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Research Paper

DETERMINATION OF QUANTITATIVE CONTENT OF LECTINS IN *DREGEA VOLUBILIS*

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Dregea volubilis plant shows the presence of lectins in roots, stem, leaves, fruitcoats and seeds. The 0-90% ammonium sulphate precipitated fractions were active in the agglutination test towards the RBCs of all the blood groups. The agglutinating activity of these fractions was inhibited specifically by α-D-glucose and α-D-mannose and all glucose derivatives. The agglutinating activity was maximally manifested from pH 4 to 7 and from 30 to 60°C. Maximum HAUs were found in the mature leaves, fruit coats of fully grown mature fruits and the mature seeds than in their younger counter parts. The agglutination activity of all the lectin fractions was found to lose activity in presence of EDTA, however the activity was restored by Mg^{++} ions.

Keywords: Dregea, Lectins, Agglutinins, Phytolectins, Plant proteins

INTRODUCTION

Plant lectins form a highly heterogeneous group of carbohydrate binding proteins due to their specificity and biological activities. Many plants accumulate lectins in large quantities especially in seeds, barks and fruits, thus these proteins were called as plant storage proteins (Suseelan K and Mitra R, 2001; Peumans W J and Van Damme, 1995). It was thought that these proteins may serve as nitrogen source for plant (Peumans W J and Van Damme, 1995). But as these plant proteins possess the ability to specifically discriminate the complex carbohydrates naturally occurring in the cell surface conjugates, thus the physiological role played by lectins in the plant was thought to be some other than storage proteins. It was suggested that they may help in transporting the carbohydrates synthesized as a result of photosynthesis to organs where they are required. Lectins are localised within the cell in considerable amount and are able to react with storage proteins. From the above observations, it was assumed that lectins make up the storage proteins more complex and convert them into a compact insoluble stage which makes their deposition easy in the storage region. In this manner, they were believed to be used for packaging of storage materials (Luiza I M *et al*., 2000). Some lectins are found to contain catalytic domain and thus are associated with enzymatic activity, e.g. Ricin, the very first lectin is found to contain RNA-N- glycosidase, which inactivates the eukaryotic ribosome by cleaving the N-glycosidic bond of rRNA (Peumans W J and Van Damme, 1995). Similarly, *Vigna mungo* lectin was found to be associated with α- and β-galactosidase activity (Suseelan K and Mitra R, 2001). Later defensive role of plant lectins against prokaryotic and eukaryotic organisms such as phytopathogenic bacteria and fungi was reported (Tulsi R B and Nadimpalli S K, 2002.

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Lectins from *H. brasiliensis* (Van Paris, 1991), *D. stramoneum* (Broekaert W F and Peumans, W J, 1986), *H. pomata* (Suseelan K and Mitra R, 2001), *C. rotundacauda* (Tulsi R B and Nadimpalli S K, 2002), *S. tuberosum* (Suseelan K and Mitra R, 2001), were reported for blocking the movement of bacteria thereby acting as safeguard of the plants against bacterial attack, WGA (Wheat germ agglutinin), (Peumans W J and Van Damme, 1995), stinging nettle lectins (Peumans W J and Van Damme, 1995), *A. caudatus* lectins (Broekaert W F and Peumans W J, 1986), *S. tuberosum* lectins, *L. auriculata* lectin (Broekaert WF, 1989), were found to possess antifungal activity. Insecticidal properties of many purified seed lectins were also reported by other authors. Seed lectins from *B. simplicifolia, B. purpurea, C. fragile, R. communis, V. villosa, P. americana* were found to inhibit growth of insect larvae (Pusztai A and Bordocz S, 1996). Lectins of *R. pseudoacacia* and *S. nigra* exert the toxic effect on predators and thus protect the plants from the rodents, deer and other animals (Peumans W J and Van Damme, 1995).

