CHAPTER III

BIOLOGICAL ACTIVITY OF LECTINS: MITOGENIC EFFECT OF RBL AND CSL ON NORMAL HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)
3.1. INTRODUCTION

Lectins are structurally very diverse class of proteins that bind carbohydrates with considerable specificity but moderate affinity [Lis and Sharon 1998, Van Damme et al. 2003]. Lectin carbohydrate interactions are involved in a wide variety of biological functions, including recognition, agglutination, cell adhesion, migration, apoptosis and proliferation and thus possess immunomodulatory properties [Varki et al. 1993; Sharon 2007; Pohlven et al. 2009; Perillo et al. 1998].

A mitogen is a substance or a protein that encourages a cell to commence cell division, triggering mitosis. Mitogens trigger signal transduction pathways in which mitogen-activated protein kinase is involved, leading to mitosis. Mitogens are often used to stimulate lymphocytes and therefore assess immune function. Most of the mitogens induce proliferation on T-lymphocytes. Plasma cells are terminally differentiated and therefore cannot undergo mitosis. Memory B-cells can proliferate to produce more memory cells or plasma B-cells [Palacios 1982; Kimura and Ersson 1981].

During recent past lectins have captured the attention of a large number of researchers on account of the various exploitable activities that they exhibit including their proliferative effects on various cell types. The ability of lectins to detect subtle variations in carbohydrate structures on the surface of cells and tissues has made them a convenient tool for detecting
protein-carbohydrate interactions. Lectins display a considerable repertoire of carbohydrate specificities. These characteristics, together with the ability to stimulate lymphocytes as well as other cells, have made lectins as an important diagnostic and experimental tool to study the various aspects of cell growth and differentiation [Ashraf et al. 2003].

The various molecular events underlying the immune response also include lectin carbohydrate interactions. Hence, lectins are used as polyclonal reagents to investigate the molecular basis and control of lymphocyte activation and proliferation. Lectins can stimulate transformation of the cells from the resting phase to blast like cells, which may subsequently undergo mitotic division. Apart from this, lectin can also be used to identify and fractionate the cells of the immune system, and as drugs [Singh et al. 2004].

The first mitogenic agent reported was PHA, the lectin from red kidney bean (Phaseolus vulgaris), by Nowell [1960]. The discovery of the lectin mediated mitogenesis led to the discovery of many other mitogenic lectins; notable examples include Con A [Haris et al. 1963], WGA [Aub et al. 1965] PWM [Brittinger et al. 1969]. These mitogenic lectins have been extensively used to study lymphocyte function, in vitro, for many years. Mitogenic lectins have been reported from various plant parts of different taxonomic groups having varied sugar specificities like lectins from tubers of Alocasia indica, Gonatanthus pumilus, and Sauromantum guttum.
Most of the lectins stimulate only T-cells and are inactive or inhibitory for the mitosis of the other class of lymphocytes [Ashraf 2003]. For example, Con A and PHA are found to be selective T-cell mitogens when compared to their effect on B-cells. However, PWM is both a T- and B-cell mitogen. Lentil lectin, which was previously considered to be solely a T-cell mitogen, has been shown to stimulate human B-cell proliferation as well [Miller 1983]. On the contrary, some lectins are selective B-cell mitogens like, lectins from the slime mould Dictyostelium purpureum [Lipsick et al. 1980] and from the crab Homarus americanus [Campbell et al. 1982], which stimulate B-cells and not T-cells from chicken tissue. Certain lectins preferentially stimulate neuraminidase-treated lymphocytes, while others may stimulate untreated lymphocytes.

In recent past fungal lectins have been receiving greater attention due to their interesting sugar specificities and the biological activities exhibited, giving rise to a wide range of potential pharmacological and biotechnological applications [Ng 2004]. Apart from the earliest recognized erythroagglutinational ability, the biological activity of fungal lectins towards organisms or cells includes phenomena such as lymphomitogenic

