BIOCHEMICAL CHARACTERIZATION OF *ABRUS* LECTINS TOXINS AND AGGLUTININS

Thesis Submitted to the
UNIVERSITY OF HYDERABAD for the
DOCTOR OF PHILOSOPHY

By

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ENROLMENT NO. 93LPPH06
1999

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Biochemical Characterization of *Abrus* lectins: Toxins and Agglutinins"** has been carried out by me under the supervision of Dr. K. **Seshagirirao** and this has not been submitted for any degree or diploma of any University.

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CERTIFICATE

This is to certify that Ms. **J. Gauthami Satyasree** has carried out the work in the present thesis under my guidance for a full period prescribed under the Ph.D. ordinances of the University. I recommend her thesis entitled **"Biochemical Characterization of *Abrus* lectins: Toxins and Agglutinins"** for submission for the award of the degree of ***Doctor of Philosophy*** of this University.

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ACKNOWLEDGEMENTS

It gives me great pleasure to express my deep sense of gratitude to my supervisor Dr. K. Seshagirirao for his guidance and constant encouragement.

I am thankful to Prof. R. P. Sharma, Dean School of Life Sciences, Prof. A. S. Raghavendra, Head of the Dept. and Prof. A. R. Reddy, former Dean for providing the necessary facilities. I am also thankful to Dr. Mohan C. Vemuri, Dr. N. Sivakumar, Dr. M. J. Swamy, Prof. M.N. V. Prasad, Dr. G. Padmaja and Dr. P. Apparao for suggestions and help rendered throughout my study.

My sincere thanks to Dr. Aparna Datta Gupta for providing me insects and insect culture room facility for my studies.

I wish to express my sincere thanks to all my present and ex-labmates Mr. Sasidhar, Mr. Narsaraju and Mr. Shankarrao for extending their co-operation during the course of my research work.

I extend my thanks to all my fellow friends Mr. Y. Rajgopal, Miss. N. Nagalakshmi, Mr. T. Ramakumar, Mr. Vinay kumar,, Miss. M. Padmavathi, Mr. Ramakrishna, Mrs. Manjula, Miss. Neeraja, Mr. Krishna Kishore, Dr. Muralidhar Reddy, Dr. Veera Reddy, Dr. Anuradha, Dr. Giridhar, Dr. Padmavathi for their timely help. I also thank Mr. Pattabhi and other non-teaching employees in the school for their help during the course of my research work.

I express my heartful gratitude to Mrs. Vasanthi for her encouraging words and continue moral support throughout my research period.

My special thanks to all my friends Mrs. Rekha, Mrs. Radhika, Mrs. Mazumdhari, Miss. Rani, Mrs. Prema, Miss. Jhansirani, Miss. Malathi, Mrs. Vanaja and all other galaxy of unsung friends for their cheerful friendship and making my stay in this campus a memorable and pleasant one.

My warm gratitude to my husband Dr. Mahadev Kalyankar for his continuous encouragement and help throughout my research period.

I am indebted to my parents, sister, brother, sister-in-law and cousins who always encouraged me in all my endeavors.

I gratefully acknowledge the financial assistance provided by CSIR, New Delhi, for my research work

Gauthami Satyasree

ABBREVIATIONS

AFSA	*Abrus fruticulosus* seed agglutinin
APA	*Abrus precatorius* agglutinin
APS	A *Abrus pulcellus* seed agglutinin
AT-Sepharose-4B	Acid treated Sepharose-4B
BSA	Bovine serum albumin
CLGG	Cross linked guargum
cm	centimeter
DEAE-Sephacel	Diethylaminoethyl-Sephacel
g	gravitational force
gm	gram
h	hour
IEF	Isoelectric focussing
M	Molarity
N	Normality
PBS	Phosphate buffered saline
µg	microgram
mg	milligram
ml	milliliter
mm	millimeter
min	minute
mM	millimolar
Mr	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
pI	isoelectric point
RIP	Ribosome inactivating protein
SDS	sodium dodecyl sulphate
TEMED	N'N'N'N' -tetramethyl ethylene diamine
Tris	Tris (hydroxymethyl amino methane)
v/v	volume/volume
w/v	weight/volume

INTRODUCTION

PROLOGUE ON LECTINS

Lectins are proteins of non-immune origin that agglutinate cells and bind specifically, and reversibly to sugar molecules or carbohydrates or carbohydrate moieties of glycoconjugates (Dixon, 1981). The term 'lectin' (Latin *Leger*: to choose) was coined by Boyd and Shapleigh, 1954, to denote a group of plant agglutinins. Lectins are ubiquitous proteins that are widely distributed in nature and are found in plants, animals, and fungi and also have been detected in other lower organisms such as bacteria. As they readily and selectively agglutinate different erythrocytes and cells of other origin they are also called as haemagglutinins and/or agglutinins (Liener *et al.*, 1986).

It was observed for the first time that toxic extracts of castor bean (*Ricinus communis*) agglutinated erythrocytes and further analysis revealed that the agglutination is caused by a protein called ricin (Stillmark, 1888). Abrin in *Abrus precatorius* and crotin in *Croton tiglinum* were two such similar proteins discovered much later by Hellin in 1891 and Elfstrand in 1898 (Toms and Western, 1971). At the same period another lectin, designated robin, was isolated by Power and Cambier in 1890 from the bark of black locust (*Robinia pseudoacacia)* (Kocourek, 1986). Following these reports Paul Ehrlich, the father of modern immunology, immediately carried out a series of experiments with abrin and ricin, which in turn established the fundamental concepts of immunology. He demonstrated for the first time the quantitative determination of an antigen and antibody complex *in vitro*. In addition, he found the specificity of immune response in animals after administration of abrin and ricin, and immunity to the toxin was transferred from a mother to the offspring through blood during pregnancy and by milk after birth.

In 1919, J. B. Sumner isolated Concanavalin A (Con A) from extracts of the jack bean (*Canavalia ensiformis*) by salt precipitation and crystallized it. It was the first time to obtain a pure lectin. In 1936, he together with S. F. Howell showed that haemagglutination by Con A was inhibited by cane sugar and suggested that this might be a consequence of the reaction of the plant protein with carbohydrates on the surface of the red cells (Sharon and Lis, 1987). In 1952, W.M. Watkins and W.J.T. Morgan showed that the agglutination of type A red cells by lima bean (*Phaseolus lunatus*) lectin was best inhibited by α-linked N-Acetyl-D-galactosamine and the agglutination of type O cells by the lectin of *Lotus tetragonolobus* was inhibited by α-linked L-fucose (Liener *et al.,* 1986). The investigations of Watkins and Morgan were incidentally, the first proof for the presence of sugars on cell surfaces (Sharon and Lis, 1987).

Phytohaemagglutinin or PHA a lectin isolated from red kidney bean *{Phaseolus vulgaris*) was shown to be mitogenic i.e. it possesses the ability to stimulate lymphocytes to undergo mitosis (Nowell, 1960). Further. that Con A acts as a mitogen and its activity was inhibited by low concentrations of D-mannose. These studies led to the conclusion that mitogenic stimulation occurs by the cell surface sugars and further established the biological role of cell surface sugars. Joseph C. Aub was the first to demonstrate the ability of lectins to preferentially agglutinate malignanT-cells (Aub *et al.,* 1965 a and b). He found that a lipase containing extract from wheat germ (*Triticum vulgare)* inhibited tumor growth, and caused agglutination of several transformed cell lines but did not appear to agglutinate their normal cell counterparts. Further studies revealed that the wheat-germ lipase preparation contained a contaminant lectin, wheat germ agglutinin (WGA) that was subsequently purified by Nagata and Burger (1972). Later it was found that Con A also agglutinated malignanT-cells

(Inbar and Sachs, 1968) and this lectin became a popular tool for studying cell surface sugars. Further Sela *et al,* (1970) found that soybean agglutinin also distinguished between normal and malignanT-cells which rekindled interest in the properties of known lectins and led to an intense search for and purification of new ones. Taking advantage of the fact that Con A reacts with dextrans, Agarwal and Goldstein (1965) developed a simple and convenient technique for the direct isolation of lectin from crude extracts of jack bean meal by specific adsorption on a column of commercially available cross-linked dextran (Sephadex) and eluted with D-Glucose. Con A was also the first lectin to crystallize and shown to be sugar-specific. This was the first lectin used for structural studies of carbohydrates, as well as for affinity purification of glycoproteins.

Since the early 1970s, the pace of research on lectins and their applications has greatly accelerated (Kocourek, 1986; Gabius and Gabius, 1993; Lis and Sharon, 1986a). Numerous lectins have been purified from plants, animals and microorganisms, and much information on their distribution, molecular properties and biosynthesis are well documented. Extensive homologies have been observed between the primary sequences of lectins from taxonomically related resources, demonstrating that these proteins are conserved throughout evolution and suggest distinct evolutionary roles. Studies on the biosynthesis of Con A have revealed a novel mechanism of protein maturation involving the rearrangement of a primary sequence by post-translational formation of a peptide bond (Bowles *et al,* 1986). Some of the plant lectins have been expressed in bacteria and work on site directed mutagenesis is in progress in several laboratories to understand their molecular mechanisms (Stubbs *et al,* 1986; Hoffman and Donaldson, 1987).

PHYSICO-CHEMICAL PROPERTIES OF LECTINS

To analyze a lectin in its usefulness as a tool in biochemical studies, it is important to establish the carbohydrate binding specificity of a lectin. Sugar-lectin complementarity is determined generally by Landsteiner hapten-inhibition technique (Landsteiner, 1962). Physical methods like equilibrium dialysis, fluorescence spectroscopy have been used widely to establish the carbohydrate specificity of lectin (Krishna Sastry *et ah,* 1986). Lectins not only interact with free sugars but also with polysaccharides and glycoproteins at their non-reducing terminal glycosyl groups. Lectins with similar specificity to monosaccharides differ in their affinity towards di- and oligosaccharides (Debray *et ah,* 1981).

Based on their sugar specificity, Goldstein and Poretz (1986) classified lectins into the following classes:

(1) Mannose/glucose-binding lectins.

(2) Galactose/N-Acetylgalactosamine-binding lectins.

(3) N-Acetylglucosamine-binding lectins.

(4) L-Fucose-binding lectins.

(5) Sialic acid-binding lectins.

The mannose/glucose-binding lectins comprise a group of agglutinins found mostly in the Fabaceae (Leguminosae) family. The mannose/glucose-binding group of lectins is the most thoroughly studied group of lectins. The amino acid sequences of some of the lectins in this group reveal extensive homology and provide new vistas for examining taxonomic and evolutionary relationships.

N-Acetylgalactosamine/galactose-binding lectins are the first plant haemagglutinins shown to display human blood group specificity. Galactose-binding toxins were identified in diverse plants including ***Ricinus communis*** (Euphorbiaceae), *Abrus*

precatorius (Fabaceae) and *Viscum album* (Loranthaceae).

N-Acetylglucosamine-binding lectins comprise a diverse group of agglutinins that exhibit a primary specificity for their monosaccharide and/or its (pl-4)-linked oligomers (chitin oligosaccharides) and in some instances glucosamine. This group includes lectins from three families of Poaceae, Solanaceae and Fabaceae.

The fucose-binding lectins were found to be useful serological reagents. In serological laboratories, the *Ulex europaeus* I lectin is widely used as it exhibits anti blood group O activity (Liener *et al.*, 1986). Fucose lectins are found in diverse organisms like *Lotus tetragonolobus, Ulex europaeus* (plants), *Anguilla anguilla* (animals) and *Aleuria amantia* (fungus).

The haemolymph and sera of most of the vertebrates are a rich source for the sialic acid binding lectins. In recent years sialic acid lectins have also been isolated from the bark of *Maakia amurensis* and different parts of *Sambucus* sp. also (Knibbs *et al*, 1991; Rojo *et al.*, 1997).

However. Peumans and Van Damme (1997) have recently proposed a new classification of lectins on the basis of their overall structure and biochemistry of the subunits viz., merolectins, hololectins, chimerolectins and superlectins (Figure 1). Merolectins are proteins that are built exclusively of a single carbohydrate-binding domain. They are small, single polypeptide proteins, and are incapable of precipitating glycoconjugates or agglutinate cells because of their monovalent nature. Examples of this group are hevein (Van Parjis *et ah,* 1991) and the monomeric mannose-binding lectins from orchids. Hololectins are also built exclusively of carbohydrate-binding domains but contain two or more such domains that are either identical or very homologous. This group comprises all lectins that have multiple binding sites and hence, are capable of agglutinating cells or precipitating glycoconjugates. Obvi-

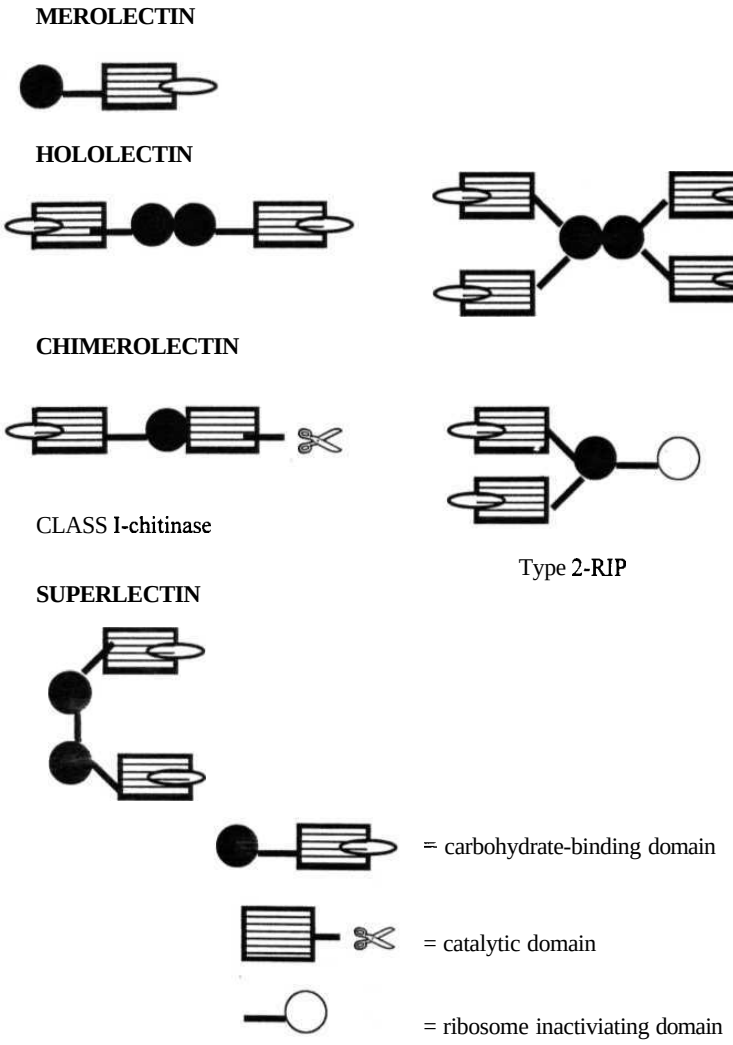


Figure 1: Schematic representation of four types of lectins merolectins, hololectins, chimerolectins and superlectins.

ously, the majority of all known plant lectins are hololectins. Chimerolectins are fusion proteins possessing carbohydrate-binding domain tandemly arrayed with an unrelated domain, which has a well-defined catalytic activity (or another biological activity) that acts independently of the carbohydrate-binding domain. Depending on the number of sugar binding sites, chimerolectins behave as merolectins or hololectins. For instance, type-2 Ribosome inactivating Proteins (RIPs) with two carbohydrate binding sites on their B-chain (e.g., ricin) agglutinate cells, whereas class I plant chitinases with a single chitin binding domain do not. Superlectins are special type of chimerolectins which built in a fusion of two tandemly arrayed carbohydrate binding domains that are structurally different and recognizes structurally unrelated sugars e.g. tulip lectins.

Some of the lectins are metalloproteins containing predominantly the divalent cations of Ca, Mg, Mn, Zn and Cu (Goldstein and Poretz, 1986). The metal ions in Con A render the lectin a high degree of stability which protects it against both high temperature and proteolytic degradation (Thomasson and Doyle, 1975; Blumberg and Tal, 1976). Legume lectins are composed of two or four identical or very similar subunits with molecular sizes ranging from 25 to 30 kDa, each with a single small carbohydrate combining site and tightly bound Ca^{2+} and Mn^{2+} ions for carbohydrate binding (Einspahr *et al,* 1986). The *Dolicos biflorus* and *Macrotyloma axillare* lectins contain Ca^{++}, Mg^{++}, Mn^{++} and Zn^{++} metal ions. The *Evonymus europaea* lectin contains Ca^{++}, Mg^{++}, Zn** and Cu^{++} ions. Lectin from *D. biflorus* binds several divalent cations but only Ca^{++} is required for the lectin to express full binding activity (Borrebaek *et al,* 1981). By employing X-ray crystallographic techniques, the three-dimensional structure, metal ion requirements and carbohydrate binding properties of related lectins have been revealed (Einspahr *et al,* 1986). The metal ions are located

close to the combining site where they help to position amino residues for carbohydrate binding but do not bind directly to the carbohydrate. In some lectins the metal ions maintain a high degree of stability, which protect them against both high temperature and proteolytic, and require for haemagglutination. In others the removal of the metal ions had no effect on the carbohydrate binding and other biological aspects (Goldstein and Poretz, 1986).

BIOLOGICAL PROPERTIES OF LECTINS

Lectins become indispensable tools in biological and medical research. They are used in wider applications such as agglutination of erythrocytes, mitogenic stimulation of lymphocytes, insulin like effect on fat cells, toxicity to cells, inhibition of fungal, bacterial and viral growth, insecticidal property, anti HIV property and recently reported to have nuclease like activity has been highlighted (Kumar *et al*, 1993; Girbes *et al*, 1996; Batelli *et al*, 1997). The biological properties of lectins are as follows:

(i) **Agglutination:** Agglutination is the most easily detectable manifestation of the interaction of a lectin with cells and is used to detect the presence of lectin in a biological source. The ability to agglutinate cells distinguishes lectins from other sugar-binding macromolecules, such as glycosidases and glycosyltransferases. Agglutination occurs when the bound lectin forms multiple cross bridges between opposing cells. It was Boyd and Shapleigh 1954 who found that lectins are blood group specific. A and B blood group specificity has been found in lectins of *Sophora japonica*, *Calpurnea aurea* and *Dolichos biflorus* (Etzler and Kabat, 1970). *Iris amara* lectin is M blood group specific, whereas *Vicia graminae* and *Bauhinia purpurea* lectins are N blood group specific (Sengupta *et al*, 1997). Lectin from *Erythrina velutina* seeds is A, B and O blood group-specific (Stojanovic *et al*, 1997) and *Parkia javanica* lectin

agglutinates RBC of rabbit and rat (Utarbhand and Akkayanont, 1995). Agglutination is affected by many factors, such as the molecular size, saccharide binding sites of the lectin and cell surface properties like number and accessibility of receptor sites, membrane fluidity and metabolic state of the cells (Nicolson, 1976). In addition, agglutination is affected by external conditions of the assay such as temperature, cell concentration, mixing etc. When agglutination does occur and is inhibited by an appropriate sugar it can be taken as an indication that carbohydrate structures for which the lectin is specific are present on the surface of the cells.

