ISOLATION AND IDENTIFICATION OF LECTIN GENE IN LICORICE, *Glycorrhiza glabra* L., PLANT IN EGYPT

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Licorice (*Glycrrihiza glabra* L.), family: *Fabaceae* is a traditional medicinal herb which grows in various parts of the world (Vivek *et al.*, 2007). Ancient Egyptian healers began using roots of licorice (*Glycyrrhiza glabra*) 4000 years ago. Considered to be non toxic and with the lowest side effects among plants (Dhiman and Chawla, 2005), Licorice (*Glycrrihiza gabra* L.) roots and rhizomes are extensively used in herbal medicines for their emollient, anti-inflammatory, anti-viral, anti-allergic, anti- oxidant, gastro-protective and anti-cancerous properties (Kitagawa, 2002). A root component of the licorice plant being generally regarded as the major biologically active principle and have been widely used in food, pharmaceutical and confectionery industries because of the presence of several bioactive compounds such as glycyrrhizin (~16%), different sugars (up to 18%), flavonoids, saponoids, sterols, starches, amino acids, gums and essential oils (Kitagawa, 2002). It has been used in cough, suppression gastric, ulcer treatment, treatment of liver disease and now for cancer sores (Nitalikar *et al.*, 2010).

Lectins can be defined as carbohydrate binding protein which binds reversibly to specific mono- di- or oligosaccharides without altering the structure of bound ligand (Jones, 1995). They are ubiquitous proteins and have been isolated from plants, animals and microorganisms (Shibamoto and Bjeldanes, 1993). The lectins are commonly called hema-agglutinins that were introduced later. Today, the term plant lectin is used to denote all plant proteins possessing at least one-catalytic domain which binds reversibly to a specific mono- or oligosaccharide (Peumans and Van Dame, 1995).

Most convenient sources of lectins are in plant. Plant lectin has been attracting much attention because of their ease to isolation and their usefulness as reagents for glycol conjugation in solution and on the cell. There is growing evidence that many crops are naturally resistant to pests

because their constituent lectins interact strongly with the surface glycosyl groups of the cells of the insect digestive tract, leading to less food assimilation, affecting survival and reproduction (Michiels *et al.*, 2010; Vandenborre*et al.*, 2010), in addition to other biological function such as serum glycoprotein turn over, innate immune response (Vijayan and Chandra, 1999), anti-tumor (Puzati, 1998), immunomodulatory (Abdulla and de Mejia, 1997) and anti-human immunodeficiency virus (HIV) (Herre *et al.*, 2004). Legume lectin is the best known lectin family. Classical legume lectins have been found exclusively in members of the leguminosae. It is found in significant quantities (as much as 2.4-5.0% of total protein) in legumes (Dobbins *et al.*, 1986) .Protein and gene sequencing ha*s* demonstrated that all legume are built up of either two or four sub units of about 30 KDa in the so called two chain legume lectins, either identical or slightly different, each with a single, small carbohydrate combining site with the same specificity. Though several lectin genes have been tested for their efficacy (Leal-Bertioli *et al.*, 2003). Also, there are many efforts to produce the DNA samples of active legume lectins using different heterologous expression systems such as *Escherichia coli* and tobacco plants. Although the licorice plant belongs to a family leguminoceae, fewer researches have been done for the isolation of lectin from its roots. Therefore, the aim of the current study was the detection and identification of legume lectin gene in the licorice plant, especially in its dried roots where, green pea *(Pisum sativum*) plant belongs to the same family (*Leguminoceae*) which lectin protein present with high level in it (Shibamoto and Bjeldanes, 1993; Jones, 1995) then, it was used in the present study and considered as a positive control for the lectin substance.

MATERIALS AND METHODS

Samples

Both dried roots of Licorice (*Glycorrhiza glabra*) and commercial seeds of green pea *(Pisum sativum*) were obtained from a local market. One gram of each sample was powdered under liquid nitrogen in a mortar with a pestle for DNA extraction.

Strains, plasmid and major reagent

Escherichia coli DH5α was preserved in laboratory. Gel extraction kit was purchased from Koma Biotech (Korea). pCR4-TOPO cloning kit and (Pperox-HTa) expression vector were obtained from invetrogen (USA). pCRclean up kit was from Maxian Biotech INC (USA). Taq DNA polymerase was from Promega (USA). PCR nucleotide mix dNTPs was from Bioron (Germany). DNA ligase and 10% ligase buffer were supplied from Sib Enzyme (Russia). All other chemical reagents were analytical purity.

