Biological Properties of the Lectin
INTRODUCTION

The widespread distribution in plant and animal kingdom and ubiquitous nature of lectins, has led to their extensive studies which has revealed that these molecules exhibit a variety of biological properties (1).

Agglutination of cells is a clear cut manifestation of the interaction of a lectin with cells. The agglutination of different cells is specific. Certain lectins are blood group specific and therefore, are used in serological laboratories for typing blood. The lectins from *Dolichos biflorus* and *Ulex europeus* have been shown to be specific for blood groups 'A' and 'H' (0) respectively (2,3). Later, *Ulex europeus* and *Lotus tetragonolobus* lectins were used in typing 'O' blood and in distinguishing $A_2$ from $A_1$ sub-groups and that from *Dolichos biflorus* was used in typing blood group $A_1$. The lectin of *Vicia graminea* serves to distinguish between 'M' and 'N' blood groups and the lectin from *Bandeiraeas simplicifolia* has been shown to be 'anti-B' reagent.

The mitogenic activity of lectins was first observed and reported by Nowell in 1960 (4), with the phytohemagglutinin (PHA) from red kidney bean. Since then many other lectins have been isolated and reported to exhibit mitogenic activity. For example, lectins isolated from
pokeweed, Phaseolus vulgaris, Canavalia ensiformis, Lens culinaris, Wisteria floribunda, Bauhinia caronni, lentil, Robinia pseudoacacia etc. are found to be mitogenic. They stimulate the transformation of lymphocytes from small 'resting cell' into large 'blast like cells', which may ultimately undergo mitotic division. This property of lectins provides an important tool for the examination of biochemical events involved in the conversion of resting cells into actively growing rapidly proliferating ones and also helps in understanding the chromosomal abnormality of genetic diseases.

Mitogenic stimulation is routinely determined by measuring the increase in the rate of incorporation of labelled thymidine into DNA after a 48-72 hr incubation of lymphocytes with varying concentrations of the lectin (mitogen) to be tested. This method gives the relative number of lymphocytes that have been transformed into blast like cells. The blast like cells can be counted under a microscope or by radioautography of cells pulsed with a radioactive isotope.

Recently, there are many reports on the involvement of lectins in the symbiosis of Rhizobia, the nitrogen-fixing bacteria, in legumes. The attachment between the rhizobia and legumes is specific. This can be explained with the following example. The rhizobia that infect and
nodulate soybeans cannot nodulate garden peas or white clover and vice-versa. The mechanism of this specificity may involve the capacity of the bacterial cell to be recognized and bound by some component, possibly a lectin(s), in the roots of the plants. That is, a lectin from a particular legume binds only to the corresponding rhizobial species and not to bacteria that infect other legumes. Fluorescein isothiocyanate (FITC) - labelled SBA binds to 22 out of 25 strains of *Rhizobium japonicum* which infect soybeans, but does not bind to any of the 23 strains from 5 species of rhizobia that infect only other legumes (5). The binding of the lectin to the bacteria was reversibly inhibited by specific inhibitors of the lectin, for example, N-acetyl galactosamine and galactose in case of SBA. Similar attachment of other legume lectins with the Rhizobia has been carried out and reported (6).

Certain lectins present in plant foodstuffs are highly toxic to experimental animals, when fed without heat treatment orally or administered parenterally. It has been proposed by Liener (7) that the toxic effects of lectins when ingested orally may be due to their ability to bind to specific receptor sites on the surface of the intestinal epithelial cells. Different lectins have been shown to react with the crypts and/or villi structure of the intestine, but at different portions of the intestine depending upon
the specificity of the lectin (8,9). The specific binding of lectins may produce profound physiological effects on cells, impair seriously the ability of intestinal cells to adsorb nutrients from the gastrointestinal tract (8,9). Lectins from Ricinus communis, Abrus precatorius, Robinia pseudoacacia, Croton tiglium (10), Adenia digitata (11,12) and Viscum album (13) are found to be toxic. Recently, other lectin meals from Phaseolus vulgaris (14), winged bean (Psophocarpus tetragonolobus) (15,16) and tomato (17) lectins have also been shown to be toxic.

Lectins appear to be widely distributed among leguminous plants which display many similar properties. Most of these studied so far are metal-ion binding tetrameric glycoproteins. Based upon their common source and upon their similar physical, chemical and biological properties, many of the legume lectins might be considered homologs. Immunochemistry provides a powerful method for determining relationships among proteins. Different proteins can be functionally similar and yet antigenically totally unrelated. However, when proteins are found to be antigenically similar there is a strong probability for both an evolutionary and a functional relationship (18).

Recently, many of the biological properties of lectins from different sources have been described by many
authors in detail (19-21).

Some of the above exhibited biological properties of the lectins have been investigated with reference to winged bean tuber lectin and are discussed in detail in this Chapter.

MATERIALS

The purified lectin sample from winged bean (*Psophocarpus tetragonolobus*) tubers was obtained as described in Chapter III by conventional chromatographic methods and has been used in all investigations except the toxicity effect.

The blood samples were obtained from the Newzealand breed rabbits, maintained in the animal house, Department of Chemistry, Karnataka University, Dharwad and human blood group samples were procured from Karnataka Medical College, Hubli. Different rhizobial strains were obtained as a gift from the Department of Microbiology, University of Agricultural Sciences, Bangalore. Rats for the toxicity effect studies were kindly supplied from the Department of Zoology, Karnataka University, Dharwad. Trypsin, Freund’s adjuvants, FITC were obtained from Sigma Chemical Company, St. Louis, USA. Con A and PHA are gift samples. Other chemicals used were all of analytical grade of highest purity.
METHODS

Determination of Hemagglutinin Activity

The preparation and trypsinisation of erythrocyte suspension was carried out as described in Chapter II. The hemagglutinin activity was measured by the serial two-fold dilution method of Liener and Hill (22) with slight modification. The usual method of assay for lectin activity was carried out by using trypsinised and untrypsinised rabbit erythrocytes. In order to investigate the human blood group specificity of winged bean tuber lectin, different blood group erythrocytes with and without trypsinisation have been used in place of rabbit erythrocytes.