The variability in the agglutination with lectins is also used in detection of changes on cell surfaces during physiological and pathological processes (Nicolson, 1976). Chemical or enzymatic modifications of lectins or modifications on cell surfaces by enzymes or mild proteolyses often have remarkable effects on cell agglutination by lectins. For instance, cross-linking of soybean agglutination with glutaraldehyde increases the haemagglutinating activity of the lectin for human erythrocytes by 100 to 200 fold (Lis and Sharon 1986b). Human erythrocytes are not agglutinated by peanut agglutinin even at a concentration as high as 1 mg of lectin/ml. Removal of N-Acetylneuraminic acid by treatment of the cell with sialidase renders them agglutinable by microgram quantities of the lectin (Lis and Sharon, 1981). Treatment of human erythrocytes with galactose oxidase greatly diminishes their susceptibility to agglutination by soybean agglutinin (Lis *et al.*, 1982).

(ii) Mitogenic stimulation of lymphocytes: One of the most dramatic effects in the interaction of lectins with cells is mitogenic stimulation, i.e. the triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation. The first mitogenic lectin to be described was PHA from red kidney bean (***Phaseolus*** *vulgaris)* (Nowell, 1960). It was 1970 by which three additional mitogenic lectins were known

i.e. a lectin from *Wistaria floribunda,* Con A and pokeweed mitogen (Lis and Sharon, 1977). Con A was the first mitogen whose activity could be readily inhibited in a reversible manner by low concentrations of simple sugars. During the past decade, several lectins have been recognized to have mitogenic activity. Recent examples include the lectins isolated from *Hura crepitans* (Falasca *et ah,* 1980), *Lathyrus sativus* (Koleberg and Sletten, 1982), *Abrus precatorius* (Olsnes and Pihl, 1982), and almost all of these mitogens are inhibited by simple sugars.

Most mitogenic lectins stimulate only the thymus-dependent population of lymphocytes (T-cells) and are inactive or inhibitory for mitosis of the other class of lymphocytes, the thymus independent or B-cells. However the lectins from chicken tissue, and from the slime mold *Dictyostellium purpureum* (Lipstick *et ah,* 1980) stimulate mouse B-cells but not T-cells. The lentil lectin previously considered as T-cell mitogen that has also been shown to stimulate human B-cell proliferation. WGA, previously regarded as a non-mitogenic lectin, under suitable conditions stimulates markedly both human B-lymphocytes (Greene *et al.,* 1981) and T-lymphocytes (Udey *et ah,* 1980). Lectin stimulated lymphocyte release a variety of biologically active polypeptides, known as lymphokines (Cohen *et ah,* 1979). The best characterized of these lymphokines are interleukin-2 (IL-2) or T-cell growth factor (Gillis *et ah,* 1982) and γ-interferon (Epstein, 1981).

Mitogenic stimulation could be resolved into two separately regulated phases. The first is mediated via a lectin-receptor interaction and represents the transition of the cell from the resting state to G|. It is manifested by morphological changes and blastogenesis by the induction of "early genes" that may be important in controlling the transit of cells through the cell cycle and by the generation of functional receptors for interleukin-2. This stage is referred to as "competence induction" or "activation".

Interaction of interleukin-2 with its specific receptors on the surface of competent cells initiates the second, proliferate phase of mitogenic stimulation, which eventually leads to DNA synthesis and cell division (Lis and Sharon, 1986a).

(iii) Induction of suppressor cells: Lectins are capable of inducing the suppressor cells that inhibit the activities of T and B-cells. Treatment of human and murine lymphocytes with various mitogens, in particular Con A, also induces the generation of potent suppressor cells capable of inhibiting activities of T and B-cells *in vitro*. The ability to develop suppressor cell activity upon the treatment with Con A, in humans seems to be a prerogative of normal lymphoid cells. The generation of suppressor cells is decreased in peripheral blood lymphocytes of patients with immunodeficiency diseases. Lectins provide a rationale for using the measurement of mitogen-induced suppressor cell activity as a convenient clinical test for assessing the level of the immune competence of patients (Lis and Sharon, 1986b).

(iv) **Toxicity:** Several lectins e.g., Con A, wheat germ agglutinin, PHA and the lectin from *Robinia pseudoacacia* are toxic to mammalian cells both *in vitro* and *in vivo* (Liener *et al,* 1986). Toxic lectins such as ricin from *Ricinus communis,* abrin from *Abrus precatorius* and modeccine from *Adenia digitata* are transported along with neuronal processes to the cell body where they inactivate ribosomes resulting in neuronal death (Wiley *et al,* 1982). This process of "suicide transport" suggests a powerful new experimental strategy for solving neurobiological problems. These toxins consist of two chains inter-linked by disulfide bonds. The heavier (B) chain possesses the carbohydrate binding site, whereas the lighter (A) chain inhibits protein synthesis in cell free systems thus representing the toxic moiety of the molecule (Olsnes and Pihl, 1982; Fulton *et al,* 1986; Endo and Tsurugi, 1987; Endo *et al,* 1987). Only the intact molecule is active on cells. The B-chain binds the toxins to receptors on the

cell surface, and the A-chain enters the cytoplasm and inactivates the ribosomal subunits affecting the activities involved in peptide elongation reaction (Endo *et al,* 1987; Hartley *et al,* 1991). Therefore, these toxins are called as Ribosome Inactivating Proteins (RIPs).

Toxic lectins are generally selective in their action on cells. In particular, transformed cells are frequently much more sensitive to the cytotoxic effects of lectins than normal cells. Based on this property attempts have been made to inhibit tumor growth by lectins *in vivo*. The protective effect of ricin (Lin *et al,* 1970; Fodstad and Pihl, 1980;), Con A (Shoham *et al,* 1970) and *Griffonia simplicifolia* I lectin (Eckhardt *et al,* 1982) against tumor growth in experimental animals has been described. The protective effect of ricin and abrin in humans (Lis and Sharon, 1986a; Olsnes and Pihl, 1982) against tumor growth and in combination with anticancer drugs has also been described.

Immunotoxins are conjugates in which cell-binding ligand couples to toxins or their subunits. If the ligand is a cell specific monoclonal antibody the immunotoxin should selectively kill target cells. Among the lectins ricin A-chain is the most frequently used RIP to prepare immunotoxins (Vallera and Myers, 1988). Studies have demonstrated that these RIPs along with glycoconjugates make excellent cytotoxic agents when targeted and internalized (Lambert *et al,* 1988). The immunotoxins were used in the treatment of autoimmune diseases such as thyropathy and myasthenia gravis (Rennie *et al,* 1983; Killen and Lindstrom, 1984; Krolick, 1989) and the killing of parasites (Villemez and Carlo, 1984; Santana and Teixeira, 1989; Teixeria and Santana, 1990).

(v) **Lectin dependent cytotoxicity of lymphocytes and macrophages:** The interaction with and killing of target cells by cytotoxic T-lymphocytes requires the specific

recognition by the effected cells, mediated by lectin, a phenomenon known as lectin dependent cytotoxicity (Parker and Martz, 1980). The mitogenic lectins shall not have immune specificity to both effected and target cells and thus promoting the proximity between the two types of cells necessary for facilitating the cytotoxic activity of the effected cells (Greene *et al,* 1981; Parker and Martz, 1980). According to Bonavida and Katz, 1985 the lectin plays no direct role in the intercellular recognition or in the activation process, but acts by binding to and modifying the surface of the target cells, rendering them recognizable by the cytotoxic lymphocytes.

Lectins from wheat germ (Kurisu *et al,* 1980), *Griffonia simplicifolia* (Maddox *et al,* 1982) and the insect *Sarcophaga peregrina* (Ohkuma *et al,* 1985) possess the ability to mediate carbohydrate specific binding of mouse macrophages and tumor cells and to induce killing of the tumor cells by the macrophages. Lectin mediated cytotoxicity may occur *in vivo,* since intraperitoneal injection of *Griffonia simplicifolia* I lectin protected mice inoculated with Ehrlich ascites tumor cells from tumor growth and subsequent death (Eckhardt *et al,* 1982).

(vi) Lectinophagocytosis: During late 1980s, it has been shown that specific recognition between phagocytes and their targets can be accomplished by lectins on the surface of the type cell that combine with complementary sugar on the surface of another cell in a lock and key manner. This type of recognition has been described as lectinophagocytosis (Sharon, 1987). Lectinophagocytosis in bacteria can occur by two major modes. In the first mode, the bacteria, which carry surface lectins, bind to complementary carbohydrates on the surface of the phagocyte cells. In the second mode, lectins that are integral components of the phagocytic cell membrane bind to carbohydrates on the bacterial surfaces. Lectin carrying bacteria also bind readily to sugars on phagocytic cells e.g., human polymorphonuclear leukocytes or human and mouse

peritoneal macrophages (Sharon, 1987). WGA markedly enhance the binding and phagocytosis of bacteria such as *Staphylococcus aureus* H, *Staphylococcus albus* and *Micrococcus luteus* (Gallily *et al*, 1984). Ingestion of the bacteria occurred with an encapsulated *E. coli* cells were attached to human polymorphonuclear leukocytes by bridging with Con A (Gallily *et al*, 1984).

DISTRIBUTION OF LECTINS

(i) Lectins in Protozoa: The occurrence of lectins has been documented in the pathogenic amoebae *Entamoeba histolytica* which causes dysentery in humans (Mirelman, 1986). Several sugars inhibit the amoebic adherence to enterocytes suggesting that the adherence be mediated by lectin carbohydrate interactions. Two distinct lectins, one specific for β1-4-linked oligomers of N-Acetyglucosamine and the other for galactose and N-Acetylgalactosamine have been isolated from *E. histolytica* (Petri *et al*, 1989).

(ii) **Lectins in Slimemolds:** Studies of slime mold lectins grew out of an interest in the endogenous functions of lectins, especially in cell-cell and cell-matrix interactions. The cellular slime mold *Dictyostelium discoideum* was taken as a model system for studying specific cellular associations. Interestingly, these cells exist as unicellular amoebae when food is available but transform into adhesive aggregating cells within eight hours of starvation. From the agglutinating cells two haemagglutinins namely discoidin I was and discoidin II were isolated and their activity was inhibited by simple sugars including N-Acetylgalactosamine, galactose and lactose (Simpson *et al*, 1974). Discoidin I is intimately involved in slime mold aggregation, through a short segment the tripeptide Arg-Gly-Asp. The same peptide is present in many other adhesion molecules, such as fibronectin, which again constitutes an important cell-binding recognition site (Ruoslahti and Pierschbacher, 1987).

(iii) Lectins in Viruses: Lectin carbohydrate recognition system is best characterized in the interaction of influenza viruses with their target cells (Paulson, 1985). The ability of the virus to agglutinate erythrocytes has been known since 1941. The viral haemagglutinin (lectin) responsible for the binding of human virus to erythrocytes and other cells by N-Acetylneuraminic acid, one of the sialic acids, present on the cell surface and the binding is prerequisite for initiation of infection. The fusion of the viral and cellular membranes allowing release of the cell membranes by sialidase abolishes binding and prevents infection. In turn reattachment of sialic acid or insertion of sialic acid containing oligosaccharides into the membranes of sialidase treated cells restores the ability of the cells to bind the virus and to be infected by it (Vlasak *et al*, 988; Weis *et al*, 1988). The lectin is made up of two subunits HA1 and HA2 having molecular masses of 36 and 26 kDa respectively, covalently linked by a disulfide bond and is located on the surface of the viral membrane (Paulson, 1985). A detailed knowledge of the sialic acid haemagglutination interaction provides a possible basis for the design of anti-viral drugs that would block viral attachments to cells (Sharon and Lis, 1989).

(iv) Bacterial lectins: Bacteria posses the ability to bind and agglutinate erythrocytes and other types of cells through their hair like appendages called fimbriae or pili which contain lectins on their surfaces. e.g. *Escherichia coli, Klebsiella pneumoniae, Vibrio cholerae,* actinomycetes sp. etc. (Mirelman, 1986; Sharon, 1987). Binding to cells and agglutination are frequently inhibited by simple sugars like galactose indicating that these activities are due to lectins present on the bacterial surfaces or due to intracellular lectins (Sharon and Lis, 1989). *E. coli* and oral actinimyces bind readily to sugars on phagocytic cell results in metabolic activation of the phagocytes, ingestion of the bacteria, and eventual bacterial death. The lectin-mediated phagocytosis

may be of clinical relevance in immuno deficient hosts and in tissues (Sharon and Lis, 1989).

(v) Lectins in animals: Several lectins have been identified and purified from a variety of animals including sponges (Bretting and Kabat, 1976; Muller *et al,* 1979), snails (Hammarstrom and Kabat, 1969; Van Der Knaap *et al,* 1982), horseshoe crabs (Marchalonis and Edelman, 1968), insects (Komano *et al,* 1980; Suzuki and Natori, 1983), sea urchins (Ryoyama, 1974; Sasaki and Aketa, 1981; Yamada and Aketa, 1982), fish (Teichberg *et al,* 1975), frogs (Robertson and Barondes, 1982), chicks (Nowak *et al,* 1977) and mammals (Hudgin *et al,* 1974), including humans (Bladier *et al.,* 1991). There are three major structurally distinct groups of animal lectins, Ca^{2+} dependent (C-type) and sulphydryl-dependent (S-type) and carbohydrate recognition domain (CRD) with an affinity for β-galactosides (galectins) (Drickamer, 1988; Ozeki *et al.,* 1991). Animal lectins play important roles in cell-cell or cell-matrix interaction by recognizing specific carbohydrate moieties of glycoproteins and glycolipids (Barondes, 1988; Sharon and Lis. 1989: Springer and Lasky, 1991).

PLANT LECTINS

Although lectins are widely distributed in nature, much of the research has been focussed on plant lectins. Lectins are found in different species of major taxonomical groups and most of them considered as seed storage proteins (Etzler, 1986; Su *et al,* 1980). Nevertheless, they are also often quite abundant in vegetative organs such as roots, leaves, rhizomes, bulbs, tubers, corms, stems, bark, flowers, fruits, phloem sap, latex and even nectar (Etzler, 1986; Peumans and Van Damme, 1995; Seshagirirao and prasad, 1995). Amongst plants the Fabaceae (legumes) and the Poaceae (Graminae) in dicots and monocots, respectively, are the most thoroughly studying families for the lectins. The legume lectins make up a class which sharing

structural and amino acid sequence homologies (Strosberg *et al,* 1986; Sharon and Lis, 1990). However, the homology between legume lectins from *Bandeiraea simplicifolia, Bauhinia purpurea, Phaseolus lunatus* and *Vigna radiata* is concomitant with a lectin from castor bean (*Ricans communes),* which is a non-legume (Hankins *et al,* 1979). This suggests that there may be a unique portion of these proteins that is evolutionarily highly conserved. In Poaceae, the lectins with similar sugar specificity and structural properties to the WGA have been isolated from rye and barley embryos (Peumans *et al,* 1982), and the biochemical studies were conducted on Triticale (Siva Kumar and Padma, 1996).

Plant lectins occur in multiple forms that possess similar biological activities and differ in their chemical properties (Lis *et al,* 1966; Agarwal and Goldstein, 1968; Gould and Scheinberg, 1970; Entlicher *et al.,* 1970; Ticha *et al,* 1970; Entlicher *et al,* 1971). These proteins are comprised of subunits, which undergo complex association and dissociation reactions, giving rise to multiple forms of the same lectins that differ in molecular size (isolectins). Such phenomenon was well observed in soybean (Catsimoolas and Mayer, 1969), garden pea (Entlicher and Kocourek, 1975), wheat germ (Rice, 1976), lentil (Howard and Sage, 1969), *Viccia graminae* (Duk and Lisowska, 1981), *Griffonia simplicifolia* (Murphy and Goldstein, 1979), *Hura crepitans* (Barbieri *et al,* (1983), *Maclurapomifera* (Bausch *et al,* 1981), *Psophocarpus tetragonolobus* (Kortt, 1984) and *Abrus precatorius* (Hegde and Podder, 1991, 1997). Post-translational modifications including cleavage and variable degrees of glycosylation account for dispersion of molecular weight as well as for most of the polymorphism (Strosberg *et al,* 1986; Hegde and Podder, 1992).

Some of the plant lectins exhibit ribosome inactivating property that depurinates the rRNA, thus damaging ribosomes and arresting the protein synthesis (Endo

and Tsurugi, 1987; Stirpe *et al,* 1992). These lectins are called as Ribosome Inactivating Proteins (RIPs). RIPs have been recently classified into three categories, type-1, 2 and 4 (Citores *et al,* 1993). Type-1 RIPs consist of a single enzymatic polypeptide chain and are non-lectins. Type-2 RIPs contain two polypeptides chains A and B linked by disulfide bonds, A being the enzymatic chain, and B a D-galactose binding chain. Type-4 RIPs are composed of two dimers associated by non-covalent forces, each dimer comprising of two polypeptide chains linked by disulfide bonds (A_2B_2). The RIPs can also be subdivided into two categories; the toxic, which are lethal to the living organisms *in vivo* and the non toxic, which possess the property of inhibition of protein synthesis *in vitro*. Since the identification of the toxic lectins by Stillmark (1888) only eight type-2 toxic RIPs have been characterized; *viz.*, ricin (Olsnes and Pihl, 1973a), modeccine (Barbeiri *et al,* 1980), viscumin (Stirpe *et al,* 1980a), volkensin (Stirpe *et al.,* 1985), three abrins (Hegde *et al,* 1991) and *Eranthys hyemalis* lectin (Kumar *et al.,* 1993). There are three type-4 RIPs characterized i.e. *Ricinus communis* agglutinin 60 and 120 kDa (Hegde and Podder, 1992) and *Viscum album* four chain agglutinin (Citores *et al,* 1993). It is interesting to note that the toxic type-2 RIPs are more than 100 times lethally potent when compared to the toxic type-4 RIPs. Recently type-2 non-toxic RIPs have been reported (Citores *et al,* 1997; Rojo *et al,* 1997; Van Damme *et al.,* 1997a). The physiological, biochemical, cellular, molecular and evolutionary evidences indicate that lectins have a role in plant defense. These lectins are stable over wide pH range, withstand that and are resistant to animal and insect proteases. Type-2 RIPs offer the plant good protection against insects and plant viruses by inactivating the ribosomes and thereby provoke a form of hypersensitive response (Gatehouse *et al,* 1990; Kumar *et al,* 1993). A major argument for this role is that the plant lectins bind to glycoconjugates of other organisms

(Peumans and Van Damme, 1995; Zhu *et al,* 1996; Rao *et al,* 1998).

The main interest on the plant lectins is due to their ability to serve as recognition molecules within a cell, between cells, or between organisms. A number of physiological roles attributed to them including recognition of nitrogen fixing bacteria at the surface of roots (Bohlool and Schmidt, 1974; Sharon and Lis, 1989; Brewin and Kardailsky, 1997), host-pathogen interactions (Broekaert and Peumans, 1986; Broekaert *et al,* 1989, Murdock *et al,* 1990; Czapla and Lang, 1990; Van Parijis *et al,* 1992) and transport of sugars, hormones and glycoproteins (Liener, 1976; Liener *et al,* 1986). There are a number of ways by which lectins interact with molecules within and outside the cells and draw considerable attention towards their potential biological activities and also their natural functions in the plant that made them. During sexual reproduction the algal *{Chlamydomonas)* cells differentiate into gametes adhere to each other only via the agglutinins present on the surfaces of their flagella (Musgrave and van den Ende, 1987). Several of the plant lectins show mitogenic activity by interacting with lymphatic cells which eventually leads to DNA synthesis and cell division (Toyoshima *et al,* 1971; Waxdal, 1974; Toyoshima and Osawa, 1975).