The lectins bind specifically with cell surface glycan ligand and induce diverse effects on cell behavior that are thought at least in part due to the differences in their sugar specificities. Reorganization of cell surface carbohydrates is shown to be required for various activities of lectins such as mitogenic stimulation and induction of apoptosis. The lectins bind specifically with cell surface glycan ligand, followed by ligand clustering and induce lymphoproliferation and myeloproliferation with balanced secretion of various cytokines, interferons, growth factors [Kilpatrick 1988; Sacchettini et al. 2001]. All these immuno stimulatory activities of lectins depend on the concentration, physical environment and the presence of accessory cells [Kilpatrik 1988]. Most of the mannose binding lectins namely Concanavalin A, *Lens culunaris* agglutinin (LCA) and *Pisum sativum* agglutinin (PSA) induce mice splenocyte to secrete pro inflammatory cytokines [Muraille et al. 1999] and human basophiles to
release IL-4 and IL-13 [Haas et al. 1999]. Whereas, the galactose binding Mistletoe lectin-1 secretes a wider range of cytokines [Gabius et al. 1992; Gabius and Gabius 1998, 1999].

Present chapter describes the interaction of two lectins RBL and CSL on normal human peripheral blood mononuclear cells (PBMCs) and the mitogenic effect executed by them. Of the two lectins tested, RBL emerged as a potent mitogen comparable to that of PHA-L, a well known mitogen.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Lectin from *Rhizoctonia bataticola* (RBL) was purified using ion exchange chromatography on CM-cellulose and affinity chromatography on asialofetuin-Sepharose 4B as described in the earlier chapter. Lectin from *Cephalosporium curvulum* (CSL) was purified using affinity chromatography on asailofetuin-Sepharose 4B.

Mucin (porcine stomach, type III), fetuin (fetal calf serum), PHA-L, Histopaque 1077, fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co., St. Louis, USA. Tritiated thymidine was procured from BRIT (Board of Radiation and Isotope Technology), India. The tissues culture flasks and 96 well plates were procured from NUNC (Denmark). Human blood samples were obtained from local blood bank. All other chemicals used were of analytical reagent grade.
3.2.2. FITC-labeling of lectins

FITC conjugated RBL (FITC-RBL) and FITC conjugated CSL (FITC-CSL) used for flow cytometry binding studies was prepared according to the procedure described by Goldman [1968]. Briefly, lectin (10 mg/ml) was incubated with FITC at the concentration of 25 μg/mg of protein in carbonate buffer (pH 9.5) with gentle stirring for 12 hr, at 4°C. Unbound excess FITC was removed by extensive dialysis against TBS (pH 7.2) and stored at 4°C till further use.

3.2.3. Preparation of periodate-BSA (p-BSA)

Periodate-BSA required for blocking non-specific lectin binding sites was prepared according to the method of Glass et al. [1981]. BSA (fraction V) in 0.1 M sodium acetate buffer, pH 4.5 (4 g/100 ml) was treated with 10 mM periodic acid for 6 hr at room temperature. Then excess of periodate was eliminated by adding glycerol to a final concentration of 10 mM, followed by extensive dialysis against 10 mM PBS and subsequently against distilled water. Dialyzed sample was freeze dried and stored at 4°C till further use.

3.2.4. Isolation of PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by density gradient using Histopaque-1077 according to Boyum [1968]. Heparinized blood (diluted with saline 2:1) was overlaid on ficoll hypaque solution in a ratio of 3:1 and was centrifuged
at 2,000 rpm, for 30 min at room temperature, to separate three distinct layers. PBMCs, appeared as opaque middle layer, were removed carefully without disturbing the lower layer of RBCs. Finally PBMCs were resuspended in complete medium (RPMI 1640 + 10% FCS).

3.2.5. Binding of lectins (RBL and CSL) to human PBMCs

Binding of lectins to human PBMCs was determined quantitatively by flow cytometry analysis using FITC-conjugated lectins. Human PBMCs isolated from the blood of healthy donors were suspended in complete medium (RPMI 1640 + 10% FCS), washed with ice cold PBS and adjusted to 1x10^5 cells/100 μl per vial. Then the cells were incubated with 3% p-BSA (200 μl) in order to block the non-specific lectin binding sites. After being washed with ice cold PBS, cells were incubated with FITC-lectin (FITC-RBL or FITC-CSL, 1 μg/100 μl) for 1 hr on ice. Then the cells were washed thoroughly with 50 mM PBS followed by fixing with 2 % freshly prepared paraformaldehyde. Finally, cell pellet was thoroughly washed and resuspended in 500 μl of cold PBS. Data was acquired for 10,000 events using FACS Vantage (Becton Dickson) equipped with 488 nm argon lasers and analyzed with cellquest-pro software for determining percent positivity and mean fluorescence intensity (MFI, an arbitrary unit for measuring the fluorescent intensity). Unstained cells processed similarly were used as negative control and the positivity for these cells was set at 1 %. Data was acquired was presented as a frequency histogram (X-axis; fluorescent
intensity and Y-axis; number of events or cells). Three independent experiments were carried out for each of the lectin.