Primer design

Specific primers for lectin gene (lec1, forward:

5'ATGGGACCAAGCAACAGAG3' and lec2 reverse: 5'ATCCTTCAAAGACACAATGTCG-3') were designed by Metabion (Germany) and used to identify the coding sequences licorice lectin gene in the present study.

DNA extraction

Total DNA extracted from the frozen powder of each the dried roots of licorice and seeds of green pea according to the method by Edwards *et al.* (1991). The total DNA was visualized on 1% agarose gel and photographed using gel documentation system (Alpha-chemimager, USA). Concentration of DNA samples were checked in UV- spectrophotometer and reading was taken at wave lengths of 260 nm and 280 nm.

Polymerase chain reaction (PCR)

DNA lectin gene in both the licorice and green pea plants was initially detected by PCR amplification with specific primers for lectin gene (lectin 1, forward: 5'ATGGGACCAAGCAACAGAG-3' and lec2 reverse: 5'ATCCTTCAAAGACACAATGTCG-3'). PCR amplification carried out in a total volume of 25 µl contains 2.5 µl 5x Green Go Taq flexi buffer, 2.5 µl (5x colorless Go Taq flexi buffer), (100 mM Tris-HCl, pH 8.8 at 25°C), (500 mM KCl), 2.5 µl $MgCl_2$ (25 mM) (Promega, USA). 2.5 µl 4 dNTPs mixture (10 mM of each), 1 µl DNA of lectin,4 µl of each primer (20 pmol/µl), 0.2 µl 2 U Taq polymerase (5 U/µl), the volume completed up to 25 µl with sterile H_2O. The reaction mixtures were subjected to be amplified as follows: initial denaturation step was at 95°C for 3min, followed by 33 cycles of amplification with denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min ending with extension at 72°C for 10 min. PCR product was run on 2% agarose gel and a desired DNA fragment was excised for cloning and sequencing.

Cloning and sub-cloning of lectin gene expression

The amplified products were cloned into (PcR-4.TOPO) vector using TA Cloning kit (Invitrogen^{TM}, USA) and transformed into competent *E. coli* cells (strain DH5α). The recombinant DNA from the transformed clones were released and sub cloned into pPROEX HTa vector (life technologies, USA) with *E. coli* by addition IPTG synthetic inducer for inducing lectin gene expression as described by Goh *et al.* (2005).

Lectin protein purification

Lectin purification was carried out by Ni-NTA resin matrix (QIAGEN Inc., USA). The induced bacterial cells were pelleted and re-suspended in 4 volumes of lyses buffer (50 mM Tris-HCl, PH 8.5 at 4°C, 5 mM 2-mercaptoethanol, 1m M PMSF). The suspension was sonicated until 80% of the cells were lysed. The cell debris was removed by centrifugation; the supernatant was transferred to a new tube (crude supernatant) for Protein purification using affinity chromatography ac-

cording to the instruction of the manufacturer of Life Technologies, (Invitrogen).

SDS-polyacrylamide gel electrophoresis

The purified recombinant protein was separated on slabs of 12% resolving gel and 5% staking gel as described by Sambrook and Russel (2001).

Western blot assay

Polyclonal antibody production for lectin protein was performed at National Research Institute, Cairo, Egypt, according to Leenaars *et al*. (1999). After separation of the recombinant lectin protein through SDS-poly acrylamide gel electrophoresis .Western blot technique was carried as describe by Towbin *et al*. (1979). Where, polyacrylamide gel-membrane sandwich was arranged on western transfer cassette (Bio-Rad, USA) using a wet sheet of nitrocellulose membrane (Costa, Bio Blot, Canada), all western blot transfer components were inserted in a buffer tank filled with western transfer buffer, PH 8.3 and left at 100 V for 1 hr then maintained at 30 V overnight. Once,the protein was transferred from polyacrylamide gel-membrane to the nitrocellulose membrane, it was coated with western blocking buffer (3% BSA) for one hour at room temperature and washed with TBS buffer, PH 7.5. Primary antibody (polysera) was added to it and incubated for one hour to bind with the lectin protein then, the secondary antibody that was labeled with alkaline phosphate supplemented onto nitrocellulose membrane in presence BCIP/NBT substrate for 10 min and photographed.