(i) Anti-insect activity: Since glycoproteins are major constituents of these membranes, the binding of a lectin to a glycoprotein receptor provokes a local or systemic deleterious effect, as a result of which the insect may be repelled and/or retarded in its growth or killed. The epithelial cells along the digestive tract of phytophagus insects are directly exposed to the contents of the diet and therefore, are possible target sites for plant defense proteins. The lectins from wheat germ, potato tubers and seeds from peanut, thorn apple *{Datura stramonium)* and storage orange *{Madura pomifera)* show an inhibitory effect on the development of larvae of the cowpea weevil (Mur-

dock *et al,* 1990). The pokeweed (*Phytolacca americana*) lectin killed larvae of the southern corn rootworm (Czapla and Lang, 1990). WGA has an inhibitory effect on development of two important maize pests, the European cornborer (*Ostrinia nubilalis*) and Southern corn root worm (*Diabrotica undecimpunctata*) (Czapla and Lang, 1990). The chitin-binding lectins from rice (*Oryza sativa)* and stinging nettle also moderately inhibited larval growth of the cowpea weevil (Huesing *et al,* 1991). Similarly, Feeding trials with purified lectins from snowdrop (*Galanthus nivalis)* and garlic (*Allium sativum)* indicated that they are moderately active against cowpea weevil and the tobacco horn worm (*Spodoptera litoralis*) (Hilder *et al,* 1995). Type-2 RIP ricin was highly toxic to the coleopteran insects *Callosobruchus maculatus* and *Anthonomus grandis* (Gatehouse *et al,* 1990). Another type-2 RIP from winter aconite (*Eranthys hyemalis)* inhibited larval growth of *Diabrotica undecimpunctata* (Kumar *et al,* 1993). Although, the mechanism of toxicity is on the basis of specific binding of the glycoconjugates to the gut of the insect, the specific mechanism has not been clearly elucidated. There are three postulations for the mechanism i.e. (i) binding of lectin to the chitin in peritrophic membrane (only for chitin binding lectins), (ii) binding to glycoconjugates exposed on the epithelial cells along the digestive tract (iii) binding of lectins to glycosylated digestive enzymes (Peumans and Van Damme, 1995).

(ii) Antiviral activity: Several plant lectins are potent inhibitors of animal and human viruses, which have glycoproteins in their virions (Sengupta *et al,* 1997). Type-2 RIP from *Eranthis hyemalis* has an inhibitory activity against plant virus (Kumar *et al,* 1993). Possible the RIP included in the virus suspension used for the infectivity test kill the wounded plant cells by inactivating the ribosomes. Plant lectins may have an indirect antiviral role also. The presence of insecticidal lectins may indirectly prevent

and/or reduce the spread of insect transmitted viral diseases.

(iii) Antibacterial activity: Plant lectins play a role in the plant defense against bacteria, and it must be through an indirect mechanism that is based on interactions with cell wall carbohydrates or extracellular glycans. Such interactions observed between several legume seed lectins and bacterial cell wall components muramic acid, N-Acetylmuramic acid and muramyl dipeptide (Ayouba *et al,* 1994). Another indirect defense mechanism involves the blocking of the movements of normally motile bacteria at the air-water interface of the thorn apple *{Datura stramonium)* seed lectin (Broekaert and Peumans, 1986). The effect was fully reversed by fetuin a lectin binding protein. The lectin-mediated block of bacterial motility *in vitro* was correlated with the rapid and highly specific release (during imbibition) of the lectin from the seed coat and the seed epidermis (Broekaert and Peumans, 1986).

(iv) **Antifungal activity:** Plant lectins cannot bind to glycoconjugates on the fungal membranes or penetrate the cytoplasm of the cells because of the presence of thick and rigid cell walls. Hence direct interference with the growth and the development is unlikely. However, indirect effects based on the binding of lectins to carbohydrates exposed on the surface of the fungal cell wall are possible. The lectin from stinging nettle (*Urtica dioica)* inhibited the growth of *Botrytis cinera, Trichoderma hamatum* and *Phycomyce blackesleeanus* (Broekaert *et al.,* 1989). Lectins do not affect the normal metabolism of the fungal cells. Only the synthesis of the cell wall appears to be affected as a result of disturbed chitin synthesis and/or deposition (Chrispeels and Raikhel., 1991; Van Parijs *et al.,* 1992). The molecular basis of recognition and adhesion is determined by specific binding of lectins and haptens on the surface of the host and the pathogen. Biochemical and molecular biological investigations have revealed that several strains of the fungus *Trichoderma* are mycoparasitic to specific soil borne

plant pathogenic fungi and here by is an effective biocontrol agent. The specifcity is due to lectins, which are involved in the recognition between *Trichoderma* and host fungi (Sengupta *et al,* 1997). Treatment of conidia of *Disculla umbriella* with different lectins resulted in inhibition of its attachment responsible for adhesion of the conidia to the host and glycoproteins are involved in recognition and attachment process (Peumans and Van Damme, 1995). Chitin-binding proteins affect the growth of organisms that contain chitin (fungi and insects) (Mirelman *et al,* 1986; Raikhel *et al,* 1984).

PERSPECTIVES AND SCOPE OF THE PRESENT INVESTIGATION

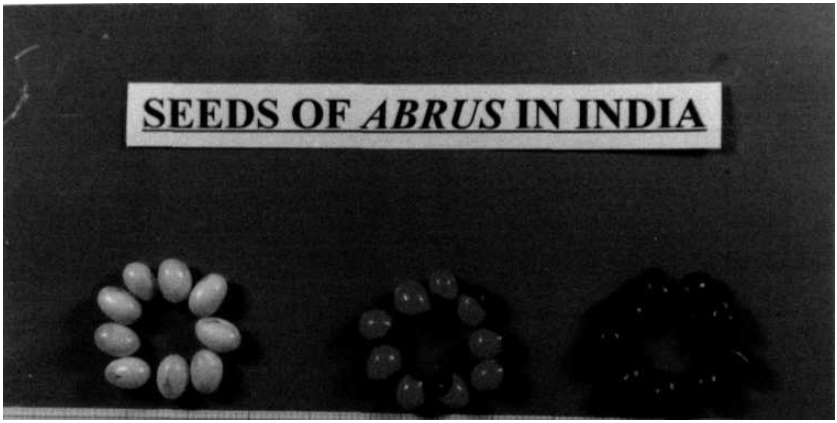
Owing to the ready availability and high protein content, the seeds of a number of Fabaceae (Leguminosae) species have become potential source for lectins and their studies (Toms and Western, 1971; Etzler, 1986). In Fabaceae the *Abrus precatorius* L. is one of the best known lectin ever since the beginning of lectinology. The studies on the lectins from *A. precatorius* are as old as the lectins from *Ricinus communis*, wherein the foundation was established for immunology and lectinology (Stillmark, 1888; Liener *et al,* 1986; Van Damme *et al,* 1997b). The toxic lectins from these two plants are type-2 RIPs and are highly lethal to humans and other mammalians (Stirpe *et al,* 1992). Interestingly the lectins from *A. precatorius* and *R. communis* belong to the galactose-binding group, but do not have immunological relationship. *A. precatorius* lectins are well known for their applications in biochemical, immunological and clinical investigations (Goldstein and Poretz, 1986; Van Damme *et al.,* 1997b; Wu *et al,* 1997). Several methods for isolating the lectins from *A. precatorius* seeds by a combination of affinity, ion-exchange and gel filtration chromatographic techniques have been reported (Table 1).

Table 1: Comparison of *Abrus precatorius* toxins and agglutinins

Protein	Purification Method	M, (kDa)	pI	(µg)	References
Abrin	Seph-4B	65	*	0.07	Olsnes and Pihl., 1973b
Abrin Agglutinin	Seph-4B + Ion-exchange	65 130	6.1 5.2	0.07 10.00	Olsnes *et al.*, 1974b
Abrin A Abrin C	Ion-exchange + Seph-4B	60.1 63.8	* *	0.6 0.2	Wei *et al.*, 1974
Agglutinin	Seph-4B	130	*	*	Wei *et al.*, 1975
Abrin Agglutinin	Seph-4B	* 132	* 4.9 5.3 5.5	0.2	Roy *et al.*, 1976
Abrin a Abrin b	Seph-4B + Ion-exchange	65 67	• *	0.25 0.625	Lin *et al.*, 1978
Abrin A Abrin C	Ion-exchange + Seph-4B	62.5 65.5	7.5 7.1 7.0	0.6 0.2	Herrman and Behnke, 1981
Abrin a Abrin b Abrin c Abrin d Agglutinin	Seph-4B + Ion-exchange	63 67 63 67 67		0.25 0.627 0.4 0.775 25	Lin *et al.*, 1981
Abrin-I Abrin -I1 Abrin- III APA-I APA-I1	Lac-Seph + Ion-exchange	64 63 63 130 128	7-8 6.6 5-6 5.4 4-5	0.55 0.06 0.25 5.0 50.0	Hegde *et al.*, 1991 Hegde and Podder, 1992, 1997

*No information; Seph-4B = sepharose-4B; Lac-Seph • Lactamyl Sepharose-4B

The *Abrus* genus belongs to Fabaceae (Leguminosae) family and representing by 12 species of tropical distribution (Willis, 1897). There are three species occurring in India i.e. *Abrus precatorius* has hard red seeds with black tips, *A. fruticulosus* Wall. with hard white seeds and *A. pulchellus* Wall. with hard black seeds (Hooker, 1876) (Figure 2). Amongst the three species *A. fruticulosus* and *A. pulchellus* are rare, and *A. precatorius* is abundant in India as well as in the tropics. Different plant parts of *A. precatorius* have also been used for the biochemical and clinical investigations (Rao,



A. fruticulosus A. precatorius A. pulchellus

1987; Sinha, 1990; Sinha and Madhur, 1990; Rajaram and Janardhanan, 1992; Ndambe *et al.*, 1994). The seeds of *A. precatorius* are rich in most of the essential amino acids and they are deficient only in cysteine and threonine, when compared to the WHO/FAO requirement pattern (WHO/FAO, 1973). Anonymous information from the medicinal plant collectors of India reveals that the seeds of *A. fruticulosus* and *A, pulchellus* are more preferred than the *A. precatorious* in the traditional medicine. Hence, the biochemical studies on the other available species are augmenting the knowledge of their utilization. Also the purification and biochemical characterization of lectins from the related species of *A. precatorius* would throw new vistas on their evolution and divergence.

The current investigation focusses on the following aspects:

1. Evaluation of cross-linked guar gum matrix, Sepharose 4B and AT-Sepharose 4B for their affinity to lectins and to customize a suitable matrix for standard purification protocols.
2. Purification and characterization of lectins from the three Indian *Abrus* species viz.. *Abrus precatorius. A. fruticulosus* and *A. pulchellus.*
3. Toxicity studies on lectins from the three *Abrus* species.
4. Immunological studies of the lectins from three *Abrus* species.
5. Studies on distribution of lectins in various parts of *A. precatorius.*
6. Insecticidal studies of the lectins on stored insect pests *Sitophyllus oryzae* (Coleoptera) and *Corcyra cephalonica* (Lepidoptera).

MATERIALS AND METHODS

MATERIALS

Abrus precatorius, A. fruticulosus and *A. pulchellus* seeds were collected from the wild vegetation of University of Hydearbad campus. The guar (*Cyamopsis tetragonolobus*) seeds were purchased from the local market. Protein molecular mass standards for SDS-PAGE (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa and α-lactalbumin, 14.2 kDa), protein molecular mass standards for gel filtration (fructose 6-phosphate kinase, 84 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa and cytochrome C, 12.3 kDa), saccharides used for the inhibition test (Methyl-β-D-Galactopyranoside (Me-β-Gal), Methyl-α-D-Galactopyranoside (Me-α-Gal), L-Fucose. α-Lactose, P-Lactose, Melibiose, Raffinose), Nonidet P-40 and Freund's adjuvants (complete and incomplete) were purchased from Sigma Chemical Company, St. Louis, USA. Sepharose-4B. DEAE-Sephacel and ampholines were obtained from Pharmacia, Uppasala, Sweden. All other chemicals/reagents used were of analytical grade manufactured from India by different firms.

Corcyra cephalonica insect larvae (early, mid and late last instar; ELI, MLI and LLI), pre pupae and pupae were reared on the feed of coarsely crushed sorghum seeds. The culture room mantained at 26±1°C temperature with 70±5% relatively humidity under a 14:10 h light and dark periods. *Sitophyllus oryzae* insects were obtained from the stored rice and reared in the culture room on the feed of rice grains.

METHODS

Protein estimation: Protein was estimated following Lowry *et al.,* (1951) with minor modifications. Reagent A was 4% sodium carbonate in 0.2 N sodium hydroxide, B was 1% cupric sulphate, C was 2% sodium potassium tartarate and D was 1N Folin's reagent (commercial). The working reagent is a mixture of A, B, C in a ratio of 23:1:1 and used within 24 h of preparation. One ml of the working reagent was added to one ml of the protein sample, mixed well and allowed to stand for 10 min. The 0.2 ml of reagent D was added rapidly while vortex the sample. After 30 min. the sample absorbance was recorded at 750 nm. BSA (Fraction V) was used as a standard protein (5-50 µg). Protein in column chromatography fractions was detected by the absorbance at 280 nm (Peterson, 1983).

Haemagglutinating activity: The haemagglutinating activity of the lectin was determined according to the method described by Lis and Sharon (1972) with minor modifications. The following are the details of the preparation of erythrocytes for the assay.

(i) Preparation of Alsevere's solution: The Alsevere's solution was prepared by dissolving 2.05 g of glucose, 0.8 g of sodium citrate and 0.42 g of sodium chloride in 80 ml of distilled water. The pH was adjusted to 6.1 with 1 % citric acid and the volume was made unto 100 ml with distilled water. The solution was autoclaved, cooled and stored at 4 °C.

(ii) Preparation of erythrocytes: Venous whole blood was added to an equal volume of Alsever's solution. The blood suspension can be stored as long as two weeks in the Alsever's solution. The erythrocytes were isolated from the stock blood suspension by centrifugation at room temperature using a table-top centrifuge (1000 x *g* for 5 min).). The packed erythrocytes at the 1000 x *g* considered as 100%. The packed cells were washed

with cold saline (0.9 % NaCl) for 3-4 times (5 ml saline for each ml of packed erythrocytes) and finally 4 % cells were made in saline.

(iii) Preparation of trypsin treated ervthrocytes: The erythrocytes were treated with trypsin on the day of assay. The 4% erythrocyte suspension incubated with 0.1% trypsin 1:250 (1000-1500 BAEE units/mg)) for one hour at 37 °C. The trypsin treated erythrocytes were washed 5-6 times with cold saline to remove the traces of trypsin and 4 % cells were made and used for the experiments.

(iv) Agglutination assay: 100 ul of the protein sample was serially diluted in a microtitre plate and 100 ul of trypsinized 4 % erythrocytes were added to each well. The agglutination was observed visually after incubation of the plate for one hour at 37 °C. The highest dilution which showed positive haemagglutination was considered as the titre. The amount of protein present at this dilution represents the minimum quantity of protein required for agglutination and is defined as one unit. Specific activity is the number of units per mg of protein.

Sugar inhibition assay: 50 ul of serially diluted carbohydrate solution was mixed with 50 ul protein containing 8 haemaglutination units in a microtitre plate and incubated at room temperature for 30 min. 100 µl of the 4 % trypsinized human O group erythrocyte suspension was added to the incubated solution and haemagglutination was recorded after incubation of the plate for one hour at 37 °C. The inhibition concentration of the sugar was recorded as the minimum concentration of sugar required for complete inhibition of 2 haemagglutination units with 2 % of the erythrocytes.

Preparation of affinity matrices:

(I) Cross-linked guar gum (CLGG) affinity matrix: The CLGG affinity matrix was prepared according to Kumar *et al,* (1982) with minor modifications. 25 g of *Cyamopsis tetragonolobus* (L.) Taub. seed powder (guar gum) was mixed with an emulsion of 7.5 ml of epichlorohydrin and 75 ml of 3N NaOH at 40 °C. The suspension was incubated for 24 h at 40 °C and then for 6 h at 70 °C to form a gel. The gel was then extensively washed with water. The gel was finally suspended in 10 mM sodium-phosphate buffer containing 150 mM NaCl, 0.02% sodium azide with pH 7.2 (PBS) and stored at 4 °C until further use.

(ii) Sepharose-4B affinity matrix: The stock Sepahrose-4B matrix was repeatedly washed to remove the fine particles and preservatives. The washed matrix was packed in the column and equilibriated with PBS.

(iii) Acid treated Sepharose-4B (AT-Sepharose-4B) matrix: The washed Sepharose-4B was treated with 0.1 M hydrochloric acid at 50°C for 3 h. After acid treatment the matrix was washed again with distilled water and equilibrated with PBS and packed in the column (Girbes *et al,* 1993).

Preparation of sample: Dry seeds were soaked over night in 5 % acetic acid at room temperature. They were then decoated and homogenized in 1:10 (w/v) of PBS. The homogenate was stirred continuously for about 3 h and centrifuged at 15,000 x *g* for 30 min at 4 °C. The supernatant was filtered through Whatman No.41 filter paper and filtered solution was designated as the crude extract.

Evaluation of CLGG, Sepharose-4B and AT-Sepharose-4B matrices for their affinity: The evaluation of CLGG, Sepharose-4B and AT-Sepharose-4B matrices was car-

ried out with the lectins from the seeds of the three *Abrus* species for their affinity efficiency. Three individual columns (1.5 x 10 cms) of CLGG, Sepharose-4B and AT-Sepharose-4B were packed and equilibrated with PBS at the flow rate of 18 ml/hr. The crude extract that contains 20 mg of protein was applied onto the affinity column and washed until the effluent absorbance was less than 0.02 at 280 nm. The bound proteins were eluted with 0.2 M lactose in PBS. All the experiments were carried out at 4°C.

Purification of *Abrus* lectins: The purification of the lectins was performed in two-steps, CLGG affinity chromatography followed by DEAE-Sephacel ion-exchange chromatography and all the experiments were performed at 4°C.

CLGG affinity column chromatographv: The crude extract was applied onto the CLGG affinity column (3 x 14 cm) and washed until the effluent absorbance was less than 0.02 at 280 nm. The bound proteins were eluted with 0.2 M lactose in PBS.

DEAE-Sephacel ion-exchange column chromatographv: The CLGG affinity eluted protein rich fractions were dialyzed against 5 mM sodium acetate buffer (pH 6.5) and applied to the DEAE-Sephacel ion-exchange column (1.5 x 32 cm), previously equilibrated with the 5 mM sodium acetate buffer (pH 6.5). The column was washed with the equilibration buffer until the effluent absorbance was less than 0.02 at 280 nm and the lectins were eluted from the column with a linear gradient of 5-200 mM sodium acetate (pH 6.5) at a flow rate 36 ml/ hour.

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE): Sodium dodecylsulphate-polyacrylamide gel electrophoresis for proteins was performed following the method of Laemmli (1970) with minor modifications. The seperation of proteins was performed in 11% resolving gel with 4% stacking gel. Both the resolving and stack-

ing gels contained 2.4% bis-acrylamide as a cross-linker and 0.1 % SDS. The final buffer concentrations were 0.45 M Tris-HCl (pH 8.9) in resolving gel and 0.2 M Tris-HCl (pH 6.7) in stacking gel. Ammonium persulphate and N,N,N,N-tetramethylethylene diamine (TEMED) were used as polymerizing reagents in final concentrations of 0.05 % and 0.1 %, respectively. The electrode buffer comprised of 6.025 g Tris and 14.4 g glycine in one litre of distilled water (pH 8.3). The sample buffer constitutes 0.062 M Tris-HCl (pH 6.7), 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol and 0.001% of bromophenol blue. The samples were incubated for 3 min in boiling water with the sample buffer and centrifuged for 5 min at 5000 x *g*. The clear sample solutions were loaded in the slab gel wells. The samples were loaded on the gel of 8 x 8 x 0.1 cm dimension which was polymerized in plain glass plates and was fixed to Broviga (India) mini-vertical slab-gel apparatus. Gels were run at room temperature at a voltage of 75 and 100 DC (direct current) for stacking and resolving gel, respectively. Electrophoresis was carried out until the bromophenol blue dye marker reached about 3-4 mm from the bottom of the gel. Then the gels were removed, fixed in the fixative (50 % methanol, 12 % acetic acid and 0.0185 % formaldehyde) and stained for detection of proteins.