Receptor mediated lectin binding to PBMCs was determined by preincubating FITC-labeled lectins (RBL and CSL) with mucin, fetuin and asialofetuin (25, 50 µg/100 µl) for 1 hr at room temperature. This lectin-sugar complex was added to the PBMCs preparation and analyzed by flow cytometry.

3.2.6. Mitogenic activity

The mitogenic activity of RBL was assessed in freshly isolated human peripheral blood mononuclear cells by tritiated thymidine incorporation assay. PBMCs were suspended in RPMI1640 containing 10% FCS and Cells (10^5 cells/100 µl/well) were plated in 96 well tissue culture plate (NUNC, Denmark) and incubated with different concentrations of RBL ranging from 0.156 µg/ml to 2.5 µg/ml for 72 hr at 37°C in an incubator supplemented with 5% CO₂. Appropriate controls were included such as PBMCs without RBL treatment as control and PBMCs stimulated with PHA-L (0.156 µg/ml to 2.5 µg/ml, Sigma Chemicals) were used as positive control. Cells were pulsed with tritiated thymidine (1 µCi per well, BRIT, India) 18 hr prior to harvesting and after 72 hrs cells were harvested and thymidine incorporation was measured as counts per minute (CPM).

Mitogenic activity of RBL was also measured for different time intervals, 24, 48 and 72 hr, using the optimum dose of RBL (1.25 µg/ml). Cells were pulsed with tritiated thymidine (1 µCi per well, BRIT, India) 18
hr prior to harvesting for each time interval (6 hr, 30 hr and 54 hr respectively) and thymidine incorporation was measured as counts per minute (CPM). PHA (1.25 μg/ml) was incorporated as a positive control and processed in a similar way.

The mitogenic activity of CSL was also assessed by thymidine incorporation method in a similar way. Briefly, PBMCs were incubated without and with different concentrations CSL ranging from 1.25 μg/ml to 10 μg/ml. PBMCs stimulated with PHA-L (0.156 - to 2.5 μg/ml, Sigma Chemicals) were used as positive control. Cells were pulsed with tritiated thymidine (1 μCi per well, BRIT, India) 18 hr prior to harvesting and after 72 hrs thymidine incorporation was measured as counts per minute (CPM).

3.3. RESULTS

To study the interaction of lectins with normal human PBMCs, the studies on binding of lectins (RBL and CSL) with normal human PBMCs were carried out using flow cytometry analysis. In order to know the lectin mediated effects upon binding to PBMCs, tritiated thymidine incorporation assay was used to evaluate the effect. Both RBL and CSL were found to be mitogenic; however, RBL exerted marked stimulatory effect when compared to CSL.

3.3.1. Flow cytometric analysis of RBL and CSL with normal human PBMCs

To study the interaction of RBL with PBMCs, cells were stained with FITC-RBL and its binding was determined by flow cytometry analysis. As
depicted in the histograms (Fig. 1A), 98.76% of the cells were positive for RBL binding, with a mean fluorescence intensity (MFI) of 82.54, in comparison with control (unstained cells) which was set to 1% positivity with a MFI of 11.65. In order to know whether the binding of RBL to PBMCs is the receptor-mediated or not, the binding of RBL was tested after pre-incubation of the lectin with different competing glycoproteins such as asialofetuin, mucin and fetuin. The results of RBL binding to PBMCs after blocking with asialofetuin, mucin and fetuin are presented in flow cytometry histograms (Fig. 1B, 1C, 1D). All the three glycoproteins, mucin, fetuin and asialofetuin (100 µg/ml) equally and effectively inhibited the binding of RBL to PBMCs. The same results are also indicated in Table 1, where the fluorescent intensity with FITC-labeled RBL was considered as 100% for calculating the inhibition of binding in presence of competing glycoconjugates.

