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A potent mitogenic lectin from the mycelia of a phytopathogenic fungus, *Rhizoctonia bataticola*, with complex sugar specificity and cytotoxic effect on human ovarian cancer cells

Nagaraja N. Nagre · Vishwanath B. Chachadi · Palaniswamy M. Sundaram · Ramachandra S. Naik · Radha Pujari · Padma Shastry · Bale M. Swamy · Shashikala R. Inamdar

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Abstract A lectin with strong mitogenic activity towards human peripheral blood mononuclear cells (PBMCs) and cytotoxic effect on human ovarian cancer cells has been purified from the mycelium of a phytopathogenic fungus, *Rhizoctonia bataticola*, using ion exchange chromatography and affinity chromatography on asialofetuin-Sepharose. The lectin, termed RBL, is a tetramer of 11-kDa subunits and has unique amino acid sequence at its blocked N-terminus. The purified RBL was blood group nonspecific and its hemagglutination activity was inhibited by mucin (porcine stomach), fetuin (fetal calf serum) and asialofetuin. Glycan array analysis revealed high affinity binding of RBL towards N-glycans and also the glycoproteins containing complex N-glycan chains. Interestingly, the lectin showed high affinity for glycans which are part of ovarian cancer marker CA125, a high molecular weight mucin containing high mannose and complex bisecting type N-linked glycans as well core 1 and 2 type O-glycans. RBL bound to human PBMCs eliciting strong mitogenic response, which could be blocked by mucin, fetuin and asialofetuin demonstrating the carbohydrate-mediated interaction with the cells. Analysis of the kinetics of binding of RBL to PBMCs revealed a delayed mitogenic response indicating a different signaling pathway compared to phytohemagglutinin-L. RBL had a significant cytotoxic effect on human ovarian cancer cell line, PA-1.

Keywords Mitogenic lectin · Cytotoxicity · Glycan array analysis · N-glycans · CA125

Introduction

Lectins are proteins or glycoproteins of non-immune origin with specific binding affinities for glycoconjugates. Some lectins recognize specific sugar moieties occurring on the cell surface, which are responsible for a variety of functions such as cell attachment, migration and invasion [1]. Such lectins have wider applications as histochemical and diagnostic reagents, especially for investigating the changes occurring on the cell membrane glycoconjugates [2, 3]. Lectins, due to their unique ability to bind to certain specific membrane glycoproteins, are known to have proliferative/antiproliferative, immunomodulatory, hypotensive and insecticidal effects [4-8]. In spite of being widely distributed amongst plants, animals and microorganisms, the vast majority of research has been focused on lectins from plant and animal sources. However, recently, fungal lectins have been receiving greater attention due to their interesting sugar specificities and biological activities, which have a wide range of potential pharmacological and biotechnological applications [9].

The present paper reports the purification and characterization of a mitogenic lectin (RBL) from the phytopathogenic

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N. N. Nagre · V. B. Chachadi · P. M. Sundaram · R. S. Naik · B. M. Swamy · S. R. Inamdar (✉)
Department of Biochemistry, Karnatak University, Dharwad 580003 Karnataka, India
e-mail: pinkisri2001@yahoo.co.in

R. Pujari · P. Shastry
National Center for Cell Sciences, Ganeshkhind, Pune 411007, India

fungus *Rhizoctonia bataticola.* These results revealed the complex sugar specificity of RBL towards N-linked glycans and glycoproteins containing complex N-glycans also the cytotoxic effect on PA-1, a human ovarian cancer cell line.

Materials and methods

Materials

Stock cultures of *R. bataticola* were maintained on potato dextrose agar slants containing 5% dextrose. Mucin (porcine stomach, type III), fetuin (fetal calf serum), PHA-L (isolated from *Phaseolus vulgaris*, red kidney bean), Histopaque-1077, *N*-hydroxysuccinimido biotin, fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co., St. Louis, USA. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio Gel P-100 was purchased from Bio-Rad Laboratories. Tritiated thymidine was procured from BRIT (Board of Radiation and Isotope Technology), India. Tissue culture flasks and 96 well plates were procured from NUNC (Denmark). Desialylated fetuin was prepared according to the method described by Spiro and Bhoyroo [10]. The affinity matrix, asialofetuin-Sepharose 4B, was prepared by coupling asialofetuin to Sepharose 4B after cyanogen bromide activation as described by March [11]. Rabbit blood used for the hemagglutination assays was procured from animal house, and human blood samples were procured from local blood bank. All other chemicals used were of analytical reagent grade.