Sequencing determination

The amplified DNA amplicon from each of licorice and green pea plants was sequenced by Sigma Company (USA). The DNA Sequences for licorice and green pea plants were accepted and received the accession numbers that was submitted into the GenBank. Sequence similarity was analyzed using BLAST (http://www.ncbi.nIm.nih.gov/BLAST/).

RESULTS AND DISCUSSION

DNA extraction from legume plants

The DNA concentration was ranged from 1.8 to 2.0 OD at wave lengths of 260 nm and 280 nm in each of licorice and green pea plants. The partial amplified fragment of Lectin gene was about 700 bp that was isolated from genomic DNA using degenerate primers Lec 1 and Lec 2 in both licorice and green pea plants as shown in Fig. (1). Our results is almost consistent with the results reported by Datta *et al*. (2000) where, total genomic DNA was isolated from cow pea and hybridized with heterogeneous pea lectin cDNA probe and The amplified fragment was 776 bp that contained 675 nucleotides ORF.

Cloning of lectin gene (700 bp) and gene expression

The fragment of about 700 bp containing the partial lectin gene was cloned into plasmid vector (PCR4-TOPO). The recombinant plasmid (positive colonies) were confirmed using universal primer (M13) PCR analysis. The product was sub

cloned into linearized pPEROXHTa expression vector and was fractionated on 2% agarosegel. Change in the electrophoretic mobility confirmed the presence of insert lectin gene into plasmid due to the change in molecular size (5450 bp) when compared with the empty circular plasmid vector pPEROXHTa (4750 bp) as shown in Fig. (2). The recombinant plasmid vector was transformed into competent *E. coli* cells (DH5α) with presence (IPTG) inducer protein. The deduced mature protein was purified using affinity chromatography and fractionated on 12% SDS-PAGE as a single band with molecular weight of 30 and 27 KDa for green pea and licorice plants, respectively, as shown in Fig. (3). Similar study was performed by Spurthin (2005) on cow pea lectin clones (psk1803A and psk1803B) and colocasia lectin clones (pskR2105A, pskR2105B and pskR2105C) which were subjected to SDS-PAGE. The protein bands corresponding to 29 KDa and 26 KDa were obtained

Western blot assay

The identity of the induced pea and licorice lectin protein was determined through Western blot technique. Positive reaction was observed as a single pink colored band with molecular weight of 30 and 27 KDa in both green pea and licorice plants, respectively, in presence specific polysera as in Fig. (4). Thomas *et al.* (1989) mentioned that in an expression experiment of bacterial cells harboring cDNA of lectin gene from the garden pea (*Pisum sativum*) that has been expressed by attaching its cDNA to an inducible promoter. An induced band was separated by SDS-PAGE with an apparent molecular mass of 23 KDa .The identity of the induced protein was confirmed to be pea lectin using western blot technique where, the position of recombinant pea lectin protein appeared with a molecular weight 71 KDa.

Sequencing and similarities of lectin gene

Sequencing of the amplified fragment of lectin gene (700 bp) for licorice and green pea plants were performed by sigma company (USA) and submitted into the Genbank under HQ337023 and HQ 337024 accession numbers for licorice and green pea plants, respectively, as shown in Figs (5 and 6). Comparative analysis between the sequences of the lectin gene for green pea and licorice plants in this study and the other published genes that is available in the Genbank showed that the nucleotide sequence of green pea is clearly shared 100% nucleotide identity with a lectin *psl* gene and *lec A* gene from green pea with their accession numbers EU825771 and T00440, respectively, also showed nucleotide sequence identity of 98% to the *P. sativum psl* lectin gene (xx66368) and pea *psl* gene encoding lectin complete cds (M18160) with identity of 92%. On the other hand, lectin gene sequence from licorice plant under study is completely related to the partial *lec 2* gene from licorice plant (AJ234389) with nucleotide sequence identity of 100%. Other study by Liu *et al.* (1995) reported

that the 832 bp amplified fragment of pea lectin gene from leaves that contain the entire coding sequence of pea lectin gene which has no intron showed the homology ratio by 99.6 and 98.9 for the nucleotide sequence and amino acid sequence of the pea lectin gene, respectively. Datta *et al.* (2000) illustrated that cow pea lectin probes and isolated a putative pea lectin of 776 bp showed about 72% homology with green pea (*Pisum sativum*) lectin. Complete organization of the pea lectin gene family was investigated by Alexandre *et al.* (1987). They found that the DNA sequences of the subcloned of lectin pea gene designated psl_1, psl_2, psl_3 and psl_4 that are considered as incomplete genes because of the presence of several stop codons in the correct reading frame of them. From our results, we can conclude that the licorice plant is considered as a good source of lectin protein as a legume plant representing in a green pea plant and licorice lectin which may be a member of the legume lectin family.