Hapten Inhibition Study of WBTL

The inhibitory effect of haptens (sugars) on hemagglutinating activity was determined using the following sugars: xylose, arabinose, D-glucose, D-galactose, fructose, mannose, L-galactose, L-fucose, lactose, sucrose, L-rhamnose, melibiose, melibiose, raffinose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, O- and p-nitrophenyl-α-C-, and β-galactopyranosides, methyl-α-C- and β-galactopyranosides and others of 0.1M solution in 0.15M NaCl. 0.01-0.1 ml of hapten solutions containing 1-10 mM haptens and 4 hemagglutinating units (H.U) of the lectin preparation in the
same solvent (0.15M NaCl) were added in a total volume of 0.2 ml, mixed thoroughly and incubated for 1 hr at 37°C. After incubation, 0.2 ml of trypsinised rabbit erythrocytes were added and once again incubated for 1 hr at 37°C. After two hours of incubation, the lowest concentration of sugar or hapten necessary to prevent agglutination was taken as the inhibitory titer of the corresponding hapten.

**Reversibility of Hemagglutination**

This was carried out by the following procedure. The lectin agglutinated RBC's were collected, washed with 0.15M NaCl for 5-6 times. Sugar solution (0.2 M, D-galactose) in 0.05M phosphate buffered saline, pH 7.2 was added to this cell suspension and kept at 37°C for about 20-30 min. After incubation, dissolution of cell clump was observed and the cells were washed repeatedly with saline (0.9% NaCl) to remove the traces of sugar used. The completely removed, galactose free RBC's were once again checked for agglutination by lectin by the usual two-fold dilution method.

**Lymphocyte Culture for Mitogenic Activity**

Human peripheral lymphocytes were cultured according to the method of Hungerford et al. (23) with slight modifications. Blood from normal healthy donors was drawn into heparinized, previously sterilized syringe and the
cells were allowed to settle for one hour at room temperature. After discarding the first few drops, the leucocytes rich plasma was withdrawn through 20 g needle. Then, the suspension was centrifuged at 185xg for 5 min at room temperature in sterile plastic centrifuge tubes with Ficoll-Hypaque solution. The medium was made with RPMI-1640, containing 50 units/ml penicillin, 50 μg/ml streptomycin and 6% fetal calf serum. The cell suspension was diluted to ~3 million cells/ml with medium. For each of the sterile culture tube (6x1.0 cm), 1.0 ml of this cell suspension was transferred followed by the addition of 0.5 ml of the lectin (20-250 μg) in sterile medium and then fitted with a tight cap. The culture tubes were incubated at 37°C for 72 hr. Controls were prepared in a similar way which did not contain the lectin sample. The percent of cells transformed was determined from Giemsa stained preparations including the control. Simultaneously, two standard mitogens, Con A and PHA were used.

Preparation of Giemsa Stained Slides

The cell suspension was sedimented at 185xg for 5 min. after the incubation period. The supernatant solution was discarded and a drop of suspension of cells was mounted on a clean, grease free slide and air dried. The slides were then stained with Giemsa stain, washed, cleaned, mounted and observed under the microscope.
Binding of Winged Bean Tuber Lectin to Rhizobium

a) Rhizobium cultures

Rhizobium japonicum strain, USDA-110 from soybean, Rhizobium leguminosum from pea, Rhizobium phaseoli strain HA, from bean group, R. meliloti strain Lucerna from alfalfa group, Rhizobium strains from cowpea group (moong and TAL-1145) and Burcin were obtained from the University of Agricultural Sciences, Bangalore and maintained in yeast extract mannitol agar (YEMA) medium.

b) Isolation of Rhizobium from Nodules of Winged Bean Plant

The 3-4 months old winged bean plants were removed, the root systems were washed under running tap water to remove the adhering soil particles. The healthy, preferably pink coloured nodules were selected and washed with distilled water. They were immersed in 0.1% acidified HgCl₂ for 4-5 min. The surface sterilized nodules were repeatedly washed with sterile distilled water and dipped in 70% ethanol. The washings were once again continued with sterile distilled water. Then, the nodules were crushed in a small aliquot of sterile water with the help of a glass rod, which is previously sterilized. The fluid from the crushed nodules is spread on the surface of YEMA plates with the help of a smooth glass rod. The inoculated plates were incubated
upto 6-7 days at 25-30°C. After 4-5 days, bacterial colonies have started growing and the younger colonies were transferred and maintained in agar slants.

The stock cultures of all the rhizobial strains were stored at 4-6°C on agar slants containing the YEMA medium of following composition. $K_2HPO_4$, 0.5 gm; $MgSO_4$, 0.2 gm; $NaCl$, 0.1 gm; mannitol, 10 gm; yeast extract, 1 gm; $CaCl_2$, 3 gm; and agar, 15 gm, for 1 litre of the medium. The cultures in liquid medium were initiated by inoculating the bacteria from the slants to 250 ml conical flasks containing the media with a similar composition as described above but without agar. Cultures were grown at 24°C (room temperature) on a rotary shaker at 80 rpm for 48-60 hr. Cultures of *Rhizobium* strains grown in the synthetic salt medium for ~60 hr were used as inoculum. The cultures were harvested by centrifugation at 20,000xg for 10 min in sterile centrifuge tubes, washed twice with autoclaved phosphate buffered saline, pH 7.2 (PBS) and suspended in sterile PBS.

**Labelling of WBTL with FITC**

WBTL was conjugated with the fluorochrome, fluorescein isothiocyanate for use as a stain in the microscopic examination of rhizobia. The conjugation of FITC to WBTL was carried out according to the following procedure (24). The lectin solution to be conjugated was dissolved in
carbonate buffer, pH 9.5 containing 0.85% NaCl with a protein concentration of 10 mg/ml. To this protein solution, 0.03 mg FITC/mg protein is added. The pH of the solution was maintained above 9.0. The mixture was gently rotated for 1 hr at room temperature and is then centrifuged (3000 rpm for 15 min at 4°C). The content was dialysed extensively against PBS in cold for 48-50 hr to separate the conjugate from the free fluorochrome.