Lectins are now shown to be present in various parts of the plant such as seeds, roots, barks, stems, leaves, flowers, inflorescence, fruits, fruitcoats, etc. Lectins may comprise up to 3% of total weight of a mature seed. Luiza *et al*., (2000) detected the lectin in the cotyledons through the entire germination process. Cotyledons in senescent stage were also found to possess lectin activity (Luiza *et al*., 2000). After germination, the lectin production takes place at a low, but constant level of lectin from two to eight weeks and then level of lectin was found to increase rapidly in next ten weeks, and then could be detected in various parts of the same plant. As compared to the leaves and stems, the lectin concentration was observed to increase very abruptly in the developing seeds (Luiza *et al*., 2000). Lectins present in various parts of the same plant may play different roles. Either, they may play defensive role by protecting these parts of the plant which serves the vital role in the survival of the plant against the attack by microorganisms, insects, pests, and even the higher animals like rodents, deer, etc (Peumans W J and Van Damme, 1995). It is also possible that lectins present in vegetative tissues may get involved in the transport of glycoproteins synthesized by these vegetative parts to the organs where they are required for various processes (Luiza *et al*., 2000). Different plant parts are also shown to have different types and levels of lectins e.g. seeds of Indian lablab beans contain two distinct lectins, which differ in their specificities, viz. The glucose/mannose specific lectin and the galactose specific lectin where as stems and leaves of the same plant were found to contain galactose specific lectin (Nathan S, 1977). Lectins of seeds, embryos, and cotyledons of peanut posses the affinity for disaccharides where as stem lectins SL-I and SL-II of the same plant possess the affinity for methyl- α-mannoside and lactose/cellobiose, respectively.

Growth of the plant is a continuous process from the germination through flowering to death (Singh R *et al*., 1997). During the growth and development of the plant, changes in the relative concentrations of lectins in vegetative parts of the plant take place (Nathan S, 1977; Kauss H and Glaser C, 1974). With this background, the present paper reports the results of the experiments carried out in order to know the possible types and changes in the relative levels or concentrations of lectins present in young, mature and old leaves, fruits, and seeds of *D. volubilis* plant.

MATERIALS AND METHODS

Chemicals

Sodium dihydrogen phosphate, disodium hydrogen phosphate, Sodium potassium tartate, Copper sulphate, Folin Ciocalteu's reagent, Bovine serum albumin, were purchased from E. Merck, Germany. Papain, ammonium sulphate was purchased from Sigma Chem. Co. USA. Rest of the chemicals were of Analytical reagent grade.

Plant Source

The plant was identified to be *D. volubilis* by University Department of Botany, RTM Nagpur University, Nagpur.

Plant Samples

Tender fresh roots, fresh green soft stems and fresh small young leaves, mature green leaves of uniform size, and old leaves, which were completely yellow, were collected.

Fruitcoats of very young (small & growing) fruits, fully grown (mature fruits) and yellowed (but not dried) fruits were collected.

Green immature (growing) seeds from very small growing fruits, mature seeds from fully grown fruits, and over mature seeds from dried fruits which were about to be dispersed in environment were collected and used.

Red Blood Cells

Papain treated RBCs of blood group A, B, and O were collected and prepared as described by (Deshpande K V and Patil M B, 2002).

Preparation of Extracts and Isolation of Lectins

Samples were washed two times with distilled water and crushed in 20mM sodium phosphate buffer containing 0.1mM NaCl (PBS), pH 7.0, Crude extracts were filtered through cheese cloth to remove debris. The filtrate was centrifuged at 9886 g for 20 min. in cold centrifuge (Remi C24). The supernatants were subjected to ammonium sulphate precipitation (0-90%) by the method of (Patil M and Shastri N V 1982). Ammonium sulphate precipitated proteins after solubilization in small quantity of PBS, dialysis overnight against same medium were designated as *Dregea volubilis* partially purified lectins (DVPPL).

Agglutination Assay

A 2% suspension of papain treated RBCs of all the blood groups were used for agglutination. Hemagglutination assays were conducted by the method using two fold serial dilutions of lectins in PBS (Suseelan K and Mitra R, 2001). Agglutination was observed after one hour under the microscope and was expressed as hemagglutination titre or hemagglutination activity units (HAU) which is defined as "the reciprocal of the last dilution of the lectin giving agglutination" (Suseelan K and Mitra R, 2001). Specific activity is defined as HAUs/mg of proteins (Suseelan K and Mitra R, 2001).