Isolation and purification of lectin

R. bataticola cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml Byrde's liquid synthetic media [12] with 2% dextrose and incubated at room temperature under stationary conditions. After 11 days, the mycelial mat was harvested and washed with distilled water on cheesecloth. The mycelial mass was subsequently freeze-dried and powdered in a glass mortar, and the fine powder was stored at −20°C for further use. Mycelial powder (10 g) was suspended in 200 ml extraction buffer (50 mM sodium acetate buffer, pH 4.3) and sonicated briefly at 4°C. The resulting homogenate was mixed with an additional 300 ml extraction buffer and stirred overnight at 4°C on a magnetic stirrer. The extract was centrifuged (9,500×g, 30 min, at 4°C), and the resulting cloudy supernatant was further filtered through glass wool and a membrane filter (0.45 μm). The clear filtrate (460 ml) obtained was passed through a CM-cellulose column (20×1.5 cm) equilibrated with extraction buffer, at a flow rate of 25 ml/h, and 3 ml fractions of the eluate were collected using a FRAC-100 fraction collector (Pharmacia). Unadsorbed proteins were removed by washing the column with extraction buffer until the absorbance reading of the eluting fractions read zero at 280 nm (double beam spectrophotometer, Hitachi 2,800). Adsorbed proteins were eluted using 50 mM sodium acetate buffer pH 4.3 containing 500 mM NaCl and the lectin activity in eluting fractions was determined by hemagglutination assay using trypsinized rabbit erythrocytes. Peak fractions containing lectin activity were pooled and dialyzed against PBS (50 mM sodium phosphate buffer, 154 mM NaCl, pH 7.2).

The lectin fraction was then further purified by affinity chromatography on an asialofetuin-Sepharose 4B column (10×1.3 cm) at 4°C. The lectin was applied to the affinity column that had been equilibrated in PBS, the column was washed with PBS until the absorbance of the eluting fractions read zero at 280 nm, and the affinity bound lectin was eluted using glycine-HCl buffer (100 mM, pH 2.0) containing 500 mM NaCl. Fractions containing lectin activity were pooled, dialyzed against PBS and stored at −20°C for further studies. Homogeneity of the purified lectin preparation was confirmed by SDS PAGE on a 15% gel. Biotinylated lectin for glycan array analysis was prepared as described by Duk [13] and Fluorescein isothiocyanate-conjugated RBL (FITC-RBL) required for flow cytometry was prepared using the protocol of Goldman [14].

Hemagglutination assay

Hemagglutinating activity of the lectin (50 μg) was routinely determined at various stages of purification by the serial two-fold dilution method using trypsinized rabbit erythrocytes in 96-well microtiter plates at 37°C [15]. The highest dilution of the extract causing visible hemagglutination was regarded as the titre and the minimum concentration of the protein required for agglutination (MCA) as one unit of hemagglutinating activity. The specific hemagglutination activity was expressed as unit mg^{-1} protein. The sugar specificity of the purified lectin was determined by a hapten inhibition assay. Inhibition assays were carried out by incubating the lectin sample with serially diluted sugar/glycoprotein prior to the addition of erythrocytes in a total volume of 50 μl, and the hemagglutination was visually observed. The lowest inhibitory concentration of the sugar/glycoprotein was taken as the inhibitory titre of the hapten. To determine the blood group specificity of purified RBL, hemagglutination assays were carried out using trypsinized erythrocytes of human A, B, and O types. Hemagglutinating activity was also determined using trypsinized erythrocytes of bovine or sheep origin and compared to trypsinized rabbit erythrocytes.

Lowry's method was routinely used to determine the protein concentrations [16] using bovine serum albumin (BSA) as the standard. Total sugar content of glycoproteins

was estimated by phenol-sulfuric acid method [17] using glucose as a standard.

Effect of pH on the stability of purified RBL

An aliquot of purified RBL (50 µg), in saline, was incubated with an equal volume of 100 mM buffer of desired pH for 12 h at 4°C and the hemagglutination activity was estimated after the adjusting the pH to 7.2 in each, using 1.0 M NaOH or 1.0 M HCL. Various buffer systems used for obtaining the desired pH are; gly-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris-HCl (pH 8.0 and 9.0) and sodium bicarbonate (pH 10.0).