SUMMARY

Lectins are a group of non-enzymatic carbohydrate-binding proteins that are present in plants, animals and microorganisms. In the present study, DNA was extracted from the dried roots of licorice and seeds of green pea plants (positive control). Specific PCR technique was employed using specific primers (*Lec1* and *Lec2*) for the amplification of Lectin gene. The amplified fragment mol. Size was about 700 bp that was cloned and sub-cloned into the pPROEXHTa expression vector. The purified proteins were separated at 27 and 30 KDa in both licorice and green pea plants, respectively, using SDS-PAGE. Sequencing of the PCR product of lectin gene was documented into the Genbank under the accession numbers HQ337023 and HQ337024 for licorice and green peas plants, respectively. The comparison of nucleotide sequence of the root Lectin gene from Licorice under study showed complete similarity (100%) to partial lectin 2 gene from Licorice that accession number (AJ234389) while, lectin sequence from seeds of green peas was clearly shared 100% nucleotide identity with a lectin *(psl*), complete cds and *lecA* genes from green pea with their accession numbers EU825771 and T00440, respectively, also, it showed nucleotide sequence identity of 98%. to the *P. sativum psl* lectin gene (xx66368) and Pea *psl* gene encoding Lectin complete cds (M18160) with identity of 92%.

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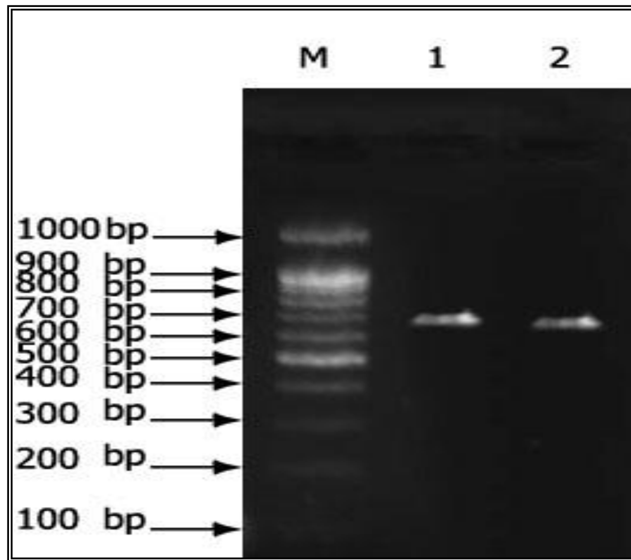

Fig. (1): 2% Agarose gel electrophoresis of PCR product for lectin gene. M: 1000 bp DNA ladder, lane 1: amplified lectin gene from licorice, and lane 2: amplified lectin gene from green pea plants.

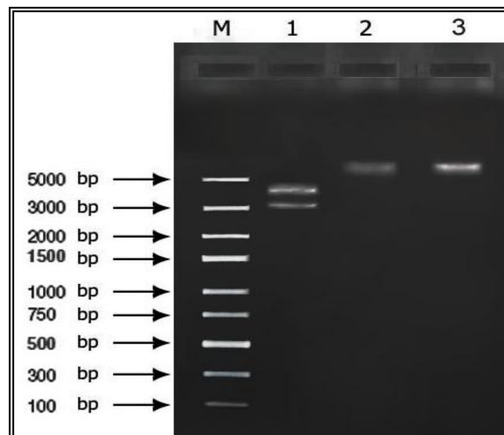

Fig. (2): 2% Agarose gel electrophoresis of plasmid containing insert of lectin gene fragment (700 bp) where, M: 5000 bp DNA ladder, Lane 1: the empty Plasmid (pPEROXHTa) vector (4750 bp) , Lane 2: lectin gene recombinant plasmid (5450 bp) from Green pea and Lane 3: lectin gene recombinant plasmid from licorice plants (5450 bp), respectively.

