–HCl buffer pH 7.8. Finally hemagglutination activity was checked in the presence and absence of 10 mM $CaCl_2$ to determine the dependency on divalent cations.

Cell culture

EAC cells were cultured in RPMI-1640 medium containing D-glucose, 2 mM L-glutamine supplemented with 10% fetal calf serum, and 1% (v/v) penicillin–streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT colorimetric assay

MTT colorimetric assay was used to determine EAC cells proliferation. Cells (5×10^5 in 200 μl RPMI 1640 media) were plated in the 96-well flat bottom culture plate in the presence and absence of different concentrations (8–120 μg/ml) of Pea lectin and incubated for 24 h at 37 °C in CO_2 incubator. After carefully removing the aliquot, 180 μl of PBS and 20 μl of MTT were added and further incubated for 8 h at 37 °C. The aliquot was removed again and 200 μl of acidic isopropanol was added into each well and incubated again at 37 °C for 1 h. Subsequently, absorbance was read at 570 nm using titer plate reader. The following equation was used to calculate the cell proliferation inhibition ratio:

$$\text{Proliferation inhibition ratio}\,(\%) = \frac{(A - B) \times 100}{A}$$

where A is the $OD_{570\,nm}$ of the cellular homogenate (control) without Pea lectin and B is the $OD_{570\,nm}$ of the cellular homogenate with Pea lectin.

Animals and ethical clearance

Adult Swiss albino mice which were used throughout the study were collected from the International Center for Diarrheal Diseases Research, Bangladesh. This research work was approved by the Institutional Animal, Medical Ethics, Bio-safety and Bio-security Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (286/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Determination of Ehrlich ascites carcinoma (EAC) cell growth inhibition in mice

EAC cells were propagated intraperitonealy in our departmental laboratory biweekly and the cells were collected from a donor Swiss albino mouse bearing 6–7 days old ascites tumors. The cells were adjusted to 3×10^6 cells/ml with the dilution of normal saline and counting by using haemocytometer. The viability of tumor cells were observed by trypan blue dye (0.4%) exclusion assay. EAC cells (in 0.1 ml saline) showing above 99% viability were injected intraperitonealy into each Swiss albino mouse. The mice were randomly distributed after 24 h into three groups with at least six mice per group. Two groups of mice were treated intraperitonealy with Pea lectin at the concentrations of 2.8 mg/kg/day (70 μg/mouse/day) and 1.4 mg/kg/day (35 μg/mouse/day) for five days. Mice in each group were sacrificed on 6th day of the lectin