Silver staining of the **gels:** The proteins were detected in the gel by silver staining procedure according to Blum *et al* ., (1987). The gel was fixed for more than 1 hr in fixative and washed 3 times with 50 % ethanol for 3 times in a 20 min. interval. The washed gel was pre-treated with 0.002 % sodium thiosulfate solution for exactly one minute and rinsed 3 times quickly with distilled water. The pre-treated gel was impregnate for 20 min. in 0.2 % silver nitrate solution contains 0.02775 % formaldehyde. The gel was rinsed 2 times with distilled water and developed for the proteins with the solution con-

tains 6 % sodium carbonate, 0.0185 % formaldehyde and 0.0004 % sodium thiosulfate. The gel was stopped for the development of protein bands in appropriate intensity with the fixative solution for 10 minutes and was stored in 50 % methanol at 4 °C.

Two-dimensional polyacrylamide gel electrophoresis: Proteins were separated by isoelectric focussing 0.3 x 10 cm long tubes, according to the method of O'Farell (1975). The second dimension slab gel for molecular weights was performed by SDS-PAGE as described previously and the gels were silver stained according to the method of Blum *et al.,* (1987).

(i) Isoelectric focussing (First dimension): 30 % polyacrylamide (1.8 % bis-acrylamide as a cross linker) tube gels (0.35 x 8 cms) in the presence of 9.5 M urea, 10% Nonidet P-40 and 2% ampholines (pH 4-6 range 1.6% and pH 3.5-10 range 0.4%) was performed for the first dimension. . The gels were subjected to pre run at 200 volts for 15 min, 300 volts for 30 min and 400 volts for 30 min with 0.03 M sodium hydroxide (NaOH) as cathode solution and 0.01 M phosphoric acid (H_3PO_4) as anode solution. After pre-run. anodic and cathodic solutions were replaced with fresh solutions. Approximately 5ug of pure protein in the sample buffer (9.5 M urea, 2% ampholines, 10% (w/v) Nonidet-40 and 5% β-mercaptoethanol) was loaded on the gels. The protein samples were overlaid with 10 µl of 8 M urea followed by 0.03 M NaOH. One tube gel was run without protein sample for measuring the pH. Electrophoresis was carried out at 600 volts for 12 h, 700 volts for 1 h and finally for 1 h at 800 volts. The gels were extruded out of the tube and equilibrated in the equilibration buffer (0.0625 Tris-HCl, pH 6.7 with 10% glycerol, 0.005 M DTT and 2.3% SDS) for 1 h. Thereafter the gels were removed and used for second dimension immediately or pH measurement. The gels can be stored at -70 °C for

long time until further use.

(ii) Second dimension: The second dimension was performed in a discontinuous SDS-PAGE and silver stained as described previously. The IEF gels were placed on stacking gel and sealed with 1 % agarose made in equilibration buffer. The standard markers were loaded in a corner side of the gel.

(iii) Measurement of pH: The isoelectric focussed gel was cut into 0.5 cm pieces and placed in individual test tubes containing 1.0 ml of double distilled water. These test tubes were vortexed and kept overnight at room temperature. Then the pH was measured with a pH meter.

Carbohydrate estimation: Total carbohydrate was estimated by the method of Dubois *et ah,* (1956). One ml of the sample was mixed well with 40 µl of 80% phenol and 2.5 ml of sulfuric acid was added rapidly. The steam of the acid was directed against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min. After which they were mixed well and cooled to room temperature. The absorbance was recorded at 490 nm. D-galactose (5-50 µg) was used as standard carbohydrate.

Toxicity studies: Wistar strain male and female rats of 90 days old weighing approximately 250 ± 10g were injected intraperitoneally with 0.5 ml of non-pyrogenic sterile normal saline containing various quantities of lectin preparations. Control animals were injected with saline without lectin. The rats were given free access to food and water, and death incidence was recorded at 12 hr intervals upto 72 hr. The LD_{50} value was then determined in five groups of four rats each that received a narrower dosage range of the lectins preparation following the method of Miller and Trainter (1944). A correction factor is applied to 0 and 100 % mortality group. The percentage of mortality values are

converted to probit values by reading the corresponding probit units from the probit table. Probit values plotted against log doses and the LD_{50} value read as the dose that corresponds to probit 5.

Determination of molecular weights for the proteins by gel filtration: The molecular weight of the lectins were determined according to the method of Andrews (1965) using Sephadex G100 gel matrix. About 4 mg of each protein in 1.0 ml of PBS with 0.2 M lactose and 0.02 M sodium azide was loaded onto the column (1.5 x 92 cm) equilibrated with the same buffer. Elution was carried out with the same buffer. The column was calibrated with the standard protein markers viz., fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kda), ovalbumin (45 kDa) and cytochrome c (12.3 kDa). 3 ml fractions were collected and the protein elution was monitored by measuring the absorbance at 280 nm.

Immunological experiments: Due to the high toxicity of the intact toxin, the immunization was started with a toxoid prepared by formaldehyde treatment of the toxic lectin (Olsnes and Pihl, 1973b). 5 mg of the toxic lectin in 3 ml of 0.05 M sodium phosphate buffer (pH 7.5) containing 1% formaldehyde was incubated for 3 days at 37°C. Then the mixture was passed through Sephadex-G25 column (1.5 x 10 cm) equilibrated with 0.05 M sodium phosphate (pH 7.5) to remove excess formaldehyde. The protein rich fractions were pooled and used for rising antibodies.

Protein (500 |ig) in saline was emulsified with an equal volume of Freund's complete adjuvant and injected into the rabbit subcutaneously in small aliquots at multiple sites on dorsal area. The rabbit was given booster dose with 500 ug by emulsifying of protein solution with equal volume of Freunds's incomplete adjuvant after three weeks

of initial injection. After one week of booster dose, blood was collected from the marginal ear vein. The blood was allowed to coagulated at room temperature and centrifuged at 5000 x *g*. The supernatant anti-serum was separated, stored in aliquots and used as antibodies . The control serum was collected from the above rabbit prior to immunization.

Western blotting: Western blotting was done according to the procedure of Towbin *el al.*, (1979) at room temperature with minor modifications. Proteins were electroblotted onto nitrocellulose using semi-dry blotting method. The membrane of the same size to the resolving gel was rinsed in water followed by soaking in the transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol).

After SDS-PAGE, the protein portion of resolving gel was washed twice with distilled water followed by transfer buffer. The semi-dry blotting was carried out in a custom made apparatus. Three sheets of buffer soaked Whatmann No. 3 papers were layered on the anode plate abd the membrane was layered over it. The gel was carefully layered on the top of the membrane and three more sheets of soaked Whatmann papers were layered. The cathode plate was placed over the Whatmann papers and the sandwitch consisting of the electrode plates, papers, membrane and gel, was tightened by clamps. The blotting was performed for 1.5 h with 0.8 mA/cm^2 constant current. After electroblotting, the membrane was washed for 5 min with 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.5 % Tween-20 (TBST) and incubated for one hour in TBST containing 5 % (w/v) low fat skimmed milk powder. The membrane was then removed and further washed five times (10 min. each) with TBST. The membrane was incubated for over night at 4°C with the antibodies of the toxic lectin (1:1000 v/v in TBST with 2 % bovine serum albumin and 50 mM lactose). Thereafter, the membrane

was washed five times (10 min. each) with TBST for 10 min each and incubated with alkaline phosphatase conjugated anti-rabbit secondary antibodies (1:1000 v/v in TBST) for one h at 37 °C. The membrane was washed five times (10 min each) with TBST. The membrane was developed with the alkaline phosphatase substrate i.e. 66 µl of nitroblue tetrazolium (50 mg/ml) and 33 ul 5-bromo-4-chloro-3 indolyl phosphate (50 mg/ml) in 10 ml buffer containing 100 mM Tris-Cl (pH 9.5) with 100 mM NaCl and 5 mM $MgCl_2$. The reaction was stopped while washing the membrane with distilled water.

Studies on distribution and localization of lectins in different parts of *A. precatorius:* The distribution of lectins in different parts was analyzed for the haemagglutinating activity as well as with Western blotting detection using the antibodies of *A. fruticulosus* toxic lectin. The seeds of *A. precatorius* were soaked overnight. The swelled seeds were surface sterilized with 0.1 % mercuric chloride for 3 min, and washed thoroughly with sterile distilled water and germinated on moistened filter papers in clean germinating boxes. The filter papers were constantly moistened with Hoagland solution and 14:10 hr of light (with 800 Lux) and dark periods maintained. The vegetative tissues (cotyledons, leaves, stems and roots) were collected from the seedlings of 12 day post-emergence. The tissues were homoginized with PBS and the homogenates were centrifuged at 15,000 x *g* for 30 min. The supernatants thus obtained were filtered through Whatman No. 41 filter paper. The clear extracts were taken as source protein. Haemagglutination assays were carried out with the different tissue protein extracts according using human O^+ type erythrocytes as described previously. The crude protein samples were resolved on SDS-PAGE, electroblotted onto nitrocellulose and the immunological localization of the lectins were made with antibodies of *A. fruticulosus* toxic lectin P-abrin as described earlier.

Insect bioassays:

(i) Insecticidal activity of abrins: The insecticidal activity of toxic lectins to stored rice insect pest *(Sitophyllus oryzae)* was tested by microincorporated feeding trials at 500, 700 and 1000 ug concentrations of abrins (Kumar *et ah,* 1993). PBS served as the control treatment. Eight insects were used each experiment. In another set of experiments were conducted with 500 and 700 µg/ml lectin concentration and the insects were introduced back into a normal diet after 3 days of exposure to the lectin treated diet. The weight and mortality were recorded at 7 days.

(ii) Larvicidal activity of abrins: The final instar larvae of *Corcyra cephalonica* were used in microincorporated feeding trials at 250 and 500 ug concentrations of toxins. Eight insects of each stage were used for each set of experiment. Mortality was recorded at the end of experimental period of 18 days.

RESULTS

EVALUATION OF AFFINITY CAPACITY OF CLGG, SEPHAROSE-4B AND AT-SEPHAROSE-4B MATRICES:

The binding capacity of all three affinity matrices viz., CLGG, AT-Sepharose-4B and Sepharose-4B was evaluated by the isolation of *Abrus* lectins and the results are shown in Table 2.

Table 2: Yield of lectins from three species on different affinity matrices

Purification step	Protein (mg)	Specific* activity	Total activity (titre x mg)	Yield (%)
Abrus precatorius				
Crude extract	20	4554	91080	100
CLGG	**7**	8484	59388	35
AT-Sepharose 4B	**4**	8598	34392	20
Sepharose 4B	**3**	8484	25452	15
*A **brus fruticulos** us*				
Crude extract	20	4746	94920	100
CLGG	**7**	8597	60179	35
AT-Sepharose 4B	**4**	8533	34132	20
Sepharose 4B	**3**	8533	25599	15
*Abrus **pulchellus***				
Crude extract	20	4552	91040	100
CLGG	**8**	8533	68264	40
AT-Sepharose 4B	**5**	8545	42725	25
Sepharose 4B	**3**	8533	25599	15

* Specific activity is expressed as titre, the reciprocal of maximal dilution of protein that gives visible agglutination with 2 % trypsinized rabbit erythrocytes.

The bound proteins were eluted as single peak from all the three matrices (Figures 3, 4 and 5). Crude protein (20 mg) was loaded on each matrix and approximately 7.0, 4.0 and 3.0 mg of *A. precatorius* lectin was obtained from the CLGG, AT-Sepharose-4B and Sepharose-4B columns, respectively. The activity of *A. precatorius* lectin deter-

Figure 3

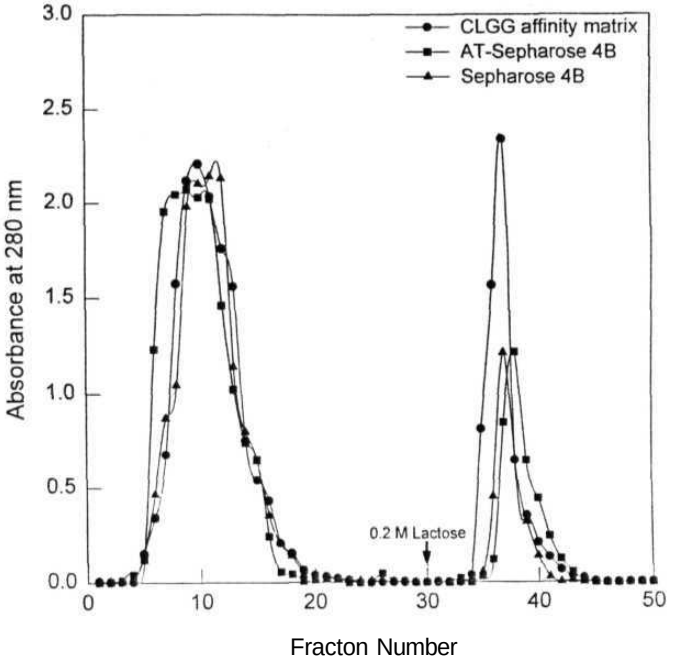


Figure 3. Affinity chromatography of *A. precatorius* seed proteins on CLGG, AT-Sepharose and Sepharose 4B columns (1.5 x 10 cms) at 4°C. The columns equilibrated with 10 mM PBS (pH 7.2) and 20 mg of protein was applied for each column. The bound proteins were eluted with 0.2 M lactose in PBS at a flow rate of 18 ml/h. Fractions of 1.5 ml were collected and evaluated.

Figure 4

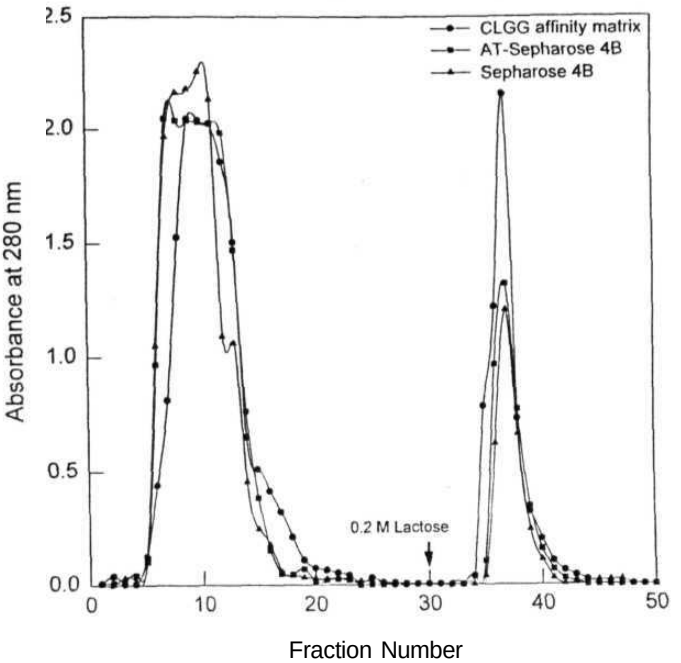


Figure 4. Affinity chromatography of *A. fruticulosus* seed proteins on CLGG, AT-Sepharose and Sepharose 4B columns (1.5 x 10 cms) at 4°C. The columns equilibrated with 10 mM PBS (pH 7.2) and 20 mg of protein was applied for each column. The bound proteins were eluted with 0.2 M lactose in PBS at a flow rate of 18 ml/h. Fractions of 1.5 ml were collected and evaluated.

Figure 5

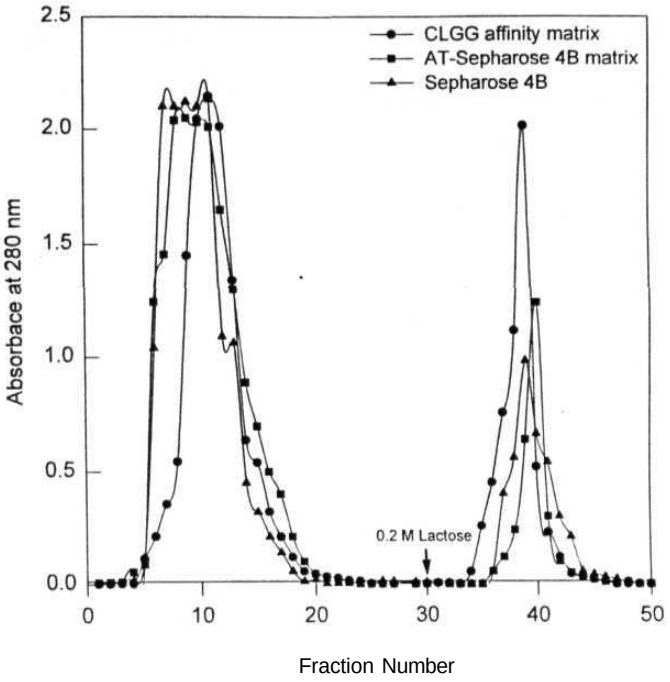


Figure 5. Affinity chromatography of *A. pulchellus* seed proteins on CLGG, AT-Sepharose and Sepharose 4B columns (1.5 x 10 cms) at 4°C. The columns equilibrated with 10 mM PBS (pH 7.2) and 20 mg of protein was applied for each column. The bound proteins were eluted with 0.2 M lactose in PBS at a flow rate of 18 ml/h. Fractions of 1.5 ml were collected and evaluated.

mined from CLGG, Sepharose-4B and AT-Sepharose-4B columns is shown to be 8484, 8484 and 8598 HU/mg/ml respectively. Similarly, 7.0, 4.0 and 3.0 mg of *A. fruticulosus* lectin was obtained from CLGG, AT-Sepharose-4B and Sepharose-4B columns, respectively and the specific activity of *A. fruticulosus* lectin obtained from CLGG, Sepharose-4B and AT-Sepharose-4B columns was determined to be 8597, 8533, 8533 HU/mg/ml, respectively. Approximately 8.0, 5.0 and 3.0 mg was the yield of *A. pulchellus* lectin from the CLGG, AT-Sepharose-4B and Sepharose-4B columns respectively and the activity of the lectin obtained from CLGG, Sepharose-4B and AT-Sepharose-4B columns was determined to be 8533, 8545 and 8533 HU/mg/ml, respectively.