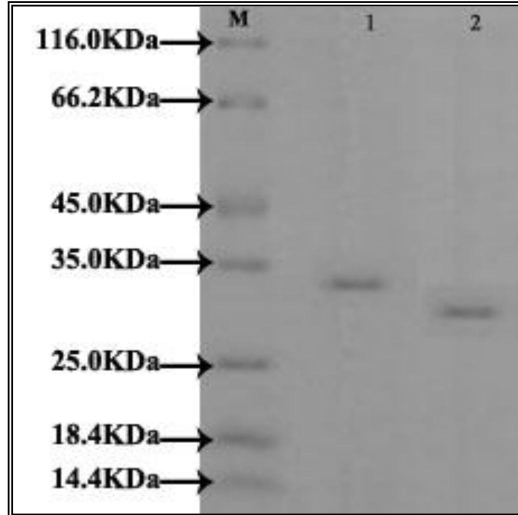

Fig. (3): 12% SDS-PAGE of purified lectin protein. M: 116 KDa protein marker, Lane 1: purified lectin protein from green pea and lane 2: purified lectin protein from licorice plants.

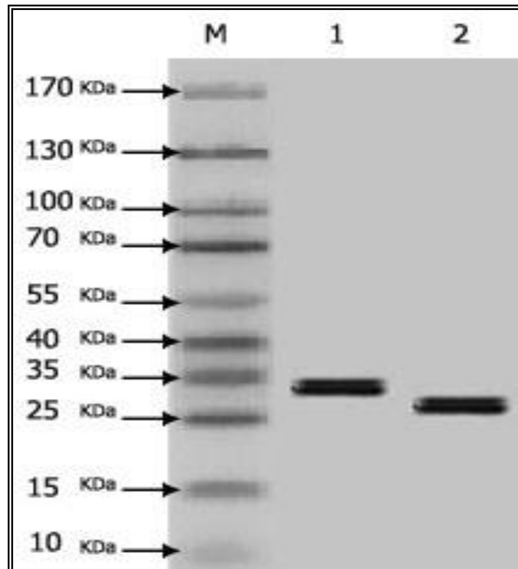

Fig. (4): Western blot for detection of lectin protein. Where, M: Protein marker, Lane 1: lectin from green pea and Lane 2: lectin from licorice plants, respectively.

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> HQ337024
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TGTAGTTAAAAATTGATATACTATTTAAACTGCGAGAGTTTTGTTTCTGAAGGTTAAAAA
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AATTAAAACAAAATATTAAATTAAACTAAAAATTTGGTGATTAATTGTGCCAAAAAAATA
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ATTGGATGAGATACAAATTTTATCATAAAATATATTAGTTATAACAATACGACCACCCTC
TCCATAAGTTTTAAATAAATATCAGCCCTAAAAAACTCTTTAAATAAATTGAAATTTAAT
GAGTCATATTTTTTTAACATATAAATTTTAATAGTTATCGTACCGAACAAAAACAGTAAT
CATGATCTAAACCGAACAACCTCGAAGAAATACAAGTTATTACATGCAAAAATATATAGT
AATAAATAAATAAACTAGTTAAACAAAATACAATATTTTT
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Fig. (5): The partial sequence of lectin gene (700 bp) for green pea (*P. sativum*) with the accession number (HQ337024) in the Genbank.

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>HQ337023
GATTTCTCGGACTTTTCAAAGACAAAGATTTCGATAAATCGAACCAAATTGTTGCCGTGGA
ATTCGACACGTTCGTTGATGAGGAGTGGGACCCACAAGGTAGACACATTGGGATTGACGTC
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TGTTCATAAGTTATGAGGCTTCCACGAAGATCTTAAGTGCCTCTTTGGTTTTTCCTTCGCG
TCAATCGAGTTATATAGTTTCCTCTGTTGTGGACTTGAAGGACGTTCTTCCGGAGTTTGTG
AGGATTGGGTTCTCAGCTACCACAGGAATATCTGAAGGCTTGTTG
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Fig. (6): The partial sequence of lectin gene (700 bp) for licorice (*Glycorrhiza glabra*) plant with the accession number (HQ337023) in the Genbank.