Glycan array analysis of RBL

To determine the carbohydrate-binding specificity of RBL, glycan micro array analysis was performed on printed glycan array slides at the Consortium for Functional Glycomics [www.functionalglycomics.org]. In brief, micro array slides (printed array version 2.1) containing 285 different covalently attached glycan structures in replicates of six were printed as described [18]. In the binding assay, micro array slides were incubated for 1 h with the biotinylated RBL (50 µg/ml), in binding buffer (20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ containing 0.05% Tween 20 and 1% BSA). Slides were washed first with the same buffer without Tween 20 followed by water, and the bound lectin was detected using fluorescently labeled streptavidin. Fluorescent intensities of sample spots were measured and analyzed using IMAGENE image analysis software (Bio Discovery, EI Segundo CA, USA).

Gel filtration chromatography

The molecular weight of RBL was estimated by gel filtration chromatography on a column (80×1.5 cm) of Bio-Gel P-100 (Bio-Rad) equilibrated with PBS. Fractions of 3.0 ml were collected at a flow rate of 12 ml/h, and the elution of the protein was monitored by measuring the absorbance at 280 nm. The column was calibrated with the following standards: cytochrome C (12.4 kDa), chymotrypsinogen A (21 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa) and bovine serum albumin (66 kDa). The elution volumes *vs.* $\log_{10}$ molecular weights were plotted to construct the calibration curve.

SDS-PAGE

The molecular weight (M_r) of RBL subunits was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli [19], on a 15% gel.

N-terminal amino acid sequence analysis

Initial attempts to determine the N-terminal amino acid by dansyl chloride method failed indicating the blocked N-terminal. However, N terminal sequence was determined after de blocking by trifluoroacetic acid vapors [20]. Purified lectin (10 µg) was applied to a 15% SDS-PAGE gel, electroblotted onto a PVDF membrane (Bio-Rad), the blots were dipped briefly in methanol and exposed to TFA vapors overnight, later analyzed on Automatic Protein Sequencer (476A-01-120, Applied Biosystems).

Binding of RBL to human PBMCs by flow cytometry

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by density gradient using Histopaque-1,077 (Sigma) and resuspended in complete medium (RPMI 1,640 + 10% FCS). The binding of RBL to PBMCs was determined by flow cytometry. Cells (1×10^5) were incubated with FITC-RBL (1 µg/100 µl) for 1 h on ice and were washed thoroughly with 1X PBS and then fixed with 2% freshly prepared paraformaldehyde. Data were acquired for 10,000 events using a FACS Vantage (Becton Dickson) equipped with a 488 nm argon laser and analyzed with Cellquest-pro software for determining % positivity and mean fluorescence intensity (MFI). Unstained cells that had been processed similarly were used as negative control.

Receptor-mediated RBL binding to PBMCs was determined by pre-incubating FITC-RBL with mucin, fetuin and asialofetuin (0.1 µg and 10 µg/100 µl) for 1 h at room temperature. This lectin-sugar complex was added to the PBMC preparation and analyzed by flow cytometry.

Binding of RBL to human ovarian cancer cell line, PA-1 by flow cytometry

PA-1 cells obtained from ATCC (American type culture collection) were grown in Minimum Essential Media (MEM), supplemented with 10% fetal calf serum and were maintained in a humidified incubator (37°C, 5% CO2). The surface binding of RBL to human ovarian cancer cell line was determined quantitatively by flow cytometry using FITC-RBL as described earlier.

Mitogenic Activity by thymidine incorporation assay

The mitogenic activity of RBL was assessed in freshly isolated human peripheral blood mononuclear cells by tritiated thymidine incorporation assay. PBMCs were suspended in RPMI-1,640 containing 10% FCS, and 10^5 cells/100 µl/well were plated in a 96 well tissue culture plate (NUNC, Denmark) and incubated with a concentration

range of RBL (0.156 μg/ml to 2.5 μg/ml) for 72 h at 37°C in 5% CO_2. PBMCs stimulated with PHA-L (0.156 μg/ml to 2.5 μg/ml, Sigma Chemicals) were used as a positive control. Cells were pulsed with tritiated thymidine (1 μCi per well, BRIT, India) 18 h prior to harvesting, and the incorporation was measured as counts per minute (CPM).

