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Anti -Hcv Lectin from Egyptian *Pisum sativum*

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Abstract: Lectins are carbohydrate binding proteins expressed in plants, animals and microorganisms and have been used to probe the surface properties of a wide range of prokaryotic and eukaryotic cells. The implication of some lectin molecules in several physiological processes has been claimed. The aim of this work is to purify the lectin from Egyptian pea (*Pisum sativum*) seeds and study its biochemical characterizations and potential as pharmaceutical compound. Egyptian Pea lectin seeds was purified by ion exchange chromatography (DEAE column) followed by Sephadex G 100 Column. Purified lectin showed Hemagglutinating activity with human and rabbit erythrocytes and appeared as one band of 32 kDa on SDS-PAGE gel, also amino acid analysis showed high content of glutamic and aspartic acids but very low levels of sulfur amino acids. The purified Egyptian pea (*Pisum sativum*) lectin showed high effect on HCV replication at concentrations of 20, 40 and 80 µg. Therefore, pea's lectins might consider as one of the natural compounds that can be used in designing a safe and effective anti HCV therapy.

Key words: Egyptian *Pisum sativum lectin*, purification, characterization, activity, pharmaceutical properties, anti HCV.

INTRODUCTION

Lectins are a group of non-enzymatic carbohydrate-binding proteins present in plants, animals and microorganisms. They can bind to glycans of glycoproteins, glycolipids or polysaccharides with high affinity Etzler, (1985) and Minnick *et al*. (1986). Lectins have been found in numerous plant species, but the best characterized are from the family Leguminosae, making up about 60% of the well-known lectins, Vasta and Pont-Lezica (1990) and Vasta (1991). In plants, number of abundant proteins accumulates, where; some of these play an important role in the nutrition of the seedling, whereas others, such as the lectins and the inhibitors of digestive enzymes of mammals and insects, Janzen (1976) and Pendland *et al*. (1988), are considered to be plant defense proteins, Joaquin Maarten (1989). Inhibitors of á-amylases and/or proteases have been classified in nine families according to their sequence identity, Garcia-Olmido *et al*. (1987). At least two of these families, the Kunitz trypsin inhibitor and the Bowman-Birk trypsin/chymotrypsin inhibitor, occur in legume seeds. Legume seeds also contain proteins belonging to the family of lectins, carbohydrate-binding proteins, lierner et.al (1986). Plant lectins are widely used as research tools, Lis and Sharon (1986) due to their specific carbohydrate-binding properties. Some seed lectins are known to be toxic to animal predators, Gatehouse *et al*. (1848), Osborn *et al*. (1988) and Puzstai *et al*. (1979). Legume lectins have been amongst the most useful and several proteins from this family have been crystallized and their structure determined. Although the number of subunits in these lectins varies, the individual subunits all have a jelly-roll tertiary structure consisting of a flat six-stranded [3-sheet and a curved seven-stranded [3-sheet interconnected by loops of various lengths, Banerjee *et al*. (1996). Sequence hypervariability in the loops which constitute the binding site is the basis of the wide range of carbohydrate specificities. The availability of crystal structures allows the possibility of rational site-directed mutagenesis to alter specificity and/or affinity, Paul *et al*. (1997). Mannose-specific lectins are widely distributed in higher plants and are believed to play a role in recognition of high-mannose type glycans of foreign micro-organisms or plant predators. Structural studies have demonstrated that the mannose-binding specificity of lectins is mediated by distinct structural scaffolds. The mannose/glucose-specific legume (e.g., Con A, pea lectin) exhibit the canonical twelve-stranded-sandwich structure, Annick *et al*. (2001). Plant lectins have been widely used to characterize variations in carbohydrate structures found on cell surface glycoproteins and glycolipids, Sharon and Lis, (1989). They have also been used, sometimes in combination with other methods, to distinguish isoenzymes that differ only in carbohydrate content, Rosalki and Foo (1994). On the other hand, lectins are also produced by mammalian cells especially the mannan binding lectin, which is a pattern reorganization molecules of the innate immune system that also bind to sugars on the surface of invading viruses and bacteria, Brown *et al*. (2007). Another type of important