Protein Estimation

Protein contents of crude extracts and ammonium sulphate precipitated fractions were determined by the method of (Lowry *et al*., 1951) using lipid free bovine serum albumin as the standard protein (Lowry O H, 1951).

Determination of Carbohydrates

Carbohydrate contents in DVPPL were determined by (Dubois *et al*., 1954).

Carbohydrate Inhibition Assay of DVPPL

Various pentoses, hexoses, disaccharides, oligosaccharides and glucose derivatives were used for carbohydrate inhibition assay by the method described elsewhere (Suseelan K and Mitra R, 2001). The inhibitory concentration of carbohydrate was determined as concentration that inhibits the lectin to agglutinate the RBCs (Suseelan K and Mitra R, 2001).

Determination of Properties of DVPPL

pH stability of DVPPL was determined by the method used by Suseelan K and Mitra R, 2001. Buffers for given pH ranges were chosen as suggested by them (Suseelan K and Mitra R, 2001).

Temperature Stability of DVPPL

DVPPL were exposed to various temperatures ranging from 5 to 100°C and agglutination assays were carried out as mentioned above.

Effect of Metal Ions on Agglutination

Effect of metal ions on agglutination was tested on demetalised DVPPL by the method of (Kawagishi, 1990).

Effect of Maturity on Lectin Content

Young, mature and senesced leaves; young, mature and senesced fruit coats and young, mature and dried seeds were tested for content of lectins as mentioned above.

RESULTS

When leaves, stems, fruit coats, seeds and roots were checked for hemagglutinating activity with papain erythrocytes of all the blood groups they were found to contain very high hemagglutinating activity, however roots were found to contain

comparatively less hemagglutinating proteins as presented in Table 1. Screening of young, mature and senesced leaves, fruit coats and seeds have shown the maximum lectin content at maturity state than the over mature or the senesced condition.

Table 1: Hemagglutinating Activities Shown by Different Parts of *D. volubilis*

Plant part	HAUs with A blood group RBCs /g fresh weight	Proteins (mg)/g of plant tissue	Specific activity
Leaf	86850	121	716
Stem	13192	109	120
Fruit coat	100570	148	678
Seed	70150	137	512
Root	8000	100	80

HAU=Hemagglutinating units. Results are mean of 3 replicates. (P< 0.05)

Blood Group Specificity

DVPPL agglutinated trypsin treated as well as papain treated RBCs at the same respective dilutions. No hemagglutination was observed with untreated RBCs. When two fold serially diluted lectin solutions were added to equal volumes of 2% suspension of either papain or trypsin treated human RBCs of blood group A, B, and O, the HAUs obtained were found to be similar for RBCs of all blood groups as shown in Table 2.

Table 2: Hemagglutinating Activity of *D. volubilis* Lectins with RBCs of Blood Groups A, B, and O

Plant source	Papain treated RBCs	HAUs/g	Proteins/g	Specific Activity
Leaf	A	86850	121	716
Leaf	B	86850	121	716
Leaf	O	86850	121	716
Fruitcoat	A	100570	148	678
Fruitcoat	B	100570	148	678
Fruitcoat	O	100570	148	678
Seed	A	70150	137	512
Seed	B	70150	137	512
Seed	O	70150	137	512

HAUs=Hemagglutinating units. Results are mean of 3 replicates. (P< 0.05)

Hemagglutination Inhibition Assay

DVPPL were found to be inhibited by α-D-glucose and α-D- mannose only. α-D ribose, α-D- arabinose, α-D-xylose, D-ribulose, α-D-galactose, α-D-fructose, α-D-maltose, α-D-lactose, sucrose, D-trehalose, D-raffinose, D-rhamnose, D-fucose, did not inhibit agglutination up to 400 mM concentration. Inhibitory concentrations of α-D-glucose and α-D-mannose were found to be 200 mM where as GluNAc was found to inhibit the agglutination at 225 mM and inhibitory concentration of Glucosamine hydrochloride, α-methyl-D- glucoside and 3-O-methyl glucose was found to be 250mM as shown in Table 3. A-d-ribose, α-D- arabinose, α-D- xylose, α-D- ribulose, α-D galactose, α-D- fructose, sucrose, D-trehalose, D-rafinose, D-rhamnose, D-fucose, did not inhibit at 400 mM concentration (Table 3).