For the time course study with RBL and PHA-L, proliferation was measured for 72 h at 24-h intervals using the tritiated thymidine incorporation assay.

Cytotoxic effect of RBL on PA-1 cells by MTT assay

The cytotoxic effect of RBL on human ovarian cancer cell line PA-1 was determined using the MTT assay [21]. PA-1 cells were grown in Minimum Essential Media (MEM) supplemented with 10% fetal calf serum in a humidified atmosphere (37°C, 5% CO_2). For cytotoxicity assays, PA-1 cells were released from the flasks by trypsinization, collected by centrifugation and then suspended in MEM (~1×10^5 cells/ml). Cells seeded in 96-well microtiter plates (5,000 cells/well) were incubated in a humidified atmosphere (37°C, 5% CO_2) for 18 h, then treated with different concentrations of RBL (0.625–50 μg/ml) and incubated for 12 h. After incubation, 20 μl of MTT (0.5 mg/ml) was added to each well, and, after 4 h incubation at 37°C, 100 μl of SDS (10% in 0.01 N HCl) was added to each well. The plates were gently shaken for 10 min. Finally the absorbance was measured at 570 nm on an ELISA plate reader against 640 nm. The percentage viability of RBL treated cells compared to control cells (no added RBL) was determined. IC_{50} values were defined as the lectin concentration needed to inhibit the cell growth by 50%.

Statistical analysis

Statistical analysis was performed using student's *t*-test and Mann-Whitney rank sum test. A p-value<0.05 was considered to be statistically significant.

Results

Isolation and purification of RBL from the mycelium of *Rhizoctonia bataticola*

Initial efforts using ammonium sulfate or alcohol precipitation to fractionate the lectin and to eliminate the polysaccharides and other interfering substances from the crude lectin extract were unsuccessful. Hence, cation exchange chromatography on CM-cellulose was used to concentrate the lectin in the crude extract. This step not only removed polysaccharides and pigments, but also eliminated some proteins. The lectin was eluted from this column with 500 mM NaCl, resulting in a recovery of 94% of the hemagglutinating activity loaded and a 17-fold concentration and a six-fold purification of the lectin. The lectin was further purified to homogeneity on asialofetuin-Sepharose 4B affinity column from which it was eluted in 100 mM glycine-HCl buffer, pH 2.0 containing 500 mM NaCl. The elution profile of the lectin on the affinity column is shown in Fig. 1 and SDS-PAGE gel of the purified protein is shown in Fig. 4 (inset). Purification results are summarized in Table 1. The purified lectin was stable over a wide range of pH 2.0–10.0 when stored at 4°C.

Purified RBL agglutinated human erythrocytes of all blood groups, indicating that it has a blood group nonspecific nature. However, it showed slightly higher activity with type A, B and AB cells compared to type O cells. The activity toward rabbit erythrocytes was eight-fold higher than toward human cells, while RBL failed to recognize bovine and sheep erythrocytes (Table 2).

Hapten inhibition studies showed that the hemagglutinating activity of RBL was inhibited by mucin, fetuin and asialofetuin (Table 3). However, mucin exhibited a strong inhibitory effect (MIC: 1.5 μg/50 μl) compared to fetuin or asialofetuin (MIC: 12.5 μg/50 μl), and none of the simple sugars tested inhibited hemagglutination.

Glycan array analysis for fine sugar specificity of RBL

Glycan array analysis using a panel of 285 glycans including 6 glycoproteins was used to identify glycans that selectively and specifically bind to RBL (Fig. 2). RBL