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mammalian cell lectins that was identified recently is type 2 C-type lectin which is expressed on liver sinusoids and showed to have high affinity to bind to HCV envelop glycoprotein E2, Ingram *et al*. (1984) and Lai *et al*. (2006). Galectin-1 is another endogenous lectin found at peripheral lymphoid organs and inflammatory sites and has shown immuno regulatory activity *in vivo* in experimental models of autoimmunity and cancer and has a novel role in regulation of monocyte and macrophage physiology, Komano and Natori (1985), Rechards and Ratcliffe (1990), George *et al*. (1990) and Barrionuevo *et al*. (2007). It was confirmed that certain N-acetylgalactosamine and N-acetylglucosamine binding lectins from diverse plant species have antibiotic effects on the development of Callosobruchus maculatus. (Cowpea weevil), larvae, Barbieri *et al*. (1993) and Endo *et al*. (1987). The aim of this study is to purify lectin from Egyptian peas (*Pisum sativum*) by ion exchange and gel filtration chromatography, characterize and test its potential as antiviral agents.

MATERIALS AND METHODS

Purification of Pea (Pisum Sativum) Seed Lectin:

Pea (*Pisum sativum*) seed were obtained from Egyptian Agricultural Research Center Cairo, Egypt. Mature seeds of pea lectin were ground and the fine powder of seeds defatted by Chloroform: according Folch method, Folch and Stenly (1957). The proteins were extracted by 0.15M NaCl, 10 % w/v for 1 hour at room temperature and then the mixture centrifuged at 5000 x g for 20 min at 4°C. The supernatant was filtered and dialyzed against distilled water and lyophilized, Richard *et al*. (1990). The crud extract was dissolved in 0.05M Tris-HCl, pH 8.0 and applied to Diethylaminoethyl DEAE-Sepharose column (1.8x 27 cm) (Amersham Pharmacia) previously equilibrated with 0.05M Tri—HCl pH8.0. the lectin was eluted from DEAE-Sepharose column with gradient NaCl (0-0.5M) in 0.05 M Tris-HCl., pH 8, at flow rate 25 ml /hr and the elution profile was monitored at 280 nm. Fractions containing hemagglutinating activity were applied to a Sephadex G100 previously equilibrated with 0.05M Tris-HCl, pH 8.0, Damico *et al*. (2003), Luzia *et al*. 2004) and Richard *et al*. (1990).

Protein Concentration:

Protein concentrations were determined by Bradford method, Bradford (1976) against Bovine serum albumin as standard. Series of Bovine Serum Albumin (BSA) were prepared in known concentrations as standard solutions. One volume of Bradford reagent was diluted with four volume of distilled water and mixed well, 100 ul of each standard and *Pisum salivum* lectin (*PSL)* samples were added to separate test tubes. To each test tube 5.0 ml of diluted reagent were added and mixed well. All tubes were incubated 5-15 min. in the same conditions and then transferred to the disposable cuvettes and the absorbance of the samples and standard were recorded at 595 nm. From standard curve the concentration of protein in the samples were calculated.

Hemagglutination Assay:

Hemaglutinating activity was conducted according to Damico *et al*. (2003) and luzia pando 2004 as follow, 50µl of *Pisum sativum* lectin (*PSL*) solution were incubated with 50µl 2% of erythrocyte suspension in phosphate buffer saline (PBS) in a microtiter plates and incubated for 1 hr at room temperature. Hemagglutinating activity was expressed as the reciprocal of the highest dilution producing agglutination. The hemagglutinating activity was assayed against human (A, B, AB and O), Rabbit and Chicken blood.