In a similar way the studies on binding of CSL with normal human PBMCs were carried out. Cells were stained with FITC-CSL and its binding was followed by flow cytometry analysis. As shown in the histogram (Fig. 2A), 99.62% of the cells were positive for CSL binding, with mean fluorescence intensity (MFI) of 411.27, in comparison with control unstained cells which was set to 1% percent positivity. The MFI of the unstained cells was found to be 76.89. Flow cytometry histograms of CSL binding to PBMCs after blocking with competing glycoproteins such as mucin, fetuin and asialofetuin are presented in Fig. 2B, 2C, 2D respectively.
Fig. 1. Binding of RBL to human PBMCs and inhibition with glycoproteins: PBMCs were stained with FITC-labeled RBL and subjected to flow cytometric analysis. X-axis represents fluorescence intensity, Y-axis represents cell number. (A) The histoplot shows profiles of the unstained cells (shadow) and cells stained with FITC-labeled RBL (bold line). Profiles of cells stained with FITC-labeled RBL preincubated with different glycoproteins: (100 pg/ml) (B) asialofetuin, (C) mucin and (D) fetuin are represented by dotted lines. The plots are representative data of three independent experiments.

Table 1- Binding of RBL to human PBMCs and inhibition of binding with competing glycoproteins.

<table>
<thead>
<tr>
<th></th>
<th>% Positive Cells</th>
<th>Fluorescence intensity-MFI (%)</th>
</tr>
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<tbody>
<tr>
<td>RBL</td>
<td>99</td>
<td>100 *</td>
</tr>
<tr>
<td>RBL + Mucin (100 µg/ml)</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>RBL + Fetuin (100 µg/ml)</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>RBL + Asialo-fetuin (100 µg/ml)</td>
<td>4</td>
<td>17</td>
</tr>
</tbody>
</table>

The table shows the % positive cells and the % fluorescence intensity in cells stained with FITC-labeled RBL in absence and presence of competing glycoproteins. The percent positivity and mean fluorescence intensity (MFI, an arbitrary unit for measurement of fluorescence intensity) in the stained cells was calculated by setting the positivity in unstained cells to 1%. * The fluorescent intensity with FITC-labeled RBL was considered as 100 % for calculating the inhibition of binding in presence of competing glycoproteins.
It was observed that, among three glycoproteins tested, mucin (100 μg/ml) potentially inhibited the binding of CSL to PBMCs. Fetuin and asialofetuin (100 μg/ml) also inhibited the binding of CSL to PBMCs.

The same results are also presented in Table 2, where the fluorescent intensity with FITC-labeled CSL was considered as 100 % for calculating the inhibition of binding in presence of competing glycoproteins.

**Fig. 2.** Binding of CSL to human PBMCs and inhibition with glycoproteins: PBMCs were stained with FITC-labeled CSL and subjected to flow cytometric analysis. X-axis represents fluorescence intensity, Y-axis represents cell number. (A) The histoplot shows profiles of the unstained cells (UNS) and cells stained with FITC-labeled CSL (CSL). Profiles of cells stained with FITC-labeled CSL preincubated with mucin, fetuin and asialofetuin (100 μg/ml) are indicated in B, C, and D respectively.
Table 2 - Binding of CSL to human PBMCs and inhibition of binding with competing glycoconjugates.

<table>
<thead>
<tr>
<th></th>
<th>% Positive cells</th>
<th>Fluorescent intensity – MFI (%)</th>
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<tbody>
<tr>
<td>CSL</td>
<td>99.62</td>
<td>100 *</td>
</tr>
<tr>
<td>CSL + mucin (100 µg/ml)</td>
<td>6.59</td>
<td>16.05</td>
</tr>
<tr>
<td>CSL + fetuin (100 µg/ml)</td>
<td>16.94</td>
<td>11.99</td>
</tr>
<tr>
<td>CSL + A. fetuin (100 µg/ml)</td>
<td>39.52</td>
<td>11.53</td>
</tr>
</tbody>
</table>

The table shows the % positive cells and the % fluorescence intensity in cells stained with FITC-labeled CSL in absence and presence of competing glycoproteins. The percent positivity and mean fluorescence intensity (MFI, an arbitrary unit for measurement of fluorescence intensity) in the stained cells was calculated by setting the positivity in unstained cells to 1%. * The fluorescent intensity with FITC-labeled CSL was considered as 100 % for calculating the inhibition of binding in presence of competing glycoproteins.