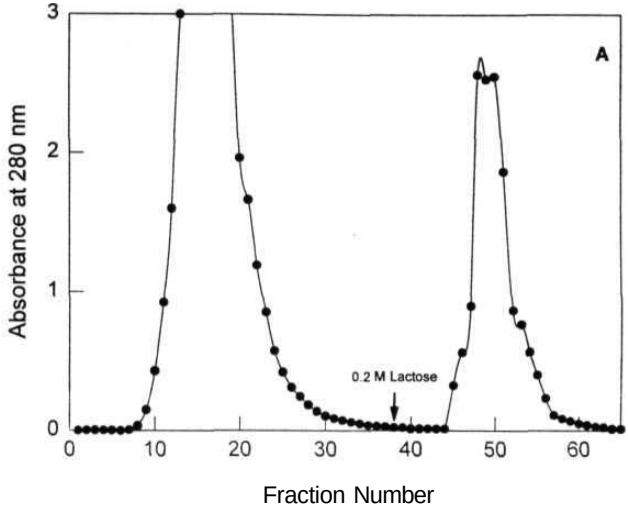
PURIFICATION AND CHARACTERIZATION OF LECTINS FROM *ABRUS* SEEDS:

A summary of the purification steps and the heamagglutinating activity for the lectins are shown in Table 3. Figures 6A, 7A and 8A illustrate the elution profile of *Abrus* lectins obtained by CLGG affinity column. The total *Abrus* lectins eluted into single peak and the non-denaturing SDS-PAGE analysis (without p-mercaptoethanol) of CLGG affinity purified protein fractions showed the presence of single band at about Mr 64, 60 and 65 kDa *for A. precatorius* (Figure 9, Lane 1), *A. fruticulosus* (Figure 10, Lane 1) and *A. pulchellus* (Figure 11, Lane 1). The affinity purified fractions were further resolved into two protein peaks I and II on DEAE-Sephacel column (Figures 6B, 7B and 8B). The non-denaturing SDS-PAGE of the peak-I showed a single band at 64, 60 and 65 kDa for *A. precatorius* (Figure 9, Lane 3), *A. fruticulosus* (Figure 10, Lane 3) and *A. pulchellus* (Figure 11, Lane 2), respectively. The denaturing SDS-PAGE (with p-mercaptoethanol) of the peak-I resolved into two bands at

Figure 6. (A) Affinity chromatography of *A. precatorius* crude seed protein on cross-linked guar gum at 4°C. The column (3 x 14 cms) equilibrated with 10 mM PBS (pH 7.2) and 415 mg of protein was applied. The hound proteins were eluted with 0.2 M lactose in PBS at the flow rate of 18 ml/h. Fractions of 10 ml were collected and protein concentration was recorded at 280 nm.

(B) DEAE-Sephacel ion-exchange chromatography of CLGG affinity purified proteins at 4°C. The column (1.5 x 32 cms) equilibrated with 5 mM sodium acetate (pH 6.5) and 120 mg of protein was applied. Proteins were eluted in a 5-200 mM linear gradient of sodium acetate (pH 6.5) buffer at a flow rate of 36 ml/h, 3 ml fractions were collected and protein concentration was recorded at 280 nm.

Figure 6


A
Absorbance at 280 nm
0.2 M Lactose
Fraction Number

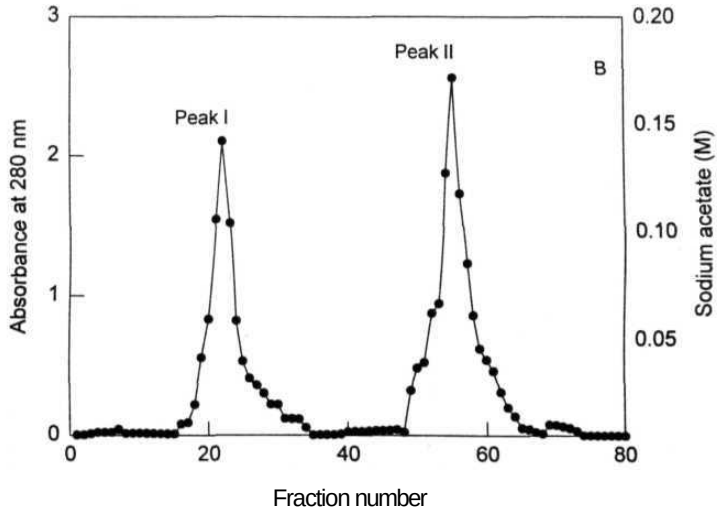

B
Peak I
Peak II
Absorbance at 280 nm
Sodium acetate (M)
Fraction number

Figure 7. (A) Affinity chromatography of *A. fruticulosus* crude seed protein on cross-linked guar gum at 4°C. The column (3 x 14 cms) equilibrated with 10 mM PBS (pH 7.2) and 438 mg of protein was applied. The bound proteins were eluted with 0.2 M lactose in PBS at the flow rate of 18 ml/h. Fractions of 10 ml were collected and protein concentration was recorded at 280 nm.

(B) DEAE-Sephacel ion-exchange chromatography of CLGG affinity purified proteins at 4°C. The column (1.5 x 32 cms) equilibrated with 5 mM sodium acetate (pH 6.5) and 138 mg of protein was applied. Proteins were eluted in a 5-200 mM linear gradient of sodium acetate (pH 6.5) buffer at a flow rate of 36 ml/h, 3 ml fractions were collected and protein concentration was recorded at 280 nm.

Figure 7

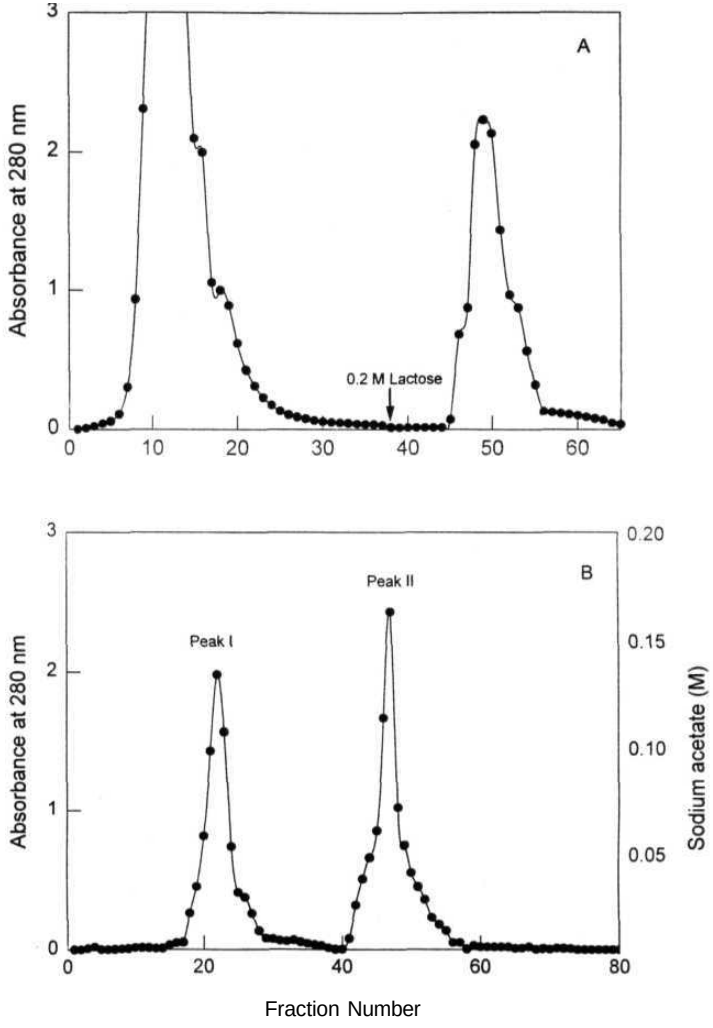

A
Absorbance at 280 nm
0.2 M Lactose
B
Peak I
Peak II
Absorbance at 280 nm
Sodium acetate (M)
Fraction Number

Figure 8. (A) Affinity chromatography of *A. pulchellus* crude seed protein on cross-linked guar gum at 4°C. The column (3 x 14 cms) equilibrated with 10 mM PBS (pH 7.2) and 418 mg of protein was applied. The bound proteins were eluted with 0.2 M lactose in PBS at the flow rate of 18 ml/h. Fractions of 10 ml were collected and protein concentration was recorded at 280 nm.

(B) DEAE-Sephacel ion-exchange chromatography of CLGG affinity purified proteins at 4°C. The column (1.5 x 32 cms) equilibrated with 5 mM sodium acetate (pH 6.5) and 128 mg of protein was applied. Proteins were eluted in a 5-200 mM linear gradient of sodium acetate (pH 6.5) buffer at a flow rate of 36 ml/h, 3 ml fractions were collected and protein concentration was recorded at 280 nm.

Figure 8

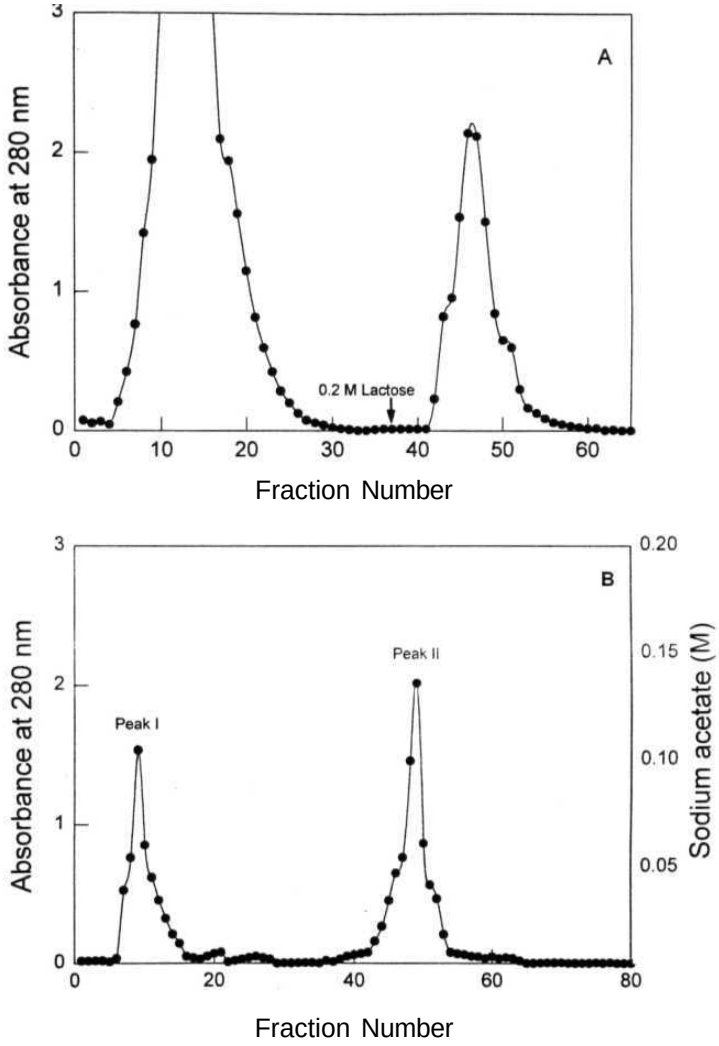

A
Absorbance at 280 nm
0.2 M Lactose
Fraction Number
B
Peak I
Peak II
Absorbance at 280 nm
Sodium acetate (M)
Fraction Number

Figure 9. SDS-PAGE of purified lectins from *A. precatorius*. Lanes 1, 2 and 3 show the CLGG affinity purified proteins, DEAE-Sephacel column chromatography peak-II and peak-I, respectively, in the absence of β-mercaptoethanol. Lane 4, 5 and 6 show the CLGG affinity purified proteins. DEAE-Sephacel column chromatography peak-II and peak-I, respectively, in the presence of β-mercaptoethanol. Lane 7 show the standard molecular marker proteins. The standards were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and a-lactalbumin (14.2 kDa).

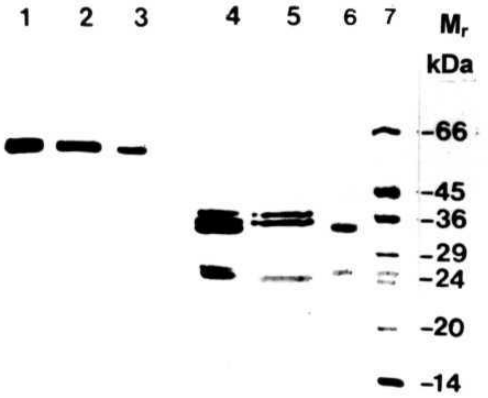

1
2
3
4
5
6
7
Mr
kDa
66
45
36
29
24
20
14

Figure 10. SDS-PAGE of purified lectins from *A. fruticulosus.* Lanes 1, 2 and 3 show the CLGG affinity purified proteins, DEAE-Sephacel column chromatography peak-II and peak-I, respectively, in the absence of β-mercaptoethanol. Lane 4, 5 and 6 show the CLGG affinity purified proteins, DEAE-Sephacel column chromatography peak-II and peak-I respectively, in the presence of β-mercaptoethanol. Lane 7 show the standard molecular marker proteins. The standards were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kda), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and a-lactalbumin (14.2 kDa).

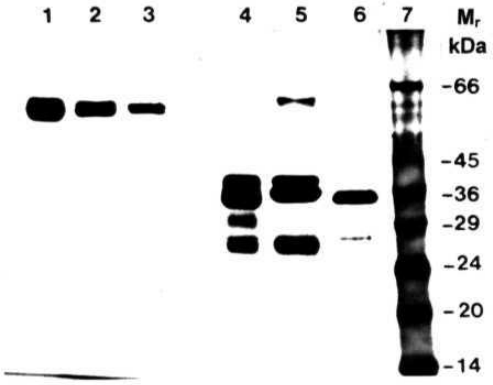
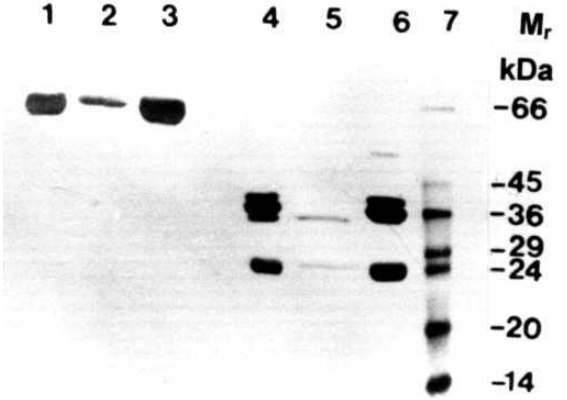

1
2
3
4
5
6
7
M_r
kDa
-66
-45
-36
-29
-24
-20
-14

Figure 11. SDS-PAGE of purified lectins from *A. pulchellus*. Lanes 1, 2 and 3 show the CLGG affinity purified proteins. DEAE-Sephacel column chromatography peak-I and peak-II respectively, in the absence of β-mercaptoethanol. Lane 4. 5 and 6 show the CLGG affinity purified proteins, DEAE-Sephacel column chromatography peak-I and peak-II respectively, in the presence of P-mercaptoethanol. Lane 7 show the standard molecular marker proteins. The standards were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and a-lactalbumin (14.2 kDa).


1
2
3
4
5
6
7
Mr
kDa
-66
-45
-36
-29
-24
-20
-14

34 and 27 kDa for *A. precatorius* (Figure 9, Lane 6), 36 and 29 kDa for *A. fruticulosus* (Figure 10, Lane 6) and 33 and 25 kDa for *A. pulchellus* (Figure 11, Lane 5). The non-denaturing SDS-PAGE of the peak-II resolved as single band at 64, 60 and 64 kDa for *A. precatorius* (Figure 9, Lane 2) and *A. fruticulosus* (Figure 10, Lane 2) and *A. pulchellus* (Figure 11, Lane 3), respectively.

Table 3: Purification of *Abrus* lectins from 10 g of decoated seeds

Purification Step	Protein (mg)	Specific activity*	Total activity (titre x mg)	Yield %	Purification factor
Abrus precatorius					
Crude protein	415	4554	1889910	100	1.0
CLGG affinity	120	8826	1059120	56	2.0
DEAE-Seph# Peak I	17.35	1280	22208	1.2	0.3
Peak II	28.73	31560	906719	48.0	7.0
Abrus fruticulosus					
Crude protein	438	4320	1892160	100	1.0
CLGG affinity	138	8334	1150092	61	2.0
DEAE-Seph# Peak I	14.48	512	7414	0.4	0.12
Peak II	30.15	28634	863315	46.0	6.6
Abrus pulchellus					
Crude protein	418	4552	1902736	100	1.0
CLGG affinity	128	8866	1134848	60	2.0
DEAE-Seph# Peak I	17.57	107	1878	1.0	0.02
Peak II	30.51	20506	625638	33.0	4.5

DEAE-Seph=DEAE-Sephacel ion-exchange chromatography
* Specific activity is expressed as titre, the reciprocal of maximal dilution of protein that gives visible agglutination with 2 % trypsinized rabbit erythrocytes.

The denaturing SDS-PAGE for the peak-II resolved into three bands at 35, 36 and 22 kDa for *A. precatorius* (Figure 9, Lane 5), 39, 36 and 28 kDa for *A.fruticulosus* (Figure 10, Lane 5) and 38, 32 and 23 kDa for *A. pulchellus* (Figure 11, Lane 6). The peak-I heamagglutinating specific activity for *A. precatorius, A. fruticulosus* and *A.*

pulchellus was 1280, 512 and 107 HU/mg/ml, respectively (Table 3). The peak-II heamagglutinating specific activity for *A. precatorius, A. fruticulosus* and *A. pulchellus* was 31560, 28634 and 20506 HU/mg/ml, respectively. Peak-I and II of the proteins agglutinated with trypsinized human erythrocytes of different blood groups and rabbit erythrocytes (Table **4).**

Table 4: Haemagglutination * of abrins and agglutinins with trypsinized human Rh' and rabbit erythrocytes

Erythrocyte source	*A. precatorius*		*A. fruticulosus*		*A. pulchellus*	
	Abrin	Agglutinin	Abrin	Agglutinin	Abrin	Agglutinin
Human A	800	11252	512	28634	107	17067
Human B	1333	27542	512	28634	177	17067
Human AB	640	6056	512	28634	107	17067
Human O	1280	31560	512	28634	107	20506
Rabbit	640	6056	178	6326	87	8534

* Specific activity is expressed as titre, the reciprocal of maximal dilution of protein that gives visible agglutination with 2 % erythrocytes.

The lectins of peak-I and peak-II were tested for toxicity on the rats. The LD_{50} of peak-I from *A. precatorius, A. fruticulosus* and *A. pulchellus* was 1.9, 3.0 and 4.98 µg/Kg body weight, respectively. The LD_{50} of peak-11 from *A. precatorius, A. fruticulosus* and *A. pulchellus* was 151, 160 and 160 µg/Kg body weight, respectively. In view of this, the heamagglutinating activity and toxicity of the peak-I was considered as toxic lectin (abrin) and the peak-II was considered as agglutinin. Agglutinins from *A. fruticulosus* and *A. pulchellus* are named as *A. fruticulosus* seed agglutinin (AFSA) and *A. pulchellus* seed agglutinin (APSA), respectively. Several laboratories have already named the agglutinin from *A. precatorius* as *A.precatorius* agglutinin (APA) (Table 1). Similarly, abrins from *A. fruticulosus* and *A. pulchellus* are named

as P-abrin and y-abrin, respectively, considering the toxin from *A. precatorius* as α-abrin.

Upon gel filtration, the a, β and γ abrins eluted in a single peak, corresponding to the molecular mass of 66 kDa (Figures 12, 13 and 14). The molecular masses for the APA, AFSA and APS A were 66, 66 and 71 kDa, respectively, on the gel filtration (Figures 15, 16 and 17). Further characterization of the abrins and agglutinins are revealed that they are glycoproteins. The carbohydrate content of APA, AFSA, APS A estimated to be 12%, 12% and 17% respectively. The carbohydrate content of a, p and y abrins were estimated to be 3.5%, 3.5% and 3.3% respectively.

CARBOHYDRATE SPECIFICITY OF THE LECTINS:

Amongst the sugars tested for heamagglutination inhibition, the Methyl-β-D-galactopyranoside (Me-β–Gal), P-Lactose, and D-Lactose inhibited the abrins strongly with a minimum concentration of 6.2 mM (Table 5). D-Lactose and β-lactose inhibited the abrins very strongly with an inhibitory potency similar to that of Me-β–Gal. Melibiose exhibited an inhibition, which was comparable to that of L-Fucose. Raffinose and α-lactose inhibited the lectins at a minimum concentration of 50 mM and the inhibitory activity was four times less potent that Me-β–gal.