**Binding of FITC-WBTL to Rhizobia Cultures**

Rhizobia growing in the medium were collected by centrifugation as described above and washed with PBS and suspended in minimum volume of PBS. FITC-WBTL conjugate (500 µl of 8 mg/ml) solution in PBS was added to each of the different strains of cell suspensions. After 15-20 min of incubation at room temperature, cells were sedimented, washed once with PBS and suspended in small volume (~500 µl) of PBS. An aliquot of this suspension was placed on a grease-free slide, covered with a cover slip and observed under fluorescence microscope.

The FITC-WBTL treated bacteria were examined under Carl-Zeiss Jena Fluorescence Microscope, Model 250-CF with photomicrographic attachment. The observations were made with both fluorescence and phase contrast optics in order to assure that any observed fluorescence was associated with
individual bacteria or clumps of bacteria, and in order to estimate the proportion of cells in a sample which are bound to FITC-WBTL. Three replicate determinations were generally made.

In addition, the biochemical specificity of FITC-WBTL binding to the bacteria was determined by hapten inhibition study of each sample. In the hapten inhibition tests, a portion of the cell suspension labelled with FITC-WBTL was washed twice with 100 mM galactose in PBS, resuspended in the galactose-PBS solution and observed again to check the reversibility of binding. Other effective hapten inhibitors like melibiose and raffinose (100 mM) have also been tried for hapten inhibition study.

**Toxicity of Winged Bean Tuber Lectin on Rats**

Albino rats of either sex weighing 60-80 gm and which had not been used previously were selected for studying the toxicity effect of winged bean tuber extract. Growing experimental rats were divided into three equal groups and were housed in metabolic cages in an environmentally controlled room temperature at 22-24°C with a 12 hr light/dark cycle. Group 1 was considered as the control and group 2 and 3 were treated as test batches and were given 30 mg and 70 mg of crude preparation of winged bean tuber lectin (30-70% (NH₄)₂SO₄ fraction) per 100 gm body weight of
experimental rats, respectively. The different doses were fed ad libitum after weighing them every day. The faeces were collected from all the groups and extracted with PBS and the supernatant was tested for hemagglutinating activity, using trypsinised rabbit erythrocytes and reactivity with anti-WBTL. After nine days of feeding, the rats were sacrificed by decapitation. During autopsy, duodenum and proximal jejunum portions of rats were removed and taken in PBS, the supernatant was screened for lectin activity. Small portions of the intestine were fixed in the Bouin's fixative solution for 24 hr. Further, the tissues were dehydrated in a graded series of ethanol, cleaned in benzene and embedded in the paraffin wax. Five to six μm thick sections were cut using a microtome, deparaffinised in xylene and stained with hematoxylin and eosin. The stained slides were observed under both low and high power magnifications.

**Immunological Cross Reaction Study of WBTL**

1) **Immunisation of Rabbits**

Immunisation of rabbits was carried out according to standard procedures. During the first week, 100 μg of the purified lectin sample (WBTL) in 0.5 ml of PBS along with 0.5 ml of complete Freund's adjuvant was injected intramuscularly into rabbits with a sterile needle and
syringe, twice at intervals of three days. During the second week, 250 pg of lectin in 0.5 ml of PBS with 0.5 ml incomplete Freund's adjuvant was given subcutaneously twice at intervals of three days. In the third week, 500 pg of the purified lectin in PBS was given subcutaneously along with 0.5 ml of incomplete Freund's adjuvant. A booster dose of 2 mg of lectin in 0.5 ml of PBS with 0.5 ml of complete Freund's adjuvant was injected intramuscularly during the forth week.

After a week, blood was collected from the ear vein into a clean, heparinized dry beaker. It was allowed to coagulate at room temperature. Antiserum was separated by centrifugation for 30 min in cold; compliments were destroyed by keeping the serum at 50°C for 30 min in a water bath. The serum was once again centrifuged at 4°C and preserved at -10°C with sodium azide until use.

ii) Double Immunodiffusion (DID)

Immunodiffusion experiments were carried out in petridishes containing a layer of solidified agar (1% agar in PBS with 0.1% sodium azide) according to the method of Ouchterlony (25). After the wells were punched in the agar plates, antisera was placed in the central well and the antigens (~1 mg/ml) to be tested for cross-reactivity were...
placed in the surrounding wells. After loading the samples, the plates were kept in a moist chamber to prevent drying of agar and incubated at 37°C for 24-48 hr depending upon the development of precipitin bands and photographed.

ii) Immunoelectrophoresis

Immunoelectrophoresis was carried out according to the standard procedure of Graber and Burtin (26). A glass plate (10x20 cm) covered with a layer of solidified agar (1% agar in 0.075M barbital buffer, pH 8.6). Two wells were punched out in the gel plate on either side at one end. The lectin samples were placed in the wells and the plate was electrophoresed using barbital buffer (0.075M), pH 8.6, in cold with a current of 300 V/plate for 12-14 hr. Before loading, the lectin sample was treated with 0.36% (w/v), formaldehyde solution for 20 min to increase the net negative charge on it and which made it to migrate to the opposite direction of the plate (27). Bromophenol blue dye was used as a marker. After the electrophoretic run, the plate was removed and a trough was made and spaced at 2-8 mm from the wells, parallel to the direction of the run. The trough was filled with anti-serum and the plate was kept in moist chamber, incubated at 37°C for the development of immuno-precipitin lines or arcs.
Immunological Cross-Reactivity of WBTL with Legume Lectins

i) Extraction of Legume Seeds

The seeds of the following plants belonging to the family Leguminoseae were collected: *Arachis hypogea* (pea nut), *Pisum sativum* (pea), *Phaseolus mungo* (gram), *Phaseolus aconitifolius* (moth bean), *Vigna catjang* (cow pea), *Cajanus indicus* (red gram), *Cicer arietinum* (bengal gram), *Dolichos biflorus* (horse gram), *Phaseolus mungo* (black gram), *Psophocarpus tetragonolobus* (winged bean) and *Ricinus communis* (castor bean) belongs to the family Euphorbiaceae. Approximately 2-4 gm of seeds were powdered and kept for extraction using PBS in cold for 24 hr. After extraction, the contents were passed through cheesecloth to remove debris and centrifuged at 3000 rpm for 30 min in cold. The clear supernatants were tested for protein and hemagglutinin activity.