3.3.2. Determination of mitogenic activity of lectins (RBL and CSL) by thymidine incorporation assay

Mitogenic activity of RBL on human PBMCs was determined by tritiated thymidine incorporation assay. RBL exerted marked stimulatory effect on the uptake of thymidine by human PBMCs with maximum incorporation occurring at 1.25 µg/ml. Similarly, PHA-L used as positive control also exhibited maximum proliferative effect at 1.25 µg/ml concentration (Fig. 3A). Time course study depicted a time dependent increase in proliferation in both RBL and PHA-L stimulated PBMCs. Both RBL and PHA-L did not reveal significant increase in proliferation after 24 hr incubation. However, after 48 hr and 72 hr incubation there was a noticeable increase in proliferation. Interestingly, the response elicited by RBL was different compared to PHA-L with respect to the fold - increase in
proliferation at 48 hr. In the case of PHA-L no further significant increase occurred between 48-72 hr but with RBL, there was a significant increase in proliferation from 48 to 72 hr post stimulation (Fig. 3B).

In similar way, the proliferative effect of CSL on human PBMCs was assessed by tritiated thymidine incorporation assay. CSL showed a dose dependent mitogenic effect with maximum proliferation occurring at a concentration of 10 μg/ml. CSL is weak mitogen compared to PHA-L, which exhibited maximum proliferative effect at 1.25 μg/ml concentration (Fig. 4).

![Graph A](image1.png)

![Graph B](image2.png)

Fig.3. Mitogenic activity of RBL: (A) PBMCs were isolated from blood of healthy donors and exposed to serial concentrations of RBL and PHA-L (0.16-2.5 μg/ml) for 72 hr and proliferation was measured by tritiated thymidine incorporation assay. (B) PBMCs were stimulated with RBL and PHA-L (1.25 μg/ml) for 24, 48 and 72 hr. Fold increase in proliferation was calculated by considering the CPM of unstimulated cells as 1 for each time point. The data are presented as mean ± SE of four independent experiments done in triplicates. * difference (p< 0.025) between RBL and PHA-L at 48 hr post stimulation, ** difference (p< 0.025) between PBMCs stimulated for 48 hr and 72 hr with RBL.
Fig. 4. Mitogenic activity of CSL: PBMCs were isolated from blood of healthy donors and exposed to serial concentrations of CSL (0.625-10 μg/ml) and PHA-L (0.16-2.5 μg/ml) for 72 hr and proliferation was measured by tritiated thymidine incorporation assay. PBMCs treated with only PBS (UNT) under similar conditions were used as control. The data presented as mean ± SD of three independent experiments carried out in triplicates.

3.4. DISCUSSION

Recognition of cell-surface carbohydrates by lectins has broad implications in important biological processes. Mitogenic stimulation is one of the most dramatic effects of the interaction of lectins with cell surface glycans. In contrast to other mitogenic antigens, lectins do not stimulate lymphocytes in a preferential manner. Lectins bind to carbohydrate receptors on the surface of all the lymphocytes which contain the same sugar moieties appropriate for the specificity of the mitogenic lectins. It has been observed that over 80% of the total lymphocytes in a homogeneous
population may be induced and stimulated with the same lectin under appropriate conditions [Hume and Weidaman 1980]. Thus lectins have a clear advantage over specific antigens as mitogenic stimulants, and they are the best polyclonal stimulants for studies on the reaction mechanism of the mitogenic transformation of lymphocytes.

In the present study we demonstrated the binding of two lectins, RBL and CSL to normal human PBMCs by flow cytometry. Flow cytometric analysis revealed that 98.76% of the cells were positive for RBL binding, with mean fluorescence intensity (MFI) of 82.54, in comparison with control unstained cells which were set to 1% percent positivity with a MFI of 11.65. Flow cytometry analysis of RBL binding to PBMCs demonstrated the receptor mediated RBL binding as it can be effectively blocked by glycoproteins such as mucin, fetuin and asialofetuin.