The inhibitory activity of carbohydrate on Hemagglutination was conducted by incubating 50µl of serial dilutions of sugar solution (100mM) with 50µl of lectin solution. After 30 min. 50 ul of a 2 % suspension of erythrocytes were added and the titer was incubated for 1 hr at room temperature. The lowest concentration of sugars which capable of inhibiting Hemagglutination was calculated based on the dilution curves.

Electrophoresis Analysis:

SDS-PAGE was carried out according to Laemmli (1970) using 12.5 % separating gel and 5% stacking gel as follow: glass plates of BioRad mini gel were cleaned with detergent and alcohol. The cassettes with spacer and comb were assembled and inserted in gel pourer. The 12.5 % separating gel was prepared, mixed well gently to avoid air bubbles and poured to the cassettes. Gently added isopropanol to ensure level surface and allowed 30 min to polymerization, then the isopropanol was remove and the gel washed with water. Stacking solution was poured and the 0.75 cm comb was added and the allowed 30 min to polymerization, then the comb was removed and the gel was transferred to electrophoresis tank and the running buffer was added to the tank. The *PSL* samples (20ul) and marker (5ul) were loaded to the gel and connected to the power supply unit at 80 volt. When dye approached end of gel the cassette was dismantled and gel processed to staining with Comassie blue and distaining.

Carbohydrate Content:

The sugar content of the purified *PSL* was determined by phenol-sulfuric acid method, Dubois *et al*. (1956) with serial concentration of glucose as standard (0.00, 10, 30, 50, 70, 90 ug/ml). 3mg sample was

added to 250 ml bottle, 200 ml of distilled water and 20 ml of 25% HCl were added. The solution mixed well for 5 seconds. The bottle placed in water bath at 100°C for four hours, then removed and cool to room temperature in 1°C water bathe for 20 min. 5.5 ml 40% NaOH was added to the bottle and mixed well for 5 S. mixture filtered by wattman No 4 Filter paper into 500 ml glass filter flask, then the solution transferred to 500 ml volumetric flask and filled to 500 ml with distilled water. To general plate assay 96 well, 25 ul of the sample and standard were transferred and added 25 ul 5% phenol and vortex for 30 Sec at minimum speed (all samples and standard assayed in triplicates). Plate was placed on crushed ice and 125 ul of conc. sulfuric acid were added to each well and vortex again for 30 sec. The plate sealed well with plastic zipper bag and worm in water bathe at 80°C for 30 min. plate was red at 490 nm on Elisa reader and the total carbohydrate concentration was determined as glucose from standard curve.

Amino Acid Composition:

The amino acid composition of purified *PSL* was carried out on Sykam® amino acid analyzer 433 according to Winder and Eggum (1966). 1mg of *PSL* was weighed into the hydrolysis vessel which, which was then fitted together and immersed in a dry ice ethanol cooling mixture; when the solution has frozen, the vessel was evacuated to 7.9Pa. To remove traces of air dissolved in the acid, the hydrolysis vessel was taken from the cooling mixture and left, after the pump has been switches off, for the frozen solution to melt slowly. As soon as bubbles begin to emerge from viscous solution, the hydrolysis vessel was immediately reimmersed in the cooling mixture and evacuation continued until 7.9Pa. The hydrolysis vessel was then isolated and placed in an oven at 110°C for 22 h. after the hydrolysis the vessel was cooled to room temperature and then connected with rotary to distilled the hydrochloric acid at 40°C. the hydrolysate was dissolved in 1ml of 0.2 N sodium citrate buffer pH 2.2 and the insoluble matter was filtered. 30ul sample is chromatographed on the column with flow rate 20 ml/h. the absorbance of the ninhydrin/eluate mixture was monitored at 440 and 570 nm.

Cell Culture:

HepG2 cells were washed twice in RPMI1640 media supplemented with 200 lM L-glutamine and 25 lM HEPES buffer; N-[2-hydroxyethyl] piperazine-N9-[2-ethanesulphonic acid] (all chemicals and media are from Cambrex,).