ii) Double Immunodiffusion (DID)

The crude extracts of the above legume seeds were tested for cross-reactivity by double immunodiffusion (DID) as described previously using 1% agar in PBS. Sugars (50 mM galactose and glucose) were incorporated into the agar, during the preparation of the plates, to prevent any interaction between lectins and carbohydrate containing serum components.
Protein Estimation

The protein estimation of the extracts was carried out routinely according to Lowry et al. (28) using bovine serum albumin as standard protein.

Determination of Trypsin Inhibitory Activity

The activity of trypsin was determined according to the method of Kakade et al. (29) by the caseinolytic procedure. To 2 ml of trypsin solution (containing 50 µg of the enzyme) in 100 mM phosphate buffer, pH 7.6, 2 ml of 1% casein solution in phosphate buffer, (100 mM, pH 7.2) was added. The contents were incubated at 37°C for 20 min; after incubation, the reaction was stopped by adding 6.0 ml of 5% TCA. After one hour incubation at room temperature, the contents were filtered and the absorbance of the filtrate was read at 280 nm in Spectronic-2000 Spectrophotometer using a suitable control. The control solution was prepared by adding casein solution after the addition of TCA to the incubation mixture.

To determine the trypsin inhibitory activity, the enzyme (40 µg of trypsin) was preincubated at 37°C for 10 min with aliquots of the lectin (50-500 µg) in 2.0 ml and the residual enzyme activity was determined by the casein digestion method as described above. The decrease in the
proteolytic activity of the enzyme was taken as an index of the inhibitory activity.

RESULTS

Hemagglutination Specificity

Hemagglutination activity of winged bean tuber lectin against different erythrocytes is given in Table 5.1. The lectin was found to agglutinate both trypsinised and untrypsinised rabbit erythrocytes and hence used routinely throughout the investigation. The WBTL concentration required for the agglutination of trypsinised rabbit or other erythrocyte sources is less than that of untrypsinised ones. With respect to blood group specificity of this lectin, WBTL was found to agglutinate trypsinised and untrypsinised human erythrocytes of blood groups 'A', 'B' and 'AB' erythrocytes non-specifically, but it does not agglutinate native and trypsinised human type 'O' erythrocytes specifically. On the other hand, it agglutinates both untrypsinised and trypsinised rat erythrocytes but not sheep erythrocytes.

Carbohydrate Specificity

The results of the hapten inhibition study are depicted in Table 3.7. The above data indicates that the lectin from winged bean tubers was found to be D-galactose
TABLE 5.1.
AGGLUTINATION OF ERYTHROCYTES OF DIFFERENT SPECIES BY
WINGED BEAN TUBER LECTIN

<table>
<thead>
<tr>
<th>Erythrocyte source</th>
<th>Minimum concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untrypsinized µg/ml</td>
</tr>
<tr>
<td>Human 'A' type</td>
<td>18.0</td>
</tr>
<tr>
<td>Human 'B' type</td>
<td>9.5</td>
</tr>
<tr>
<td>Human 'AB' type</td>
<td>10.0</td>
</tr>
<tr>
<td>Human 'O' type</td>
<td>n.a*</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.0</td>
</tr>
<tr>
<td>Rat</td>
<td>30.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

* n.a - No agglutination
specific; its hemagglutination activity was effectively inhibited by D-galactose and D-galactose containing oligosaccharides. The oligosaccharide specificity and D-galactose derivative specificity exhibited by WBTL has been described in Chapter III in greater detail.

The ability to bind mono- or oligosaccharide in a specific manner is a characteristic property of lectins. It is this property that is responsible for their biological importance. The reversibility of hemagglutination by WBTL gave positive result. Once used or agglutinated erythrocytes can be reused for further agglutination with the lectin by treatment with galactose followed by repeated washings.

Mitogenicity of WBTL

The purified lectin preparation from winged bean tubers was found to be mitogenic. Figure 5.1 shows the mitogenic transformation or stimulation of lymphocyte cells in presence of WBTL. The different concentration of the lectin samples when treated with lymphocytes at 37°C for different incubation periods, it was observed microscopically that the normal lymphocyte cells were transformed into new "blast like cells." More than this, the mitotic cell division observed was found to be increased with respect to cell count, implying that the lectin appears to be mitogenic. This type of observation was also made by treating standard
mitogens like Con A and PHA along with this lectin.

**Binding of FITC-WBTL to Rhizobia**

Cultures of various *Rhizobium* strains were grown in a defined liquid medium (YEM) for approximately 60 hr, then harvested and washed as described previously and used for binding assay. Shortly after washing, suspensions of living cells were incubated with purified FITC-WBTL and examined under microscope for binding assay to the bacteria (Table 5.2). FITC-WBTL showed binding specificity to all the strains of *Rhizobium* tested and fluorescently labelled rhizobial cells were observed under microscope. The photographs for the FITC-WBTL binding to rhizobial cells are given in Figure 5.2. Figure 5.3 shows the young winged bean plant containing the nodules in the root system. The rhizobial cells isolated from the winged bean plant nodules were also found specifically bind to FITC-WBTL as seen from Figure 5.4. Thus FITC-WBTL binds non-specifically to the following rhizobial cells - *Rhizobium japonicum*, *Rhizobium phaseoli*, *Rhizobium leguminosum* and *Rhizobium meliloti* from different legumes.