Amongst the sugars tested for heamagglutination inhibition Me-P-Gal and β-Lactose and D-Lactose strongly inhibited the activity of agglutinins at a minimum concentration of 12.5 mM. Methyl-α-D-galactopyranoside (Me-α–Gal), a-Lactose, galactose, raffinose and fucose also inhibited the agglutination at higher concentrations (Table 5).

Figure 12

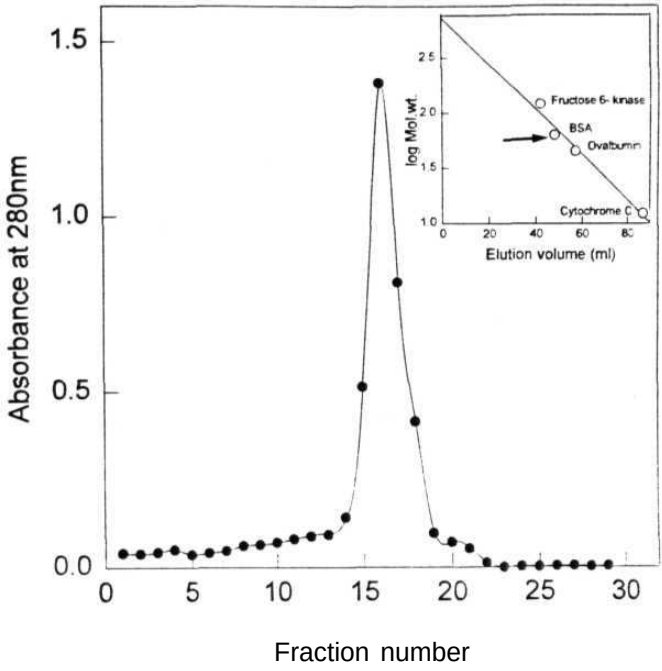


Figure 12. The molecular mass of the toxin (α-abrin) from *A. precatorius* by gel filtration on a Sephadex G-100 column. a-abrin was eluted as a single peak corresponding to 66 kDa (inset-arrow). Fructose-6-phosphate kinase (84 kDa), BSA (66 kDa), ovalbumin (45 kDa), cytochrome C (12.3 kDa) were used as standard protein **markers.**

Figure 13

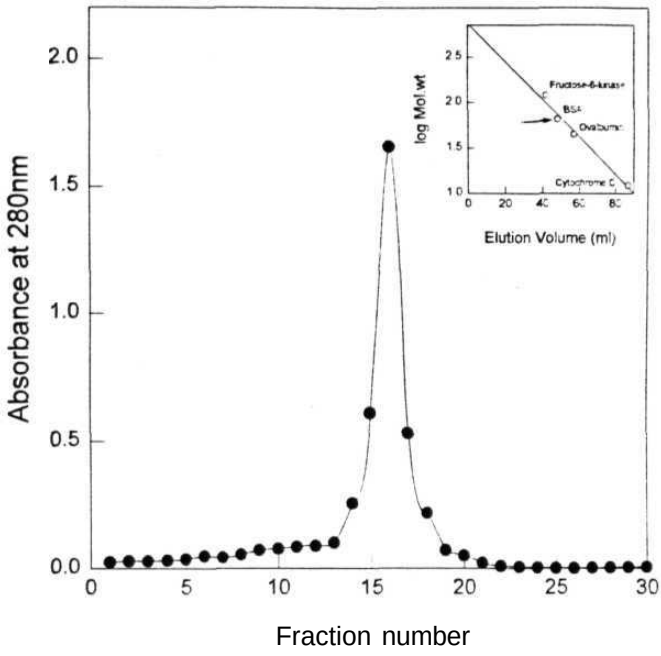


Figure 13. The molecular mass of the toxin (P-ahrin) from *A fruticulosus* by gel filtration on a Sephadex G-100 column. α-abrin was eluted as a single peak corresponding to 66 kDa (inset-arrow). Fructose-6-phosphate kinase (84 kDa), BSA (66 kDa). ovalbumin (45 kDa), cylochrome C (12.3 kDa) were used as standard protein markers.

Figure 14

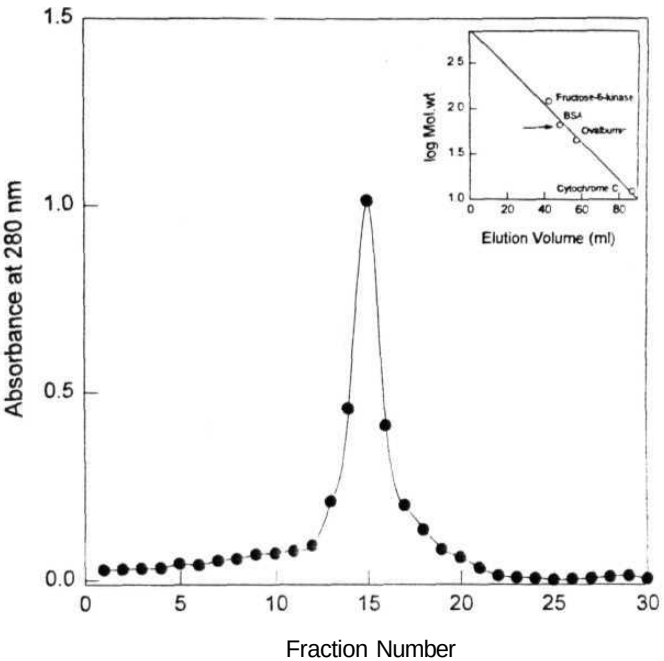


Figure 14. The molecular mass of the toxin (γ-abrin) from *A. pulchellus* by gel filtration on a Sephadex G-100 column. α-abrin was eluted as a single peak corresponding to 66 kDa (inset-arrow). Fructose-6-phosphate kinase (84 kDa). BSA (66 kDa). ovalbumin (45 kDa), cytochrome C (12.3 kDa) were used as standard protein markers.

Figure 15

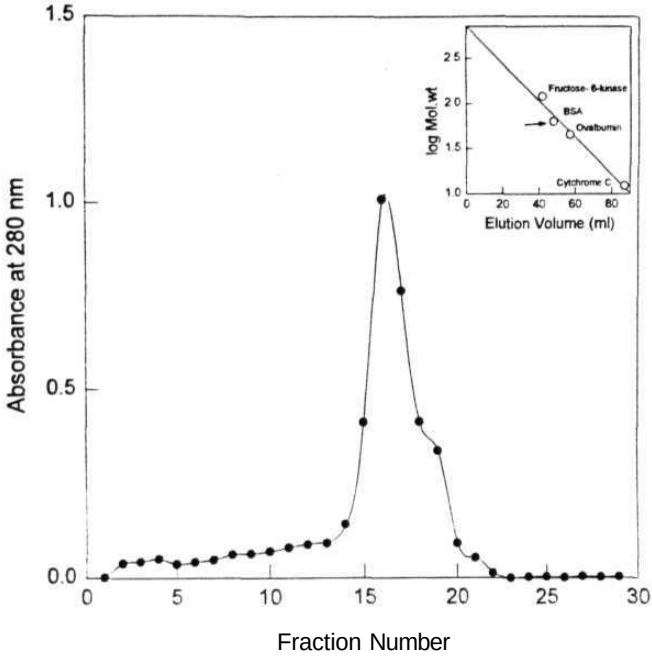


Figure 15. The molecular mass of the agglutinin (APA) from *A precatorius* by gel filtration on a Sephadex G-100 column. APA was eluted as a single peak corresponding to 66 kDa (inset-arrow). Fructose-6-phosphate kinase (84 kDa). BSA (66 kDa), ovalbumin (45 kDa). cytochrome C (12.3 kDa) were used as standard protein markers.

Figure16

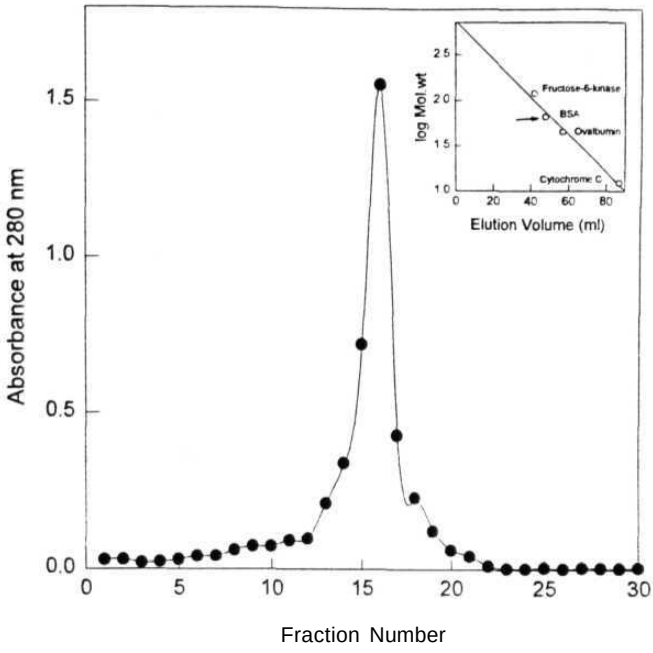


Figure 16. The molecular mass of the agglutinin (AFSA) from *A. fruticulosus* by gel filtration on a Sephadex G-100 column. AFSA was eluted as a single peak corresponding to 66 kDa (inset-arrow). Fructose-6-phosphate kinase (84 kDa), BSA (66 kDa). ovalbumin (45 kDa), cytochrome C (12.3 kDa) were used as standard protein markers.

Figure17

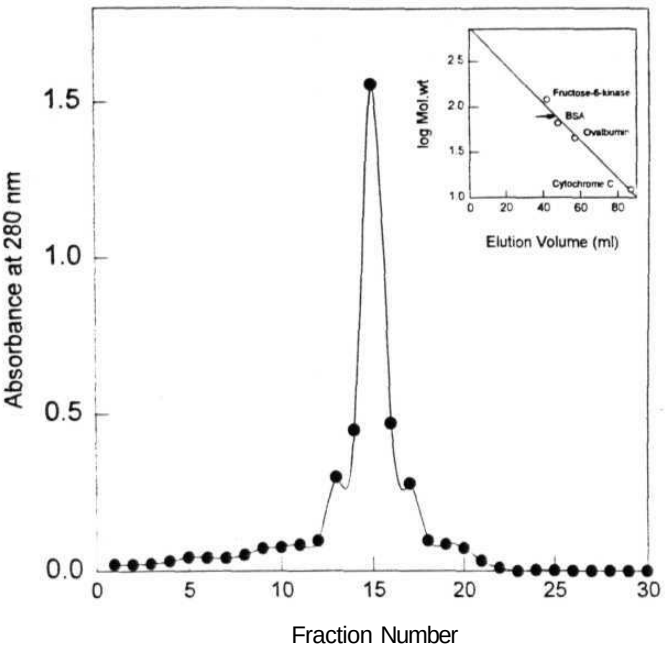


Figure 17. The molecular mass of the agglutinin (APSA) from *A. pulchelus* by gel filtration on a Sephadex G-100 column. AFSA was eluted as a single peak corresponding to 71 kDa (inset-arrow). Fructose-6-phosphate kinase (84 kDa), BSA (66 kDa), ovalbumin (45 kDa), cytochrome C (12.3 kDa) were used as standard protein markers.

Table 5: Abrin and agglutinin haemagglutination inhibition by various sugars*

Sugar	*A. precatorius*		*A. fruticulosus*		*A. pulchellus*	
	Abrin	Agglutinin	Abrin	Agglutinin	Abrin	Agglutinin
GlcNAc	NI	NI	NI	NI	NI	NI
D-Arabinose	NI	NI	NI	NI	NI	NI
L-Arabinose	NI	NI	NI	NI	NI	NI
D-Fructose	NI	NI	NI	NI	NI	NI
L-Fucose	50	50	50	50	50	50
D-Galactose	6.2	25	25	25	25	25
D-Glucose	NI	NI	NI	NI	NI	NI
α-Lactose	50	25	50	25	50	25
β-Lactose	6.2	12.5	6.2	12.5	6.2	12.5
D-Lactose	6.2	25	6.2	25	6.2	25
Melibiose	50	100	50	100	50	200
D-Mannose	NI	NI	NI	NI	NI	NI
Me-α-Gal	12.5	25	12.5	25	12.5	25
Me-p-Gal	6.2	12.5	6.2	12.5	6.2	12.5
Raffinose	50	50	50	100	50	25
Rahmnose	NI		NI		NI	
Sucrose	NI		NI		NI	
Xylose	NI -		NI		NI	

* Lowest inhibiting concentration (mM) of sugar for complete inhibition of 2 haemagglutinating units with 2 % human O^+ trypsinized erythrocytes.

NI - No inhibition observed with the highest concentration of 200 mM sugar

GlcNAc = N-acetylglucosamine; Me-a-Gal = Methyl-α-D-Galactopyranoside; Me-β-Gal = Methyl-β-D-Galactopyranoside.

IMMUNOLOGICAL CROSS REACTIVITY BETWEEN THE ABRINS AND AGGLUTININS:

Immunological cross-reactivity was studied by western blotting between abrin and agglutinins by using P-abrin antibodies. The p-abrin antibodies cross-reacted with all abrins and agglutinins (Figures 18, 19 and 20). The antibody cross-reaction was very strong with the smaller subunit (A-chain) of a-abrin (Figure 18, Lane 6), p-abrin (Figure 19, Lane 7) and γ-abrin (Figure 20, Lane 5). A moderate reactivity was ob-

Figure 18. Western blot analysis of purified lectins from *A. precatorius*. Upon completion of the SDS-PAGE, the proteins were transferred to nitrocellulose membrane and subjected to immuno detection with the primary antibody raised against β-abrin, followed by alkaline phosphatase conjugated secondary antibody. Lanes 1, 2 and 3 show the immunological analysis with the CLGG affinity purified proteins, APA and a-abrin respectively, in the absence of β-mercaptoethanol. Lane 4, 5 and 6 show the CLGG affinity purified proteins, APA and a-abrin respectively, in the presence of β-mercaptoethanol. Molecular weight markers were resolved at the right margin.

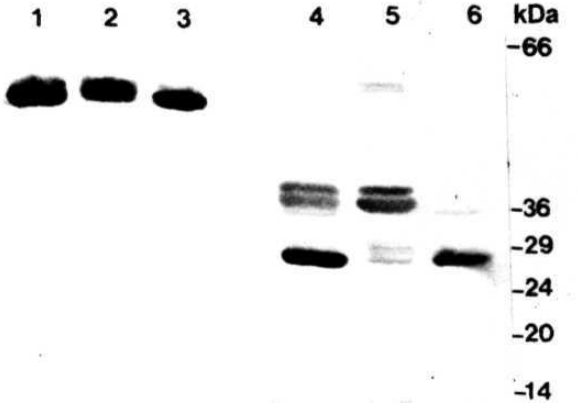

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Figure 19. Western blot analysis of purified lectins from *A. fruticulosus*. Upon completion of the SDS-PAGE, the proteins were transferred to nitrocellulose membrane and subjected to immuno detection with the primary antibody raised against P-abrin, followed by alkaline phosphatase conjugated secondary antibodies. Lanes 1, 2 and 3 show the immunological analysis with the CLGG affinity purified proteins, AFSA and P-abrin respectively, in the absence of β-mercaptoethanol. Lane 4, 5 and 6 show the CLGG affinity purified proteins, AFSA and P-abrin respectively, in the presence of β-mercaptoethanol. Molecular weight markers were resolved at the right margin.

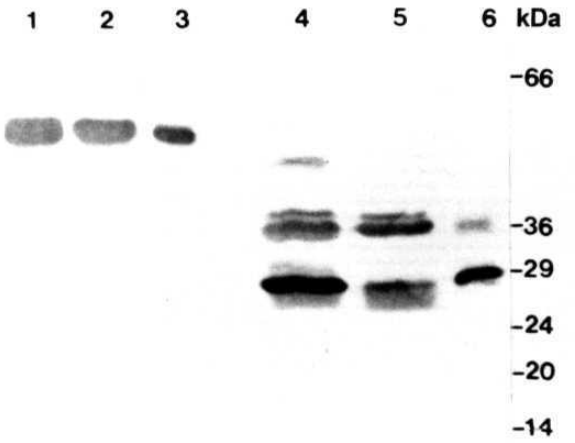
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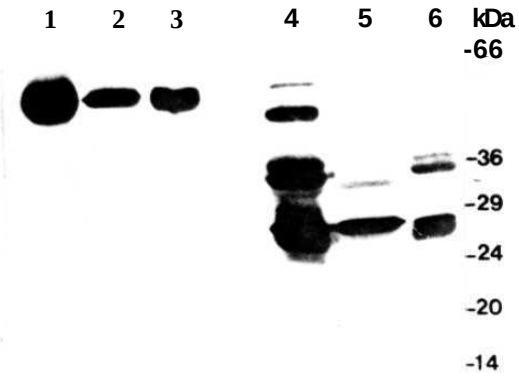
Figure 20. Western blot analysis of purified lectins from *A. pulchellus*. Upon completion of the SDS-PAGE, the proteins were transferred to nitrocellulose membrane and subjected to immuno detection with the primary antibody raised against β-abrin, followed by alkaline phosphatase conjugated secondary antibodies. Lanes 1, 2 and 3 show the immunological analysis with the CLGG affinity purified proteins, y-abrin and APSA respectively, in the absence of β-mercaptoethanol. Lane 4, 5 and 6 show the CLGG affinity purified proteins, y-abrin and APSA respectively, in the presence of β-mercaptoethanol. Molecular weight markers were resolved at the right margin.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served between p-abrin antibodies and the larger subunit (B-chain) of all the abrins. The p-abrin antibodies also cross-reacted with the agglutinins with high specificity towards the larger subunits of the APA (Figure 18, Lane 5), AFSA (Figure 19, Lane 5) and APSA (Figure 20, Lane 6).

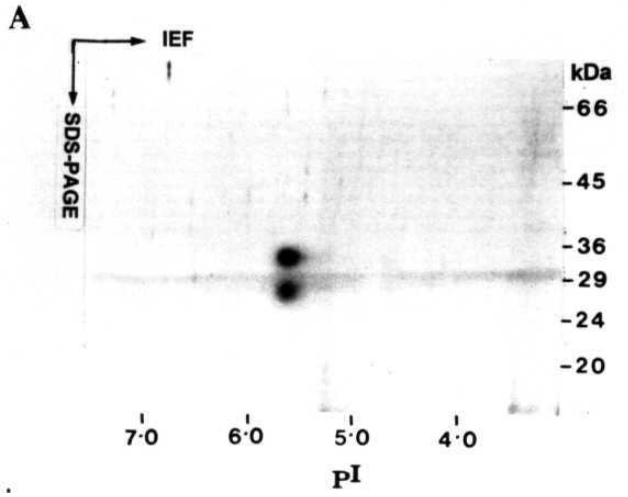
TWO-DIMENSIONAL GEL ELECTROPHORETIC ANALYSIS OF THE LECTINS:

The two-dimensional gel electrophoretic analysis revealed the homogeneity of the lectins. The pI values were 5.6 for α-abrin (Figure 21A), 5.75 for P-abrin (Figure 22A) and 5.8 for γ-abrin (Figure 23A). The pI for APA was approximately 7.3 (Figure 21B). However, the pI for both AFSA (Figure 22B) and APSA (Figure 23B) was 7.3. There was no significant difference in the subunit pattern of the abrins and agglutinins from all the three species on SDS-PAGE and 2-D IEF.