The cells were suspended at $2x10^5$ cells/mL in RPMI culture media (RPMI supplemented media, 10% fetal bovine serum (FBS); GIBCO-BRL). The cells were left to adhere on the polystyrene 6-well plates for 24 h in an incubator (37°C, 5% CO2, 5% humidity). The cells were washed twice from debris and dead cells by using RPMI supplemented media, El-Hawash *et al*. (2006).

Qualitative in-vitro Anti-HCV Screening:

Qualitative in-vitro anti-HCV screening HepG2 cell culture was prepared, then infected with 2% HCV-infected serum in RPMI culture medium containing 8% FBS. Each of the tested compounds was added at concentrations of 10, 20, 40 and 80 µg/ml. Positive and negative control cultures were included. After 96 hr of incubation at 37°C, 5% CO2 and 95% humidity another dose of the test compound was added. The cells were incubated for another 96 h at 37°C, 5% CO2 and 95% humidity, followed by total RNA extraction. The positive strand and its replicating form (negative strand) were detected by RT-PCR using HCV specific primers to the 5'-untranslated region of the virus El-Hawash *et al*. (2006).

RNA Extraction and RT-PCR of HCV RNA:

Total RNA was extracted from HepG2 HCV-infected cells as well as from HepG2 infected cells that are treated with the test compounds using the method described by El-Awady *et al*. (1999). The complimentary DNA (cDNA) and the first PCR reaction of the nested PCR detection system for the HCV RNA was performed in a 50 µl volume single-step reaction using the Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA), PCR of the cDNA was performed according to the method described by El Awady *et al*. (1999).

RESULTS AND DISCUSSIONS

Lectin Purification from Pisum Sativum Seeds:

A lectin was purified from Egyptian Peas (*Pisum sativum)* seeds using two steps, the first step by Ion Exchange Chromatography on DEAE column and the elution profile of lectin obtained by linear gradient of NaCl (0-0.5M) (Fig 1). It's revealed that the lectin bound with DEAE and all other proteins eluted in wash step by 50mM Tris-HCl buffers and followed by gradient NaCl which elute lectin from DEAE column. The first fraction which showed the hemagglutinating activity was purified by Sephadex G-100 Column, the elution profile obtained one main peak showed hemagglutinating activity with human erythrocytes (A, B, AB, O) and Rabbit erythrocytes.

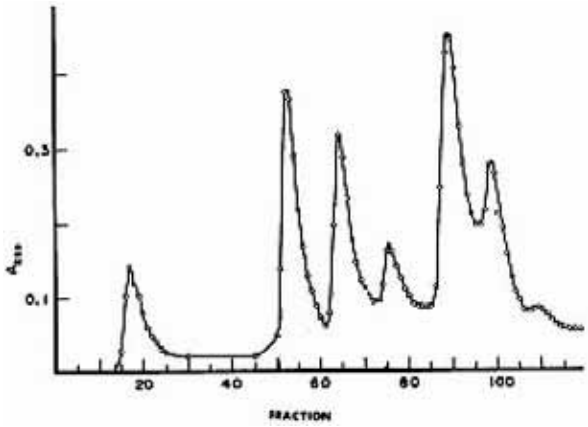

Fig. 1: Ion exchange chromatography of crude extract of Pea seeds on a DEAE-cellulose column (1.5 x 28 cm) equilibrated with 50mM Tris-HCl buffer (pH 8) and eluted with a linear gradient (0-0.5M NaCl) containing 50mMTris-HCl pH 8. Fractions of 4 ml were collected at a flow rate of 24 ml/hr and monitored at 280nm.