The effect of the monosaccharide hapten, D-galactose on FITC-WBTL binding was examined. D-galactose is an effective monosaccharide hapten which acts as an effective and specific inhibitor of WBTL during hemagglutination assay.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>FITC-WBTL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhizobium japonicum USDA-110</em></td>
<td>Soybean</td>
<td>+</td>
</tr>
<tr>
<td><em>Rhizobium leguminosum</em></td>
<td>Peanut</td>
<td>+</td>
</tr>
<tr>
<td><em>Rhizobium phaseoli HA_4</em></td>
<td>Kidneybean</td>
<td>+</td>
</tr>
<tr>
<td><em>Rhizobium meliloti Lucerna</em></td>
<td>Alfalfa</td>
<td>+</td>
</tr>
<tr>
<td><em>Rhizobium sp.</em></td>
<td>Cowpea Moong</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cowpea TAL-1145</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Cowpea Burcin</td>
<td>+</td>
</tr>
<tr>
<td><em>Rhizobium sp.</em></td>
<td>Winged bean</td>
<td>+</td>
</tr>
</tbody>
</table>

* + - Fluorescent cells were observed
(described previously), which is next only to N-acetyl-D-galactosamine. Both these sugars inhibit the binding of WBTL to rhizobial cells at concentrations of 100 mM of D-galactose and N-acetyl-D-galactosamine. The specificity of WBTL binding to rhizobial cells was also examined by testing other inhibitory oligosaccharides such as melibiose and raffinose. These sugars are also effective haptons of FITC-WBTL binding to rhizobial cells but next only to N-acetyl-D-galactosamine.

**Toxic effects of Winged Bean Tuber Lectin**

The two constant doses of crude preparation of WBTL were given *ad libitum* and continued for 9 days to two groups of rats. As the dose of WBTL increased the food intake of the rats was significantly lowered compared to the control group. Besides, a decrease in the body weight was also observed. During the course of feeding, faeces were collected and extracted with PBS. The clear supernatant of the faeces was found to contain hemagglutinin activity, but was less than the total activity fed orally per day sample. These extracts were cross-reacted with anti-WBTL, which gave precipitin lines as shown in the Figure 5.5.

**Effect of WBTL on the Morphological Appearance of the Rat Small Intestine**

Figures 5.6 and 5.7 show the photomicrographs of the small intestine taken from the rats of groups 1 and 2
FIGURE 4.3
GEL ISOELECTRIC FOCUSING PATTERNS OF NATIVE AND MODIFIED LECTIN SAMPLES.
a, native lectin sample; b, acylated WBTL; c, succinylated WBTL.
FIGURE 4.4

DOUBLE IMMUNODIFFUSION PATTERNS OF NATIVE AND MODIFIED LECTIN SAMPLES.

Antiserum was placed in the center well. Well 1, native WBTL; well 2, acetyl-WBTL; well 3, succinyl-WBTL; well 4, maleyl-WBTL; well 5, citraconyl-WBTL; well 6, reductive methylated-WBTL; well 7, 1,2-cyclohexanedione treated WBTL; well 8, NEM-treated WBTL.
FIGURE 4.8

DOUBLE IMMUNODIFFUSION PATTERN OF WBTL MODIFIED WITH NBS AND N-ACETYLMIDAZOLE.

Center well, antiserum; well 1, native lectin; wells 2 and 3, lectin treated with 10 and 20-fold molar excess of NBS at pH 6.0; wells 4 and 5, lectin treated with 10 and 20-fold molar excess of NBS at pH 4.0; well 6, lectin treated with N-acetyl imidazole.
FIGURE 5.1

PHOTOMICROGRAPH OF TRANSFORMED LYMPHOCYTES BY TREATMENT WITH WBTL.
FIGURE 5.2

FITC-LABELED WBTL BOUND BY *R. JAPONICUM* STRAIN.
FIGURE 5.3

WINGED BEAN PLANT WITH NODULES IN THE ROOT SYSTEM
FIGURE 5.4

FITC - LABELED WBTL BOUND BY RHIZOBIUM CELLS
ISOLATED FROM THE SAME PLANT.
FIGURE 5.5

IMMUNODIFFUSION PATTERN OF EXTRACTS FROM THE FAECES OF RATS FROM GROUP 1 AND 2.

Antiserum was placed in the centre well. Wells 1 and 3 contain faeces from the group 1 and 2 respectively; wells 2 and 4, crude and purified WBTL preparations respectively.
FIGURE 5.6 i
PHOTOMICROGRAPH OF CROSS SECTION OF THE SMALL INTESTINE TAKEN FROM CONTROL GROUP RATS.

FIGURE 5.6 ii
PHOTOMICROGRAPH OF CROSS SECTION OF THE SMALL INTESTINE TAKEN FROM CONTROL GROUP RATS AT HIGHER MAGNIFICATION.
FIGURE 5.6
PHOTOMICROGRAPH OF CROSS-SECTION OF THE SMALL INTESTINE TAKEN FROM GROUP 1 RATS.

FIGURE 5.6a
PHOTOMICROGRAPH OF CROSS SECTION OF THE SMALL INTESTINE TAKEN FROM GROUP 1 RATS AT HIGHER MAGNIFICATION, SHOWING THE MORPHOLOGICAL ABNORMALITIES OF THE VILLI.
FIGURE 5.7
PHOTOMICROGRAPH OF THE CROSS SECTION OF THE SMALL INTESTINE TAKEN FROM GROUP 2 RATS.

FIGURE 5.7a
PHOTOMICROGRAPH OF THE CROSS SECTION OF THE SMALL INTESTINE TAKEN FROM GROUP 2 RATS AT HIGHER MAGNIFICATION, SHOWING THE MORPHOLOGICAL ABNORMALITIES OF THE VILLI.
FIGURE 5.8


Antiserum was placed in the center well. Wells 1 and 3 contained the crude extracts of tubers and seeds of winged bean; wells 2 and 4, highly purified lectin (WBTL).
FIGURE 5.9

IMMUNOELECTROPHORETIC PATTERNS OF PURIFIED WBTL.