Table 3: Inhibition of Agglutination and Inhibitory Concentration of Carbohydrates of *D. volubilis* Lectins

Carbohydrate	Root lectin	Stem lectin	Leaf lectin	Fruit-coatlectin	Seed lectin
α-D-glucose	200mM	200mM	200mM	200mM	200mM
α-D-mannose	200mM	200mM	200mM	200mM	200mM
N-acetyl-D-glucosamine	225mM	225mM	225mM	225mM	225mM
Glucosamine hydrochloride	250 mM	250mM	250mm	250mM	250mM
α-D-methyl-D- glucoside	250mM	250mM	250mM	250mM	250mM
3-o-methyl glucose	250mM	250mM	250mM	250mM	250mM

Results are mean of 3 replicates. (P< 0.05)

Temperature and pH Stability

Exposure of DVPPL from 5 to 100°C retained activity till 60°C. The agglutination activities of *D.volubilis* lectins was found to be stable from pH 4 to 7 only, whereas at pH below 4 and above 7, lectin activities were found to be lost drastically (data not shown).

Effect of Metal Ions

Effect of metal ions on activity of EDTA treated lectin of *D. volubilis* has been shown in Table 4. On treatment with EDTA DVPPL were found to

lose activity completely, where as when tested in presence $MgCl_2$ and $MgSO_4.7H_2O$, (1mM) hemagglutinating activity was found to be restored.

Table 4: Effect of EDTA and Metal Ions on Agglutination of *D. volubilis* Lectins

Metal salts (1mM concentration)	Agglutination
Control 1(EDTA treated lectins)	--
Control 2 (RBCs+ PBS, pH=7)	--
Ca++	--
Mn++	--
Mg++ (MgCl2 and MgSO4.7H2O)	+
Hg++	--
Ba++	--
Fe+++	--
Ag++	--
Sb++	--
(--) = No agglutination; (+) = Agglutination	

Table 5: Effect of Maturity on Lectin Content in *D. volubilis*

Plant part	HAUs/g	Protein mg/g	Specific activity
Young leaf	32724	60	540
Mature leaf	86850	121	716
Senesced leaf	9372	43	220
Young fruit coat	33264	63	528
Mature fruit coat	100570	148	678
Senesced fruit coat	9175	45	203
Immature seeds	27300	70	390
Mature seeds	70150	137	512
Dry seeds	67761	136	498
Results are mean of 3 replicates. (P< 0.05)			

DISCUSSION

D. volubilis, a plant belongs to the family Asclepiadaceae. Young green tender stems, green and actively photosynthesizing mature leaves, green fruit coats of fully grown fruits and fully grown fresh seeds were found to contain highest amount of hemagglutinating activity than their immature counterparts. Similarly the tender fresh roots were also found to contain fewer amounts of lectins than the older and hard roots. As the plant part grow and achieve maturity the lectin content increases because the HAUs of mature leaves and fruit coats were found to be higher than that in the young stage of leaves and fruit coats. Similar results were also reported for *D. biflorus* leaf and seed lectins (Tulsi R B and Nadimpalli S K, 2002). Many other plants are also studied for presence of lectins in different parts (Luiza I M *et al*., 2000; Nathan S, 1977). This increase in the lectin content may contribute for the extra protection to mature leaves and fruits. Leaves perform the most vital function of photosynthesis and synthesize the energy which is supplied to the growth & development of plant. Fruitcoats serve as safeguard/shield to the seeds by protecting the seeds of the plant, which can give rise to new plant thereby conserving the species. Thus the plant leaves, fruit coats and seeds play an important role in the survival of the plant. Peumans and Van Damme, (1995) reported one of the most important functions of the plant lectins which protect the tissues or organs thereby playing a key role to keep the plants alive (Peumans W J and Van Damme, 1995. Leaves and fruitcoats were found to contain more HAUs than that of seeds. Similar types of result were also reported for WGA (Mishkind M *et al*., 1982). WGA is primarily localised in the actively growing parts of plant such as roots and leaf bases. In peanut, stem lectin (SLII) was also found to be higher in stem than in the seeds (Nathan S, 1977). As originally proposed by Howard, (1972), high lectin content in the vegetative parts of *D. volubilis* may play a role in the regulation of growth (Howard I K *et al*., 1972). The significant level of lectin in over mature seeds may play a vital role in the protection of seeds which are most attractive to the potential predators and parasites as they lack defence system due to their inactive metabolic state with their role as a storage proteins (Peumans W J and Van Damme, 1995).