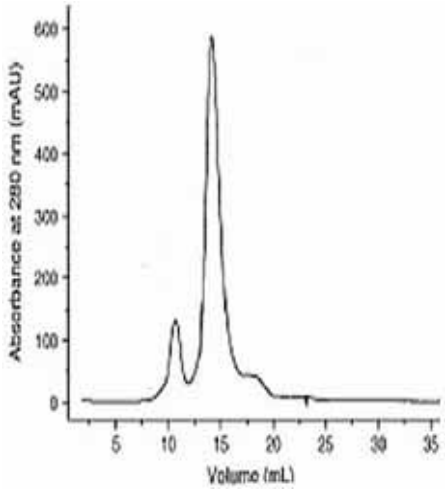

Fig. 2: Gel filtration of the on Superdex-G100 equilibrated and eluted with 50 mM Tris-HCl buffer pH 8 at 0.5 ml/min collected on 1.5-ml fractions. The eluate absorbance was monitored at 280 nm.

Protein Concentration:

Protein concentration in purified *PSL* was determined according to Bradford method using BSA as standard and the protein concentration was 42.6 mg/ml.

Electrophoresis Analysis:

The purified lectin showed a single polypeptide band around 32 kDa on SDS-PAGE. And the molecular weight of native lectin was estimated by gel filtration and found that, its molecular weight was around 120 kDa (Data not shown). *Pisum sativum* lectin purified from seeds by ion exchange chromatography yielded pure one single band on SDS-PAGE with molecular weight 32 KDa. This is indicating that, the pea seeds lectin consists of four subunits were bound together to yield a tetramer and each subunit has a carbohydrate binding sites. These results agreed with Damico *et al*. 2003 and luzia pando 2004 who's mentioned that, lectins from legumes consists of two or four subunits with relative molecular weight of 30 kDa and one carbohydrate binding site per subunit (Figure 3).

Hemagglutinating Activity and Carbohydrate Specificity:

Hemagglutinating activity of Egyptian pea lectin was conducted and the results showed that, Pea lectin agglutinated the human (A, B, AB and O), Rabbit erythrocytes but not showed any hemagglutinating activity with Chicken erythrocytes. Titration of the pea lectin with human, rabbit and chicken erythrocytes were 92µg/ml for all types of human erythrocytes, 55µg/ml for rabbit erythrocytes.

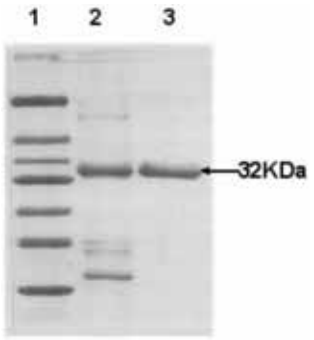

Fig. 3: SDS-PAGE of crude and purified Pea seed lectin, Commassie stained; 1) Mol wt markers from top to bottom, 66, 45, 36, 29, 24, 20.1 and 14.2 kDa respectively; 2) Crude extract; 3) Purified Lectin

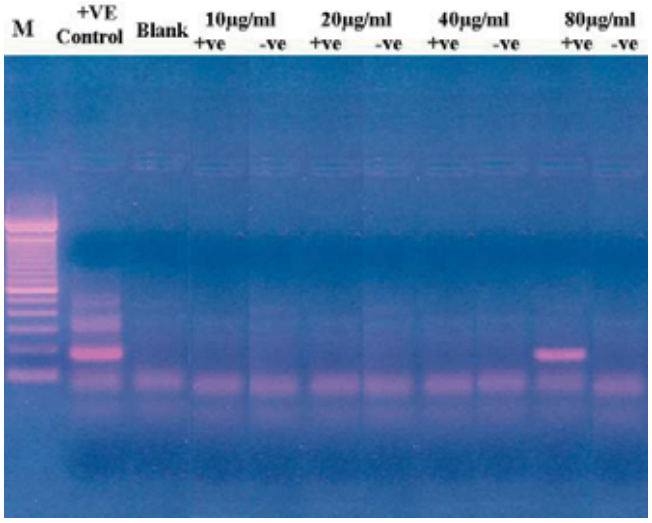

Fig. 4: RT-PCR of HCV RNA (+) and (-) strands in the presence of Lectin. Lane 1: molecular weight 100bp marker (M). Lane 2: +ve control and lane 3: blank. Lanes 4, 6, 8, 10 represent the effect of Lectin (10, 20, 40, or 80 ìg/ml respectively) on the HCV RNA (+) strand. Lanes 5, 7, 9, 11 represent the effect of Lectin (10, 20, 40, or 80 ìg/ml respectively) on the HCV RNA (-) strand after 8 days of treatment.