DISTRIBUTION OF LECTINS **IN VARIOUS** PARTS **OF** *A.* ***PRECATORIUS*** **PLANT:**

The heamagglutinating activity was high in cotyledons and significantly less by about 40 times in other parts such as roots, stems and leaves of the 12-day-old seedlings (Figure 24). The specific activity measured in cotyledons was 605 HU whereas in the roots, stem and leaves the activity was 15.5, 12.5 and 8.3 HU, respectively (Figure 24). The western blot analysis with the crude proteins of the different vegetative parts revealed cross-reactivity with p-abrin antibodies to their seed counterpart. In roots and cotyledons, antibody cross-reactivity was observed with the proteins of same molecular weights of all lectins whereas in stem and leaves, the cross-reactivity was observed with the lower molecular weight protein A-chain (Figure 25).

Figure 21. Two-dimensional electrophoretic analysis of α-abrin (A) and APA (B). Approximately 5 μg of protein was loaded onto a tube gel containing 30% acrylamide, *2%* ampholine and 9.5 M urea. Upon isoelctric focussing, the gel was layered onto 11% SDS-PAGE for the second-dimension and the proteins were visualized by silver staining. Molecular weight markers were represented at the right margin.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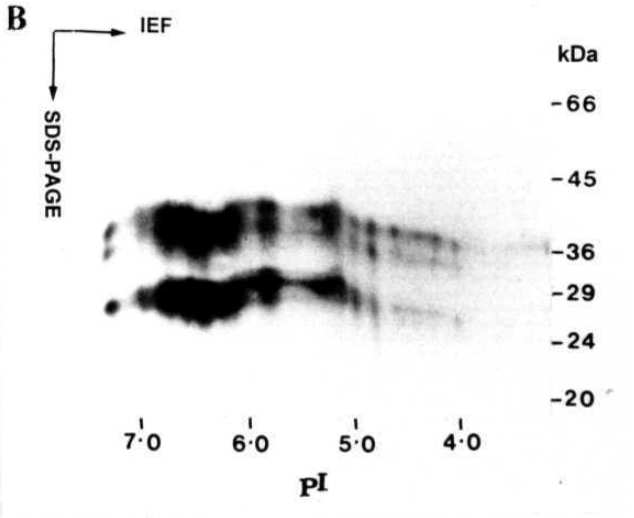
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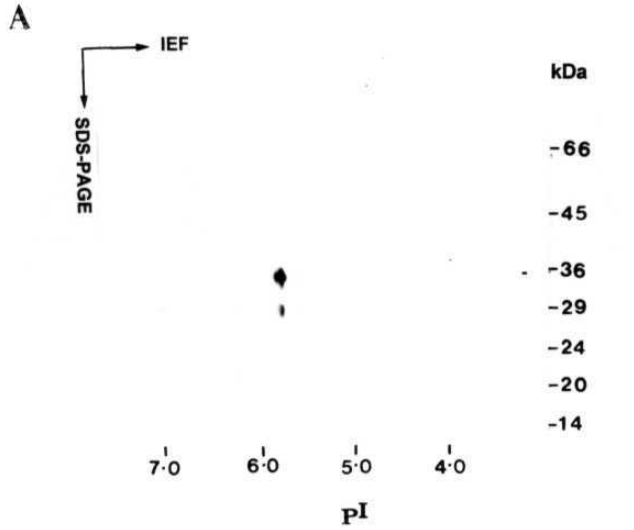
Figure 22. Two-dimensional electrophoretic analysis of β-abrin (A) and AFSA (B). Approximately 5 μg of protein was loaded onto a tube gel containing 30% acrylamide, 2% ampholine and 9.5 M urea. Upon isoelectric focussing, the gel was layered onto 11% SDS-PAGE for the second-dimension and the proteins were visualized by silver staining. Molecular weight markers were represented at the right margin.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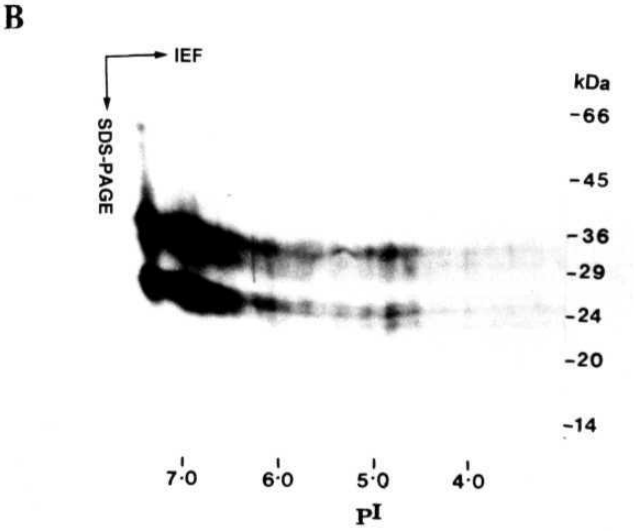
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Figure 23. Two-dimensional electrophoretic analysis of y-abrin (A) and APSA *(3)* Approximately 5 μg of protein was loaded onto a tube gel containing 30% acrylamide, 2% ampholine and 9.5 M urea. Upon isoelectric focussing, the gel was layered onto 11% SDS-PAGE for the second-dimension and the proteins were visualized by silver staining. Molecular weight markers were represented at the right margin.

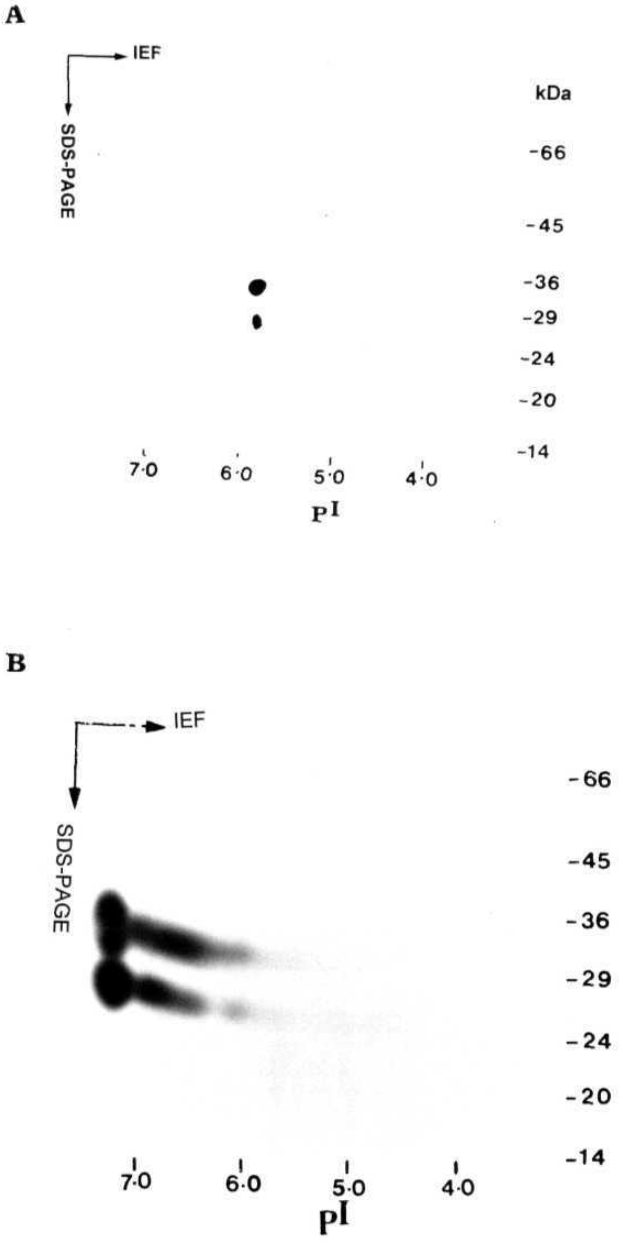
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Figure 24

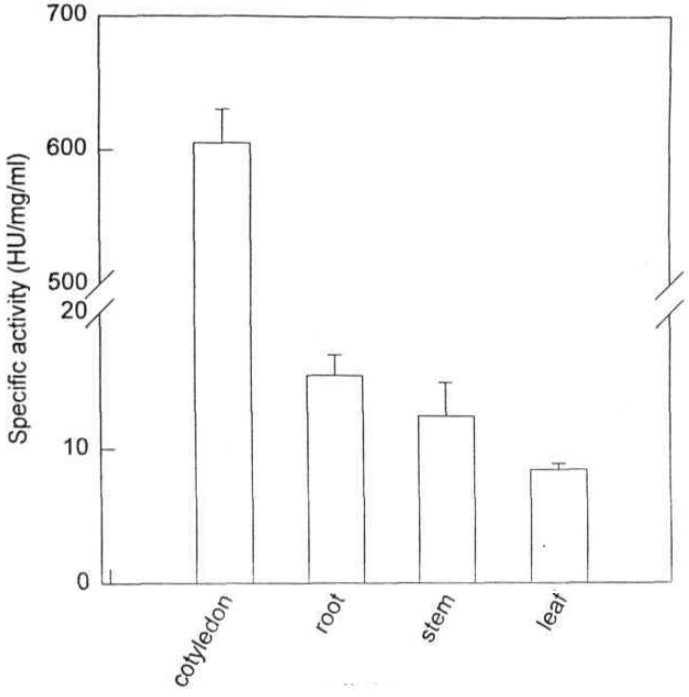
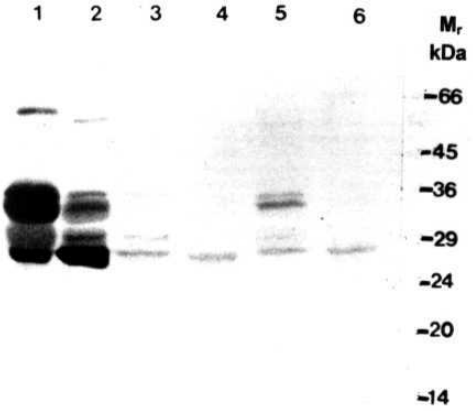


Figure 24. Distribution of lectin activities in various tissues of 12 days old seedlings. The seeds were germinated on sterile filter papers moisturized with Hoagland solution medium. 14:10 hrs light and dark period was maintained. The vegetative tissues (cotyledons, leaves, stem and roots) were collected on 12 day post-emergence. The tissues were homogenized with PBS and centrifuged at 15,000 x g for 30 min. The supernatants were filtered through Whatmann No. 41 filter paper and used for haemagglutination.

Figure 25. Western blot analysis of lectin distribution in different parts of young seedlings (12 days old). Approximately 4 μg of affinity purified seed lectin and 30 μg of crude protein from different parts of the *A. precatorius* plant were loaded on 11% SDS-PAGE. Upon completion of SDS-PAGE the proteins were transferred to nitrocellulose membrane and subjected to immunodetection with the primary antibody of P-abrin followed by alkaline phosphatase-conjugated secondary antibody. Lane 1, affinity purified seed lectin; Lane 2 crude seed protein; Lane 3 root; Lane 4 stem; Lane 5, cotyledon; Lane 6 leaf. Molecular weight markers were resolved on the right margin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INSECT BIOASSAYS

Insecticidal activity of abrins:

The toxicity of a, p and y-abrins to *Sitophyllus oryzae* (rice weevil, Coleoptera) and *Corcyra cephalonica* (rice moth, Lepidoptera) larvae, prepupa and pupa have been assayed through feeding trials. Toxicity of the abrins was found to be significantly variable against the insect pests. The mortality of insects was observed with 500, 700 and 1000 ug of abrins/10 gm feed. At the dose of 500 and 700 ug of a-abrin, 50 and 100% mortality was observed, respectively at the end of experimental period. However, 40 and 80% mortality was observed in the insects, which were fed with 500 and 700 µg of P-abrin, respectively (Figure 27). In the case of γ-abrin, 30 and 90 % mortality was observed with 500 and 700 ug concentrations, respectively (Figure 28). Further, at 1000 ug concentration 100 % mortality was observed after 3 days of treatment with all the three abrins (Figure 26, 27 and 28).

Larvicidal activity of abrins:

The final instar of rice moth larvae was used in the experiments. All the developmental stages were followed in the present study to determine the mortality. It is interesting to note that the abrins from all the three species curtailed the growth of the larvae and eventually lead to death (Figures 29, 30 and 31). More than 80% mortality was observed in the larvae fed with 500 ug of abrins (Figures 32, 33 and 34). At 250 ug concentration 75, 60 and 50% mortality was observed with a, β and y-abrins, respectively (Figures 32, 33 and 34).

Figure 26

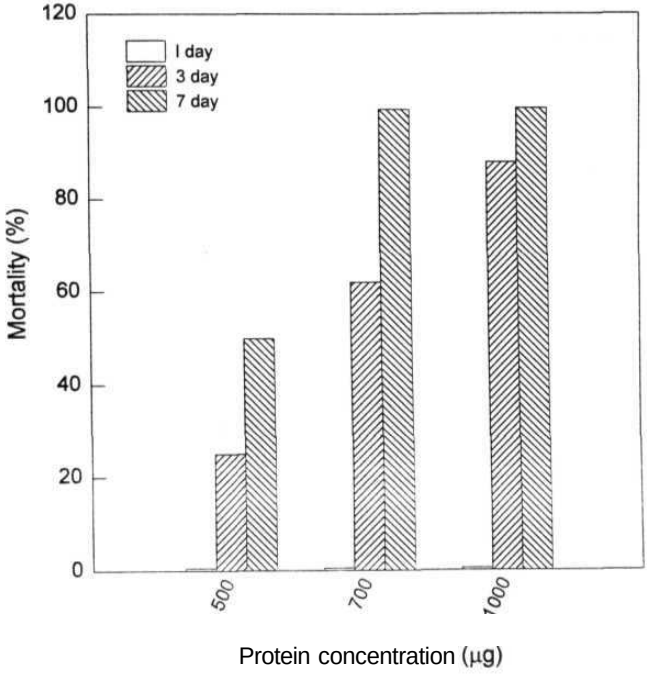


Figure 26. Toxic effect of a-abrin on *Sitophyllus oryzae* insects. The insects were reared on the 500, 700 and 1000 µg/10 gm feed of rice grains. Mortality of the insects was recorded until 7 days of experimental period.

Figure 27

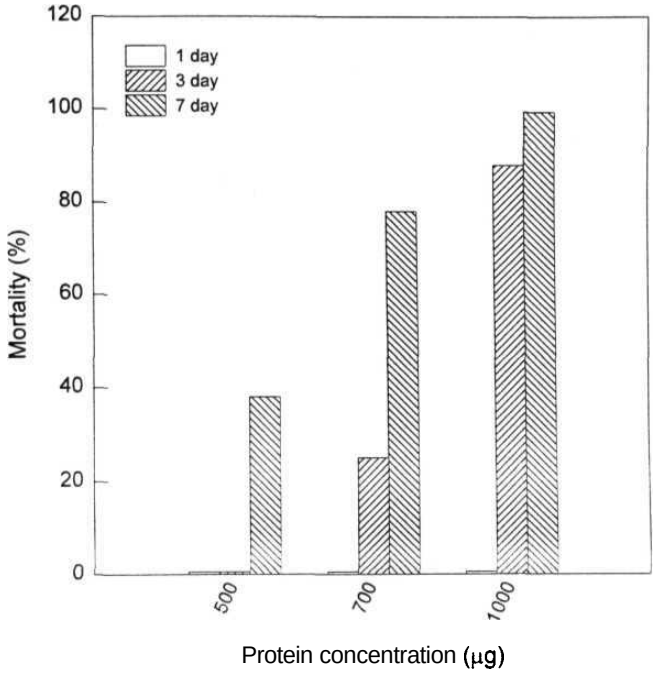


Figure 27. Toxic effect of B-abrin on *Sitophyllus oryzae* insects. The insects were reared on **the** 500, 700 and 1000 μg/10 gm feed of rice grains. Mortality of the insects was recorded until 7 days of experimental period.

Figure 28

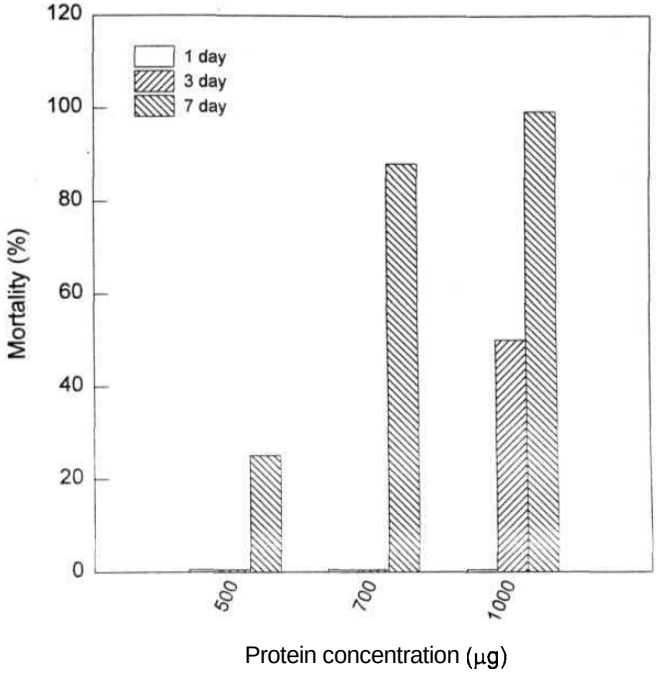
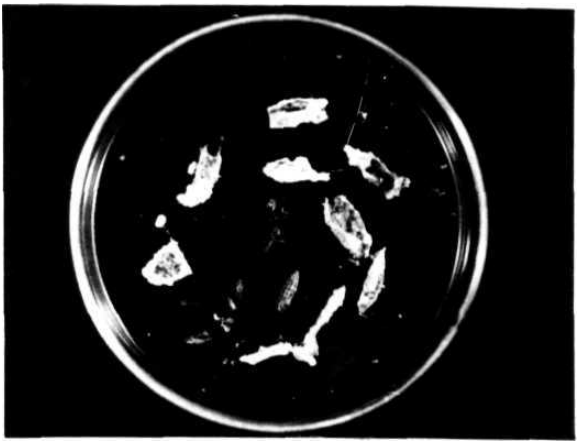


Figure 28. Toxic effect of y-abrin on *Sitophyllus oryzae* insects. The insects were reared on the 500, 700 and 1000 µg/10 gm feed of rice grains. Mortality of the insects was recorded until 7 days of experimental period.

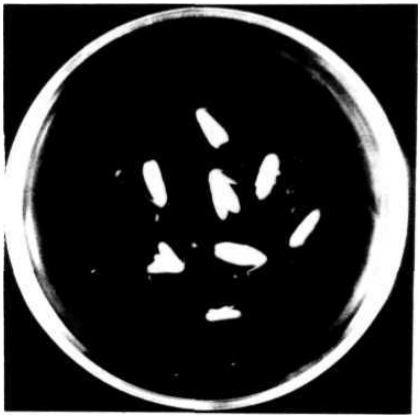
Figure 29. Toxic effect of a-abrin on *Corcyra cephalonica* insects. The early last-instar larvae were reared on 250 and 500 µg/gm feed of crushed sorghum seeds. (A) Control larvae were metamorphosed in to adult insects (imago) after 18days. (B) Treated insects were died at pupa stage.

Control

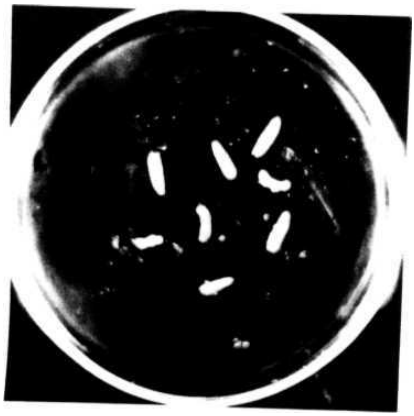


Treated

Figure 30. Toxic effect of P-abrin on *Corcyra cephalonica* insects. The early last-instar larvae were reared on 250 and 500 µg/10 gm feed of crushed sorghum seeds. (A) Control larvae were metamorphosed in to adult insects (imago) after 18days. (B) Treated insects were died at pupa stage.