Lectins of *D. volubilis* tested positive for the carbohydrate contents, indicating that they are glycoprotein in nature. Many other lectins which

are glycoprotein in nature have been reported e.g. rice lectin (Indravathamma P and Sheshadri H S, 1980), jack fruit lectin (Sureshkumar G *et al*., 1982), *D. lablab* lectin (Sivakumar N and Rajgopal Rao, 1988), *B. purpurea* lectin, etc (Balasubramanium N K, 1993).

D. volubilis lectins showed identical hemagglutination pattern with papain and trypsin treated RBCs of all blood groups. No hemagglutination was observed with untreated red cells. The reason may be that the glycoconjugate receptors in untreated erythrocytes remain masked in the protein-lipid bilayer and are not available for binding with DVPPL and therefore no agglutination was observed with untreated red cells. When these RBCs were treated with proteolytic enzymes like trypsin or papain, the glycoconjugate receptors could become available for binding thereby agglutinating papain treated or trypsin treated RBCs (Sampio A H, 1998).

D. volubilis lectins agglutinated the papain treated human RBCs of blood groups A, B, O with same efficiency. It indicated the nonspecificity of DVPPL towards blood groups. Other blood group nonspecific reported lectins are wheat germ agglutinin (Nagata Y and Burger M M, 1972), soybean lectin (Suseelan K and Mitra R, 2001), blackgram lectin (Suseelan K and Mitra R, 2001), *A. integra* lectin (Sureshkumar G *et al*., 1982), rice lectin (Indravathamma P and Sheshadri H S, 1980), and peanut lectin (Suseelan K and Mitra R, 2001).

α-D- glucose and α-D- mannose was found to inhibit agglutination. This indicated that lectins of *D. volubilis* were α-D- glucose, α-D-mannose specific lectins. Other inhibitory sugars include derivatives of glucose such as GluNAC, Glucosamine hydrochloride, α-methyl-D-glucoside, 3-o-methyl glucose. Various glucose specific lectins reported by other scientists include WGA (Nagata Y and Burger M M, 1972), rice lectin (20), peanut root lectin (Suseelan K and Mitra R, 2001), etc.

Like all other proteins *D. volubilis* lectins require a specific range of pH from 4 to 8 for agglutination. Similar pH range was also reported for *Vigna mungo* lectins (Suseelan K and Mitra R, 2001) and *H. barasiliensis* (Van Paris J *et al*., 1991). Heating above a particular temperature destroys the three dimensional structure of carbohydrate binding site (Tulsi R B and Nadimpalli S K, 2002), thus DVPPL activities were also lost at 100°C completely. *Dregea volubilis* lectins were stable and retained activity till 60°C only. Similar temperature stability was shown by lectins from *T. procubans* (Ramteke A and Patil M B, 2005).

D. volubilis lectins were found to require Mg++ ions for agglutination. EDTA treatment lost the agglutinating activity of all the lectins of *D. volubilis*, indicating the lectins to be dependent on Mg++ ions for activity. Similar lectins for Mg++ ions dependence were reported to be Con A (Concanavalin A isolated from *Canavalia ensiformis*) and D. lablab lectin (Tulsi R B and Nadimpalli S K, 2002).

All results are mean of 3 replicates. The data obtained was analysed using ANOVA technique as given by Ronald Walpole, (1982). Significance was set at $P<0.05$ (Ronald E Walpole, 1982).

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