In the similar way, binding of CSL to human PBMCs were analyzed by flow cytometry, 99.62% of the cells were positive for CSL binding, with mean fluorescence intensity (MFI) of 411.27, in comparison with control unstained cells which were set to 1% percent positivity. The MFI of this 1% cells was 76.89. This binding was inhibited by competing glycoproteins such as mucin, fetuin and asialofetuin where mucin being the potential inhibitor, suggesting involvement of lectin receptors in the process of binding.

These two lectins, RBL and CSL exhibited proliferative effect up on binding to normal human PBMCs. RBL emerged as potent mitogen with
maximum proliferation occurring at a concentration of 1.25 µg/ml and the observed effect is comparable to that of PHA-L, a well known mitogen. Like PHA-L, RBL showed a dose-dependent increase in proliferation of PBMCs. The proliferation was effectively inhibited with asialofetuin, mucin and fetuin, suggesting receptor mediated mitogenic response. Time course kinetic studies depicted the delayed response of RBL for mitogenic stimulation compared to PHA-L. In the case of PHA-L, no further significant increase occurred at 72 hr, but with RBL, there was a significant increase in proliferation from 48 to 72 hr post stimulation. These results suggested that RBL might have a different mechanism of induction of proliferation compared to PHA-L.

The other lectin, CSL also showed a significant dose dependent proliferative effect on normal human PBMCs. Maximum stimulatory effect was observed at a concentration of 10 µg/ml suggesting its weak Mitogenicity when compared to PHA-L (2.5 µg/ml) and RBL (1.25 µg/ml).

Many of fungal lectins are reported to be mitogenic. For example, lectin from *V. volvacea* [She et al. 1998], *Boletus santanas* Lenz [Licastro et al. 1993], *F. velutipes* [Tsuda 1979], *Ganoderma lucidium* [Kawagishi et al. 1997], *Lentinus edodes* [Jeune et al. 1990] and *Agrocybe cylindracea* [Wang et al. 2002] exhibit potent mitogenic activities towards lymphocytes from different species. A group of lectins including *V. volvacea* lectin, *F. velutipes* lectin and *G. lucidium* lectin collectively designated as fungal
immunomodulatory proteins (FIPs), possesses potent mitogenic activities towards human lymphocytes, thus deserving special attention [Hsu et al. 1997; Ko et al. 1995; Lin et al. 1997]. The present study revealed that RBL markedly stimulated the maximum proliferation of human PBMCs at a concentration of 1.25 μg/ml, suggesting a potent mitogenicity. To the best of our knowledge there are no reports on fungal lectins that can induce the mitogenic effect at a concentration as low as 1.25 μg/ml.

The discovery that certain lectins are potent mitogens has opened up a new arena for scientists to study the probable role of lectins in cell growth and development. The present understanding of the relationship between chromosomal abnormality and human diseases is because of the extensive use of proliferative ability of mitogenic lectins and this in turn has improved the diagnosis. The study of lectin-lymphocyte interaction has also made a substantial contribution for elucidating the mechanism of lymphocyte activation, its control and thereby contributing to our knowledge of cell growth and development [Ashraf 2003].

To date, few well known plant mitogenic lectins such as PHA-L, Con A and PWM (Pokeweed mitogen) are commercially available that have enormous clinical applications. These lectins are being used as tools to study lymphocyte differentiation as models of antigen activation, initiation of cell division and growth and also to know the immune status of an individual suffering from the immune disorders. For example jacalin is strongly
mitogenic for human CD4$^+$ T-lymphocytes and this property has made it a useful clinical tool for the evaluation of the immune status of patients infected with HIV-1 [Lafont et al. 1996]. In view of this, the potential of RBL and CSL as novel mitogenic lectins will be of promising clinical significance. However, the molecular mechanism involved in the signaling leading to the RBL mediated mitogenic effect is yet to be established.

The studies so far confirmed the potential of RBL and CSL as mitogenic lectins. However, RBL was found to be a more potent mitogen compared to CSL. Hence further studies were focused exclusively on RBL which discussed in next chapter.