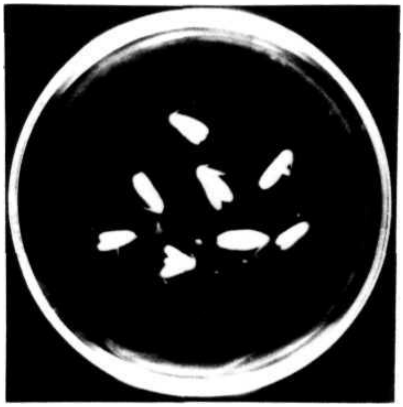


Control



Treated

Figure 31. Toxic effect of γ-abrin on *Corcyra cephalonica* insects. The early last-instar larvae were reared on 250 and 500 μg/10 gm feed of crushed sorghum seeds. (A) Control larvae were metamorphosed in to adult insects (imago) after 18days. (B) Treated insects were died at pupa stage.



Control

Treated

Figure 32

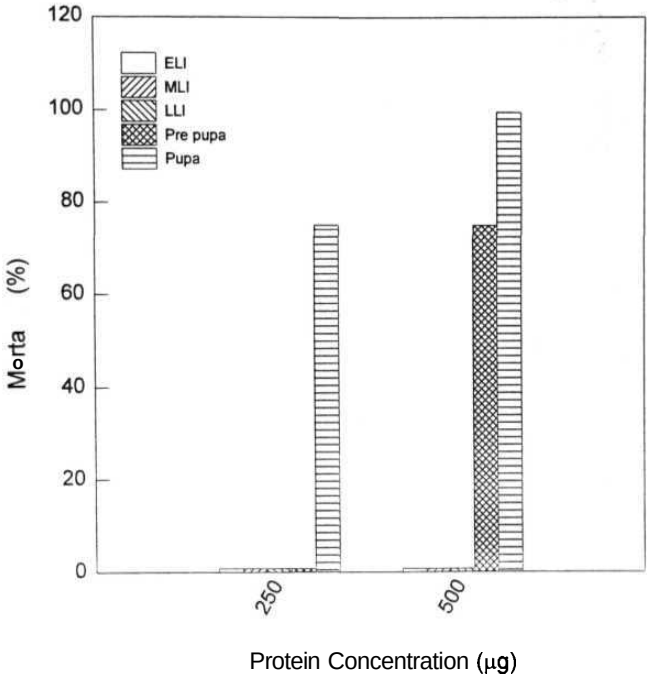


Figure 32. Toxic effect of a-abrin on *Corcyra cephalonica* insects. The early last-instar were rear on 250 and 500 µg/10 gm feed of crushed sorghum seeds. The observations on the early last-instar (ELI), mid last-instar (ML), late last-instar (LLI), pre-pupa and pupa stages were made until 18 days where the larvae metamorphosed into adult insects. Mortality of the insects was recorded.

Figure 33

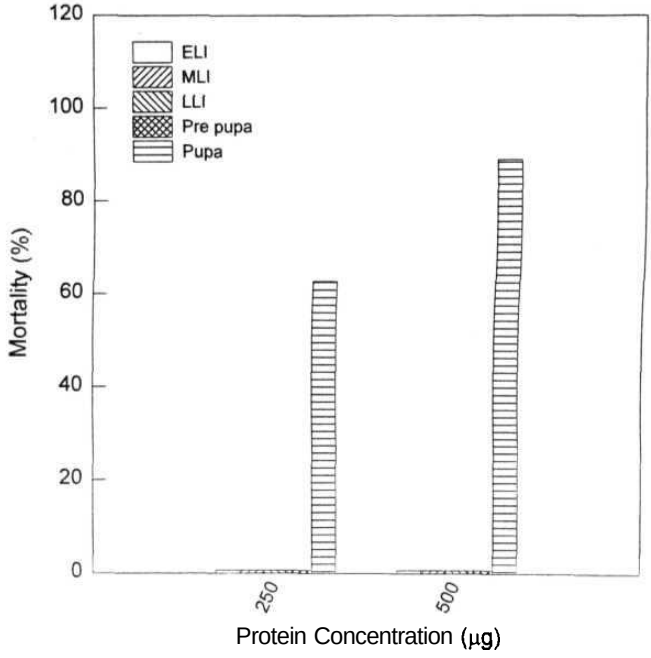


Figure 33. Toxic effect of p-abrin on *Corcyra cephalonica* insects. The early last-instar were reared on 250 and 500 µg/10 gm crushed sorghum seeds. The observations on the early last-instar (ELI), mid last-instar (ML), late last-instar (LLI), pre-pupa and pupa stages were made until 18 days where the larva metamorphosed into adult insects. Mortality of the insects was recorded.

Figure 34

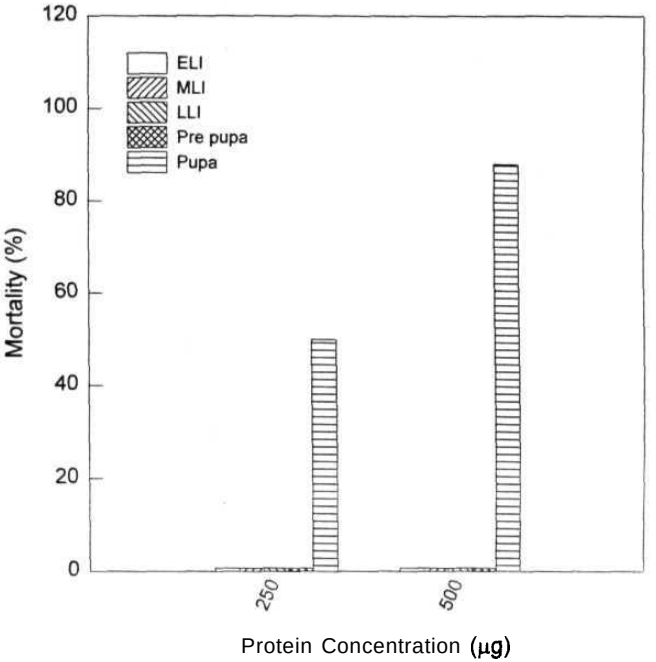


Figure 34. Toxic effect of y-abrin on *Corcyra cephaionica* insects. The early last-instar were reared on 250 and 500µg/gm feed of crushed sorghum seeds. The observations on the early last-instar (ELI), mid last-instar (ML), late last-instar (LLI), pre-pupa and pupa stages were made until 18 days where the larva metamorphosed into adult insects. Mortality of the insects was recorded.

DISCUSSION

In the present finding it was found that CLGG is the ideal matrix for the purification of *Abrus* lectins. In the affinity evaluation study, it was found that about 35% lectin was obtained from 20 mg of total protein loaded on to the CLGG affinity column. Purification of lectins using Sepharose-4B and AT-Sepharose-4B matrices resulted in a recovery of 15 and 20 % lectins out of the total proteins (20 mg) loaded. In the earlier, reports it was found that the CLGG was the best affinity matrix when compared to other affinity matrices for the isolation of several galactose-specific lectins. It is interesting to note that CLGG has a high binding capacity for both a and β anomer specific lectins (Appukuttan *et al,* 1977; Kumar *et al,* 1982). This may be due to the increased expression of galactose residues in the gel compared to those in Sepharose. Moreover, the cost of CLGG preparation was found to be very economical i.e. 100 g matrix costs US$ 0.25 (about 10 Indian rupees) when compared with other commercial affinity matrices.

Several methods have been followed in the purification of lectins from *A. precatorius* and their properties were studied (Table 1). The physico-chemical properties of the purified lectins from different laboratories are much similar, but different names were adapted for each lectin isolated by various procedures. Olsnes *et al,* (1974b) isolated a toxin and an agglutinin on DEAE-cellulose. Further agglutinin resolved into two peaks on CM-cellulose column chromatography which were not characterized. Wei *et al,* (1974) isolated two abrins, abrin A and C for the first time by different ion-exchange chromatographies. Abrin A was further characterized by Hermann and Behnke (1981) who showed that Abrin A contains three isoforms. Roy *et al.,* (1976) reported three isoforms for *Abrus* agglutinin, while Olsnes *et al.,* (1974)

and Wei *et al,* (1974b) reported only one. Later, Lin *et al,* (1978) reported four isoabrins and one agglutinin. Recently, Hegde *et al,* (1991 and 1992) isolated three abrins and two agglutinins from *A. precatorius* by lactamyl-Sepharose affinity chromatography followed by DEAE-Sephacel ion-exchange chromatography. In the present study, one toxin and one agglutinin could be purified to homogeneity from all the three *Abrus* species by using CLGG-affinity chromatography followed by DEAE-Sephacel ion-exchange chromatography.

The SDS-PAGE of the abrins in the presence and absence of β-mercaptoethanol revealed that the abrins consist of two different molecular weight subunits, A-chain and B-chain, which are linked by a sulphydryl bond. The SDS-PAGE analysis of agglutinins revealed single protein and multiple subunits in the absence and presence of β-mercaptoethanol, respectively. The abrins and agglutinins agglutinated human erythrocytes of all types and their binding to human erythrocytes was inhibited by galactose and galactose containing sugars suggesting that both the lectins bind to the same or similar sites on the blood cells or erythrocytes. On the other hand, the binding of *Abrus* lectins to rabbit erythrocytes could not be inhibited by any of the carbohydrates tested. This is consistent with earlier report on *A. precatorius* seed lectins (Olsnes *et al,* 1974b). Among the carbohydrates tested, β-lactose and Me-β-Gal were the most potent inhibitors of the lectins (Table 5). The data presented clearly shows that the primary requirement for carbohydrate interaction with *Abrus* lectins is the presence of an axial hydroxyl group at the C-4 position. This is borne out by the fact that the sugars recognized by these lectins such as Me-a-Gal, Me-β–Gal, galactose, lactose and fucose which have an axial hydroxyl group at the C-4 position. Both the lectins, clearly, prefer the P-anomer of galactose, since it

binds Me- β–Gal with twice greater affinity than Me-α-Gal. The low specificity of fucose as compared to galactose indicate that the C-6 hydroxylmethyl group in the galactose configuration contributes positively to the binding. Unlike galactose, which has a CH_2OH moiety attached at the C-5 position, fucose has a methyl group in the corresponding position. This implies that fucose denies any favorable hydrogen bonding interactions involving the hydroxyl of the primary alcohol moiety that might exist in lectin-Gal complexes. This is borne out by the fact that this sugar is a weaker ligand for the lectins than galactose itself. Among the disaccharides too, those with β-configuration for the terminal non-reducing galactose residues served as better ligands. p-Lactose binds with approximately the same affinity as Me-β-Gal and 8 times higher than melibiose (Galα1,6Glc) for abrins and agglutinins of *A. precatorius* and *A. fruticulosus*. P-Lactose binds with the affinity approximately 8 and 16 times better than melibiose (Galα1,6Glc) as in the case of y-abrin and APSA respectively. The trisacchride, raffinose is weaker than galactose. This is consistent with the lectins showing higher preference for the p-anomer of galactose, because the galactose moiety at the non-reducing terminus in raffinose (Galα1,6Glcβ1,2Fru) is in the α-configuration. Comparable binding of Me β Gal and lactose clearly indicates that the *Abrus* lectins preferentially binds the p-anomer of galactose. It was also reported that in galactose specific lectins the hydroxyl groups at C-4 on sugars are the primary binding sites for their recognition by lectins (Khan *et a!.,* 1981a and b; Shaanon *et al,* 1991; Komath *et al,* 1996; Elgavish and Shaanon, 1997).

Studies on the toxicity of the lectins to rats and dose dependence were performed. The LD_{50} dose was found to be 2-5 fig/Kg for abrins and 151-160 μg/Kg for agglutinins clearly suggesting that the abrins are toxic proteins. It was already re-

agglutinins clearly suggesting that the abrins are toxic proteins. It was already reported that abrin from *A. precatorius* was a cytotoxic or type II ribosome inactivating protein (type-2 RIP) (Olsnes and Pihl, 1973b; Olsnes *et al.,* 1974a; Olsnes and Pihl, 1982; Endo *et al,* 1987; Stirpe *et al,* 1992). Abrin, a type-2 RIP was found to possess N-glycosidase activity and inactivates the 60s ribosomal subunits by cleaving a specific adenine residue from rRNA and thereby arresting protein synthesis (Endo and Tsurugi, 1987). In intact animals, inhibition of protein synthesis most likely accounts for the lethal effect of the abrins.

It was interesting to note from the data of western blots that the p-abrin antibody reaction was strong to A-chain of abrins and high molecular weight subunits of agglutinins. This suggests that the immuno-epitopes are present more on A-chain of abrins and high molecular weight subunits of agglutinins, indicating their structural similarity, which corroborate their functional similarity. A significant immunoreactivity was also found between p-abrin antibodies and agglutinins, which supports the growing evidence that abrins and agglutinins share a certain degree of similarity in their primary structure. The immunological relatedness among these proteins suggested that there might be a unique portion of these proteins that is evolutionarily highly conserved.

The data obtained from two-dimensional electrophoretic analysis of the subunits of abrins and agglutinins of all the species revealed that the subunits of one protein were not shared with that of other protein. Based on this result, it can be predicted that abrins and agglutinins are products of homologous genes expressed independently of one another. Subunits of both the lectins in all the species existed as single forms and no isoforms were found as confirmed by two-dimensional electro-

could be due to post translational modifications including proteolytic cleavage and variable degrees of glycosylation (Strosberg *et al.*, 1986; Hegde and Podder, 1992). A number of storage proteins from a variety of plant seeds are initially synthesized as precursor polypeptides containing more than one subunit type, and processed post-translationally (Youle and Huang, 1978; Turner *et al*, 1981; Barton *et al*, 1982; Brieneger and Peterson, 1982; Cray *et al.*, 1982.)-

Lectin distribution in vegetative tissues of 12 day old seedlings *A. precatorius* was studied using a combination of haemagglutination assay and immunoblot analysis. The relative abundance of lectins in the seed and its concomitant decline after germination is in accordance with the probable role of seed lectins as storage proteins. The results of immunoblot analysis indicate that the leaves and stems contain lectin similar to abrin whereas the cotyledons and roots contain both abrins and agglutinins of seed. This could be the possibility for the enormous difference in the heamagglutinating activity between upper and lower parts of the seedlings. This study on differential distribution of lectins indicates that different tissues for different functions may utilize lectins. The preferential accumulation of abrins in the storage organs is certainly indicative of their possible role in defense against pests and diseases. Resting storage organs and seeds are particularly vulnerable, since they are most attractive to potential parasites and predators, and may lack an active defense system because of their inactive metabolic state. Taking into account the evolutionary adaptation of plants, it can be reasonably argued that they have developed defense system to protect their storage organs and seeds. From this point of view the preferential accumulation of lectins in typical storage organs is certainly indicative.

To evaluate the role of the anti-insect activity of the abrins, studies were conducted on the coleopteran insect *Sitophyllus oryza* and lepidopteran insect larvae of *Corcyra cephalonica*. As the final instar of lepidopteran larvae is the most active stage in its life cycle, the larvae feed enormously and synthesizes large number of biomolecules to be used as reserve energy material during the non-feeding pupal stage. However, when the larvae were fed on diet containing abrins, the insects were died at the pupal stage. All the three abrins were almost similar in their toxic effects on both the coleopteran and lepidopteran species displaying insecticidal property but their mechanism of action is unknown.

An intriguing hypothesis would be that the insecticidal effect of toxin is directly related to the ability of the putative lectin like B-chain to recognize and bind to carbohydrate ligand (s) on the cells lining the midgut wall of the insect. Precedence for such a mechanism comes from the demonstration of specific binding of the lectin *Phaseolus vulgaris* to the epithelial cells of the bruchid beetle (*Callosobruchus maculatus*) mid gut. culminating in the mortality of the insect demonstrating that the mechanism of toxicity was analogous to that mammals (King *et ah,* 1980; Gatehouse *et ah,* 1984). Arcelin-1, is a lectin like protein displays insecticidal activity and protects the seeds from predation by larvae of various bruchids (Osborn *et ah,* 1980). This protein that displays an intrinsic specificity in binding complex glycans and might explain the mode of insecticidal action of Arcelin (Gatehouse *et ah,* 1987; Fabre *et al.*, 1998). Arcelin-4 was found to be toxic to larvae of *Zabrotes aubfasiatus* and its antimetabolic effect was speculated to be due to its indigestibility by gut proteases in the insect as proposed by Minney *et ah,* 1990. Haider and Ellar (1987) proposed mechanism of cytolytic effect of *Bacillus thuringeinsis* crystal 8-endotoxin on

Bombyx mori cell lines. The initial interaction of the toxin with the unique receptor of the host determines the specificity of the toxin following which, cell death occurs by a mechanism of colloidal osmotic lysis. Recent reports by Nagamatsu *et al,* 1998 also suggest that the toxin act on the brush border membrane by binding to midgut receptor, subsequently breaking its infolding structure and causing cell lysis. Therefore the receptor specificity may be the mode of insecticidal action for abrins following which, cell death occurs by the inhibition of any of the metabolic pathways.

Another possible mechanism of action is that abrins are RIPs and hence might, therefore inhibit protein synthesis of insects eventually leading to their death. This is in corroboration with the result of Gatehouse *et al,* 1990 who have reported that ricin to be highly toxic to the Coleopteran insects *Callosobruchus maculatus* and *Anthonomus grandis.* Another type-2 RIP a lectin from the bulbs of *Eranthis hyemalis* (Kumar *et al,* 1993) was found to be very toxic to larvae of *Diabrotica undecimpunctata,* a major insect pest of maize. The present study offers evidences, which suggests that the abrins might act as inhibitors of protein synthesis leading to death of insects. The accumulation of toxic lectins in the seeds has been considered to be a protective mechanism (Peumans and Van Damme, 1995). The results support the hypothesis that genes of the legume lectin family encode proteins that function in plant defense against herbivores.

CONCLUSIONS

In conclusion, this study has revealed the following biochemical properties of abrins and agglutinins from three different *Abrus* species, *A. precatorius, A. fruliculosus* and *A. pulchellus*.

1. CLGG was found to be an ideal matrix for the isolation of *Abrsu* lectins compared to other commercial affinity matrices such as Sepharose-4B, AT-Sepharose-4B.
2. The heamagglutinating activity of abrins and agglutinins was inhibited by galactose and galactose containing sugars. The β-linkage sugars inhibited their agglutinating activity more effectively than the α-sugars.
3. SDS-PAGE of the abrins and agglutinins with and without reduction in the presence and absence of [3-mercaptoethanol revealed that the abrins and agglutinins of all the three species consists of peptide chains of nearly the same size.
4. The immunological cross-reactivity of abrins and agglutinins with antibody raised against abrin from *A. fruticulosus* revealed that the proteins have similar molecular identity and immunological determinants.
5. Isoelectric focussing of abrins and agglutinins of all the three species revealed their homogeneity. There was no overlap between abrins and agglutinins within same seeds of all the species.
6. Both the lectins existed as single forms and no isoforms were found as conformed by two-dimensional gel electrophoresis. The complete presence or absence of isoforms of lectins could be due to post-translational modifications.
7. Western blot analysis of different plant parts revealed and ascertained the fact that the lectins are major storage proteins.

8. The abrins show significant toxicity to rats. This suggests that abrins might be type-2 RIPs.
9. The abrins also showed toxicity to Coleoptera and Lepidoptera insects, suggesting that these lectins can be used in the pest control.

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