Antiserum was placed in the center trough. Wells 1 and 2, highly purified WBTL isolated by conventional and affinity chromatographic (gal-Sepharose 6B) methods respectively.
**FIGURE 5.10**

IMMUNOLOGICAL CROSS-REACTIONS BETWEEN OTHER CRUDE LEGUME LECTINS AND ANTISERA AGAINST WBTL.

Antiserum was placed in the center well (A).

Wells 1, A. hypogaea; 2, C. indicus; 3, C. aritinum;
4, D. biflorus; 5, P. tetragonolobus; 6, P. mungo;
7, P. radiatus; 8, P. sativum; 9, P. aconitifolius;
10, R. communis; 11, V. catjang.
FIGURE 5.11

IMMUNOLOGICAL CROSS-REACTIVITY WITH ANTI-WBTL AND LECTIN FROM Euphorbia nivulia Buch Ham LATEX.

Antiserum was placed in the center well. Wells 1 and 3, crude extract of winged bean tubers; well 2, purified WBTL; well 4, lectin from Euphorbia nivulia Buch Ham latex.
FIGURE 5.12

IMMUNOLOGICAL CROSS-REACTIVITY WITH ANTI-WBTL AND WBTL ISOLATED BY CONVENTIONAL AND AFFINITY CHROMATOGRAPHIC METHODS.

Antiserum was placed in the center well. Well 1, WBTL isolated by conventional methods; well 2, WBTL isolated by gal-Sepharose 6B column; and well 3, WBTL isolated by cross-linked guar gum column.
Effect of WBTL on Trypsin Activity

The effect of WBTL on the protease activity was studied with trypsin. The lectin exhibits trypsin inhibitory activity inactivating approximately 15 percent protease activity at 50 µg concentration of WBTL (Table 5.3). However, keeping the enzyme concentration constant (40 µg), increase in the concentration of WBTL gradually results in the increase in the inhibition profile. But, at the concentration of WBTL (200 µg) about 32 percent of inhibition of trypsin activity was observed.

Cross-Reactivity of WBTL with other Legume Lectins

The results of the cross-reactivity studies of WBTL with the (crude) lectins from the seeds of legumes are summarised in Table 5.4. Figure 5.10 shows the results of cross-reaction of anti-WBTL with crude extracts of the legumes. It was observed that the crude extracts of *A. hypogea*, *C. indicus*, *C. aritinum*, *D. biflorus*, *P. mungo*, *R. communis* and *P. tetragonolobus* cross-reacted with anti-WBTL, giving precipitin lines. On the other hand, the crude extracts of *P. sativum*, *P. radiatus*, *P. aconitifolius* and *V. catjang* were failed to give precipitin lines. In all cases where a cross-reaction was observed, the precipitin arcs were present indicating that the cross-reacting lectin was
respectively. Similarly, Figures 5.6a and 5.7a show the small intestine at a higher magnification of rats fed from group 1 and 2 respectively. A significant decrease in the villus height and the development of abnormal forms in the villi were observed in these small intestinal sections. These morphological changes were more pronounced in the sections of group 2 animals as compared to group 1 and the control group.

**Immunological Cross-Reactivity**

The immunological cross-reactivity of WBTL, is given in Figure 5.8. The native lectin (WBTL), crude extract of tubers and the crude extract of winged bean seeds were cross-reacted with the anti-WBTL, which gave precipitin bands. The above data clearly indicates that WBTL is almost similar to the winged bean seed lectin immunologically by having similar antigenic sites.

The homogeneity of WBTL isolated from conventional and affinity chromatographic (galactose-Sepharose-6B) methods was once again confirmed by immunoelectrophoresis. Both the lectin preparations gave a single precipitin arc by cross-reacting with anti-WBTL at pH 8.6, as shown in Figure 5.9.
### TABLE 5.3

**INHIBITION OF CASEINOLYTIC ACTIVITY OF TRYPsin BY WBTL**

<table>
<thead>
<tr>
<th>Concentration of WBTL (µg)</th>
<th>Percentage of inhibition</th>
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</thead>
<tbody>
<tr>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>100</td>
<td>19</td>
</tr>
<tr>
<td>150</td>
<td>21</td>
</tr>
<tr>
<td>200</td>
<td>32</td>
</tr>
<tr>
<td>250</td>
<td>26</td>
</tr>
<tr>
<td>300</td>
<td>12</td>
</tr>
<tr>
<td>400</td>
<td>8</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>


**TABLE 5.4**

**IMMUNOLOGICAL CROSS-REACTIONS BETWEEN LEGUME LECTINS**

<table>
<thead>
<tr>
<th>Lectin source</th>
<th>Family</th>
<th>Anti-WBTL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arachis hypogaea</em></td>
<td>Leguminoseae (P)*</td>
<td>+</td>
</tr>
<tr>
<td><em>Cajanus indicus</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Cicer arietinum</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Dolichos biflorus</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Phaseolus aconitifolius</em></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>Phaseolus mungo</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Phaseolus radiatus</em></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>Psophocarpus tetragonolobus</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><em>Ricinus communis</em></td>
<td>Euphorbiaceae</td>
<td>+</td>
</tr>
<tr>
<td><em>Vigna catjang</em></td>
<td>Leguminoseae (P)</td>
<td>-</td>
</tr>
</tbody>
</table>

Leguminoseae (P)* - Family Leguminoseae and sub-family Papilinaeae

+ - Precipitin band observed
- - No precipitin band observed
immunologically similar, but not identical to the homologous lectin. The crude lectin preparations which gave precipitin arcs were found to be D-galactose-specific similar to WBTL which is a D-galactose-specific lectin. In contrast to this, the D-glucose-specific lectins, which are heterologous to WBTL, did not cross-react with anti-WBTL. However, another D-galactose specific lectin from the latex of Euphorbia nivulia Ham was failed to cross-react with the anti-WBTL, as shown in Figure 5.11.