The carbohydrate binding specificity of the pea lectin was carried out by incubating the pea lectin with different sugars in the hemagglutinating assay. The inhibition of hemagglutinating activity was determined by increasing of concentration of different sugars (Table 1). From the results illustrated in table 2 the pea lectin specific with D-glucose and D-mannose. On the other hand, the other sugars didn't appear any effect in concentrations less than 100 ug/ml. It is revealed that the Pea lectin specific with mannose and glucose in its carbohydrate binding sites.

Table 1: Inhibition of hemagglutinating activity of pea lectin with different sugars.

Sugar	Inhibitory Concentration (mM)
D-Glucose	25
D-Mannose	28
D-Fractose	>100
D-Fucose	>100
D-arabinose	>100
D-Xylose	>100
D-Galactose	>100

Sugar Content:

The total sugar content of pea lectin was determined by phenol-sulfuric acid method, Dubois *et al*. (1956) and found that, the pea lectin containing 8.75 % as total sugars.

Table 2: Amino acids composition of pea (Pisum *sativum*) seed lectin.

Amino Acid	Conc. µg/ ml
Aspx	22
Glux	25
Ser	13
Thr.	11
His	3
Tyr	7
Pro	-
Val	-
Meth	-
Leu	12
Isolu	9
Phe	13
Thr	25
Cys	-
Arg	15

Amino Acids Composition:

The pea lectin showed a high content in alanin, glycin, asparagin, glutamine and poor in sulfuric amino acids (Table 2). These results agreed with Luzia *et al*. (2004) and generally like most legume lectins are rich in Aspartic, asparagin, glutamic, glutamine and poor in sulfuric amino acids.

Qualitative in-vitro Anti-HCV Screening:

The purified Egyptian pea lectin showed significant effect on HCV replication in the In Vitro system used with concentrations of 10, 20 and 40 µg/ml but showed negative result when 80µg/ml used. This considers a much promised results in pharmaceutical properties of lectins.

The purified Egyptian pea lectin investigated for their In Vitro action as anti-HCV using the hepatocellular carcinoma HepG2 cell line infected with the hepatitis C virus. Monitoring of the HCV viremia before and after antiviral therapy through the detection of viral (+) and/or (–) RNA strands by the use of qualitative RT-PCR has become the most frequently used, reliable and sensitive technique. Recently, it has been reported that the detection of the (–) strand HCV-RNA using the RT-PCR is a very important tool for understanding the life cycle of the HCV. It provides a reliable marker for the diagnosis of HCV and monitors the viral response to antiviral therapy, Sharon and Lis (1987) and Sharon and Lis (1989). Based on these facts, the adopted method in the present study contributes to the simultaneous detection of the (+) and/or (–)-HCV-RNA strands in HepG2 hepatoma cells infected with HCV. Inhibition of viral replication was detected by amplification of viral RNA segments using the RT-PCR technique, both in the cultivated infected cells alone (as a positive control) and in the presence of variable concentrations (10, 20, 40 and 80 µg/ ml) of the test compound at optimal temperature. The test compound is considered to be active when it is capable of inhibiting the viral replication inside the HCV infected HepG2 cells, as evidenced by the disappearance of the (+) and/or (–)-strands viral RNA-amplified products detected by the RT-PCR (compared to positive control).

Finally we can conclude that, the purified lectin from Egyptian *Pisum sativum* might consider as one of the natural compounds that can be used in designing a safe and effective anti HCV therapy.

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