Figure 5.12 shows the immunological cross-reactivity of lectin preparations purified from three different methods. All the three lectin preparations were found to cross-react with the anti-WBTL, which clearly proves the immunological identity of all the preparations.

DISCUSSION

The winged bean tuber lectin was found to be non-specific towards human erythrocytes. In this respect, it resembles many of the lectins so far isolated, such as Canavalia ensiformis, R. communis, V. faba, Lens ensculenta, P. vulgaris etc. In contrast to this, there are certain lectins which are specific towards different blood group erythrocytes. Boyd and Reguera (3) have reported that lima bean lectin agglutinates human blood group 'A' but not 'B' and 'O' erythrocytes. The lectin from the seeds of
L. tetragonolobus, C. sessilifolius, and L. alpinum have been shown to be specific for type 'O' group, while the lectin from the seeds of B. simplicifolia is blood group 'B' specific (30,31). Recently, another blood group 'B' specific lectin has been isolated from Plecoglossus altivelis fish eggs by Sakakibara et al. (32).

Though WBTL is non-specific, it agglutinates both trypsinised and untrypsinised rabbit, rat and human type 'A', 'B' and 'AB' erythrocytes nonspecifically but, does not agglutinate human type 'O' and sheep erythrocytes. In this respect, WBTL appears to be very similar to the basic lectin isolated from winged bean seeds by Kortt, and Higuchi and Iwai (33,34), which exhibits similar blood group specificity. Such differences in the extent of agglutination with different human blood group erythrocytes for certain lectins have been reported in other cases.

In many cases the trypsinised RBCs are agglutinated to a greater extent than the untreated ones (35,36). SBA agglutinates trypsin treated RBCs >200 times more than the untreated cells. Similarly, the lectin isolated from pearl millet (Pennisetum typhoides) agglutinates trypsinised cells to a far greater extent than the untrypsinised cells (37). With WBTL, enhanced agglutinin titer was observed with trypsinised rabbit erythrocytes. The increased sensitivity
of trypsinised cells is said to be due to the closer distribution of lectin binding receptor sites on their cell surface and not due to an increase in the number of receptor sites on the erythrocyte cell surface (1,31).

The carbohydrate specificity of WBTL is directed towards \( \alpha \)-linked galactopyranosides. \( \sigma \)- and \( \pi \)-Nitrophenyl \( \alpha \)-D-galactopyranoside, N-acetyl-D-galactosamine are the most potent inhibitors of this lectin. The inhibitory profile of WBTL appears to resemble with that of basic lectin from winged bean seeds (33,34). The details regarding this has been discussed in Chapter III and IV.

The interaction of lectins with cells is the triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation, mitogenic stimulation. Most of the lectins so far reported to be mitogenic, are usually D-glucose, D-mannose and N-acetyl-D-galactosamine specific (10, 38-41). Barbieri et al. have reported that the galactose specific lectins are, however, non-mitogenic (42). Recently, Borrebaeck and Rouge (43) have studied the mitogenic properties of 10 phytogenetically related *Lathyrus* lectins and observed the close structural resemblance and similar carbohydrate specificities of these lectins. SBA which is specific for D-galactose and N-acetyl-D-galactosamine, is non-mitogenic. However, SBA stimulates
lymphocytes after the sialic acid has been cleaved from the cell surface, by treatment with neuraminidase. It is postulated that the transformation induced by SBA in desialyated lymphocytes is caused by the binding of the lectin to the newly exposed galactosyl residues. The sialic acid moiety in the glycoprotein is known to be glycosidically linked to galactose or N-acetyl-D-galactosamine (44). The mitogenic property exhibited by WBTL, a D-galactose or N-acetyl-D-galactosamine specific lectin appears to be due to the binding of internal glucosyl residues or Gal β1→3 GalNAc sequences which occur in the glycolipids and may act as receptors for mitogenic stimulation (1). It has also been reported that the galactose-specific mitogens interact with the α-glycosidically linked sequence Neu NAcα (2→3) Gal B (1→3) GalNAc-Ser (Thr). Recently, Kilpatrick et al. (45) have shown that the tomato (Lycopersicon esculentum) lectin was non-mitogenic for human lymphocytes in culture and suppressed spontaneous DNA synthesis. It also inhibited the transformation of human peripheral blood lymphocytes. The inhibition of transformation by this lectin could be abolished by the simultaneous addition of oligomers of N-acetyl glucosamine.

Most of the studies of lectin binding by rhizobia have used lectins isolated from legume seeds. Winged bean (Psophocarpus tetragonolobus) tubers contain a lectin, which almost resembles the basic lectin from the seeds of the title
It is observed that WBTL was found to react with certain strains of Rhizobia non-specifically. This result is consistent with the lectin recognition hypothesis (1,46). However, such interaction studies between the seed lectins of winged bean and Rhizobium have not been made so far.

The association between nitrogen-fixing bacteria such as Rhizobia and legume species is specific. Legume species or cultivars which are nodulated by some Rhizobium isolates are not nodulated by others. For example, rhizobia that infect and nodulate soybeans cannot nodulate garden peas or white clover and vice versa. Similarly, fluorescein-labelled SBA binds to 22 out of 25 strains of *R. japonicum* which infect soybeans, but does not bind to any of the 23 strains from 5 species of rhizobia that infect only other legumes (5). In contrast to the above statement, WBTL is non-specific towards rhizobial cells which are tested for binding. This observation may be due to the variations in the culture and assay conditions used. Similar contradictory observations have been made in case of some species of soybean which bind to cow pea strains (6,47). Recently, Ho et al. (48) have isolated a soybean lectin from cultures of soybean cell lines and this lectin is responsible for the binding of *R. japonicum*. 
The binding between the rhizobial cells and FITC-WBTL was inhibited or reversed by N-acetyl-D-galactosamine and D-galactose, for which WBTL is highly specific. Such reversal of binding study of conjugated SBA with *R. japonicum* has been investigated by the above hapten sugars, which are specific to that lectin also (6,49). Recently, Shantaram (50) has studied the pole bean lectin binding to rhizobia. It was observed that this lectin binds to the extracellular polysaccharide (EPS) of all the rhizobial species tested non-specifically.

The non-specific binding of FITC-WBTL to strains of rhizobial cells tested indicate that either the binding of WBTL to the other strains is irrelevant and coincidental to their interactions, with winged bean, or that the lectin-mediated recognition of these strains by winged bean, if it occurs, does not result in nodulation. Further studies are needed to distinguish between these possibilities.

The impairment of growth in experimental animals such as rats etc. by purified lectin preparations has been known for a long time (51). The winged bean tuber lectin was found to be toxic to the rats. Body weight of the experimental rats was decreased. At the intestinal level, duodenal villi were shortened, while duodenal and jejunal crypts were significantly lengthened; such observations
have also been made with other lectins, such as, SBA (52), WGA and Con A (53), Phaseolus acutifolius lectin (54), PHA (14, 55) and also winged bean seed lectin (15, 16, 56). Recently Pusztai et al. (57) has discussed the effect of diets containing lectins on oral cells and gastrointestinal tract, absorption of lectins, local and systemic immune response of dietary lectins and some general physiological effects of absorbed lectins in detail.

The anti-WBTL gave precipitin band against the faecal extracts from the rats ingested with the lectin and the native lectin. As these lines become (or get) fused with each other, it was concluded that the antigenic similarity of the faecal material is identical with that of the native lectin preparation, which was administered orally. Besides, this extract also exhibits hemagglutinating activity. All these parameters clearly indicate that a part of the ingested lectin fraction remains intact in the gastrointestinal tract to bind to the intestinal mucosa and the rest being excreted in the faeces in the same form maintaining both the hemagglutinating activity and antigenicity.

The decrease in the body weight of rats or the alterations of the villi structure may also be due to a significant decrease in the activities of several intestinal enzymes such as, sucrase, maltase, alkaline phosphatase,
leucine amino-peptidase, -glutamyl transferase etc. Such observations have been made and their activities have been determined in case of winged bean seed lectin (15). Recently, similar toxic effects of kidney bean lectin has been reported on rats' gastrointestinal tract by Rouanet et al. (14).

The immunological cross-reactivity studies of proteins gives the homogeneity criteria and homologous nature of the proteins. The crude extract of the winged bean seeds and tubers gave precipitin bands with the anti-WBTL. Hence it is evident that the lectin present in the tubers of the winged bean plant is antigenically similar to the lectin present in the seeds of the same plant. The antigenic similarity of WBTL may be due to the similar carbohydrate specificity and molecular properties of the winged bean seed basic lectin (33,34). Such similarities in sugar specificity and molecular properties have been observed in white clover (58) and soybean (59). However, Lew and Strijdom (60) have reported that Lotononis bainesii root extract does not appear to contain material antigenically related to the seed lectin nor did the seed extract contain material that cross-reacted with the root lectin.

The legume lectins which have been characterised can be classified into two main groups with respect to their carbohydrate binding specificities;
i) D-galactose and its derivatives and
ii) D-glucose or mannose and its derivatives.

Most of the lectins from legumes are D-galactose specific. The results presented based on immunological cross-reactivity study, indicate that there is extensive immunological homology among the lectins in the D-galactose group. Among the different legume lectins tested for cross-reactivity with anti-WBTL, only 7 out of 11 legume lectins have cross-reacted. The non-reacted lectins are usually specific to D-glucose and its derivatives.

In addition to the immunological cross-reactions among most legume lectins, cross-reactivity was also observed between certain legume lectins and a lectin from a dicotyledonous plant (castor bean), which is outside the family leguminosae. The castor bean lectin is a galactose specific lectin and has many common properties with the legume lectins which are also isolated from seeds. This property may be due to the evolutionary and functional homology of a unique lectin species occurring widely in legumes and perhaps even in dicot plants in general. Such evolutionary relationships has been reported by Foriers et al. (61). On the other hand, a D-galactose specific lectin from the latex of Euphorbia nivulia Ham. did not cross-react with the anti-WBTL may be due to the fact that it belongs to the family Euphorbiaceae.
The immunological cross-reactivity of the legume lectins can be correlated with following properties: All plant lectins have similar chemical and physical properties, almost all are glycoproteins, all are from the same plant family and isolated from seeds. Thus, some of the galactose specific lectins from leguminous family are homologous. The other parameters such as amino acid sequence analysis and $\text{NH}_2$-terminal amino acid sequences of legume lectins are also strikingly similar which reflect the taxonomical relationships of the plants in this family (62-64). Recently, Kochibe (65), has studied the immunological cross-reactivity of D-mannose binding lectins. He has shown that, anti-*Vicia tetrasperma* seed lectin cross-reacted with the lectins from *V. cracca*, *P. sativum* and *Lens culinaris* (mannose-specific lectins), and *V. faba*, *V. hirsuta* and *V. angustifolia* (analogous species). The formation of a spur in the diffusion showed the slight difference in the immunological identity from *V. tetrasperma* lectin (65).

WBTL exhibits trypsin inhibitory activity. The co-existence of agglutinins and proteinase inhibiting functions in one molecule has been reported for the *H. pomatia* lectin by Uhlenbruck *et al.* (66). Habets *et al.* (67) have also described the existence of these two properties in *Arion empiricorum* lectin molecule; this may become relevant because
two aspects of malignant cells are involved. Malignant cells are agglutinated by several lectins (68), which also exhibit elevated proteinase production (69). Lectins as well as proteinase inhibitors can re-establish density-dependent inhibition of growth in cultures of transformed cells (70). Growth inhibiting properties of proteinase inhibitors in experimental cancers have been reported (71,72).
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