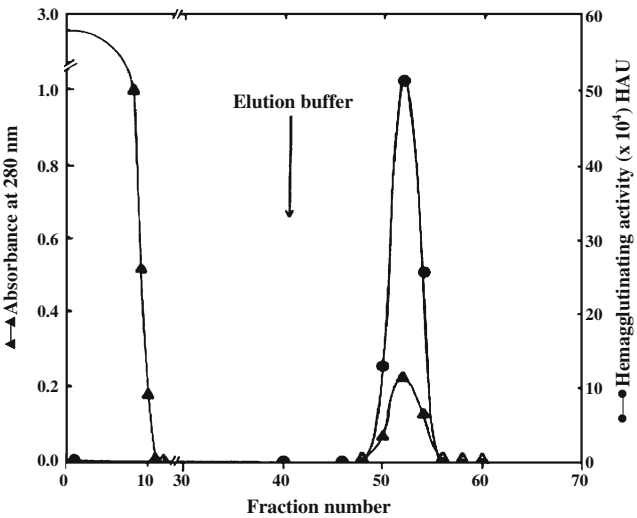

Fig. 1 Affinity purification of *R. bataticola* lectin on asialofetuin-Sepharose 4B column**:** CM-cellulose eluted lectin peak sample (20 ml) was passed through the affinity column and equilibrated in PBS, and the affinity-bound lectin was eluted with 100 mM glycine-HCl buffer, pH 2.0 containing 500 mM NaCl. Fractions of 3.0 ml were collected at a flow rate of 12 ml/h. ▲—▲ Absorbance at 280 nm; ● ● Hemagglutinating activity. HAU: hemagglutination activity unit

Table 1 Purification of RBL from mycelial extract

Sample	Volume (ml)	Protein (mg)	Sugar (mg)	MCA[a] (μg/50μl)	Activity units[c] (x1000)	Specific activity[d] (x1000)	Fold purification	Recovery of activity (%)
Original extract	460	92.0	243.8	0.0625	1,472	16.0	–	100
Lectin peak CM-cellulose column	27	14.6	21.6	0.0106	1,377	94.3	5.9	93.55
Affinity chromatography	18	0.97	ND[b]	0.00078	1,244	1282.6	80	84.51

[a] Minimum concentration of protein required to agglutinate erythrocytes

[b] Not detected by phenol-sulfuric acid method

[c] Total activity or activity units: hemagglutinating activity of lectin in total protein

[d] Specific activity: hemagglutination activity mg^{-1} protein

showed maximum affinity with high mannose and complex type N-linked glycans (Table 4). The greatest binding was seen with the glycan #51, a glycan with Man_3-$GlcNAc_4$. Among the glycoproteins tested, RBL showed greater affinity towards ceruloplasmin (glycan #4).

Glycans with the highest affinities for RBL are listed in Table 4. A complete list of glycans and designations of spacer arms are available at http://www.functionalglycomics.org. RFU values are presented along with standard deviation (SD), standard error (SE) and % CV values after statistical analysis (http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_843).

Determination of molecular weight

The purified *R. bataticola* lectin was eluted from gel filtration Bio Gel P-100 column at an elution volume corresponding to an apparent molecular weight of 43,000±2,000 Da (Fig. 3).

Table 2 Agglutination activity of purified *R. bataticola* lectin towards trypsinized erythrocytes of different origins

Erythrocytes	Hemagglutination titre[a]
Human	
Type A	128
Type B	128
Type AB	128
Type O	64
Rabbit	1,024
Bovine	Nil
Sheep	Nil

[a] The highest dilution of the extract causing visible hemagglutination was regarded as the hemagglutination titre

Nil-The hemagglutination activity of RBL towards bovine and sheep erythrocytes is nil

Or- RBL did not show any hemagglutinating activity towards bovine and sheep erythrocytes

In contrast, the relative molecular weight (M_r) calculated for the subunits of RBL by SDS-PAGE was 11,000±500 Da, indicating that the lectin is a homo tetramer (Fig. 4).

N-terminal amino acid sequence

The partial N-terminal sequence of the first 10 amino acids was defined as KKKAYSSRII. The sequence differed considerably from the known sequences for previously reported fungal lectins. Repetitive cycle yields for each run varied from 98%-92%.

Binding of RBL to human PBMCs and human ovarian cancer cell line, PA-1

To study the interaction of RBL with PBMCs, cells were stained with FITC-RBL and RBL binding was followed using flow cytometry. In total, 98.76% of the cells were positive for RBL binding, with mean fluorescence intensity (MFI, an arbitrary unit for measuring the fluorescent intensity) of 82.54, in comparison to an MFI of 11.65 in unstained control cells

Table 3 Hapten inhibition studies with the purified RBL

Glycoprotein	Minimum concentration required for inhibition(MIC) in μg/ 50μl
Mucin (from porcine stomach)	1.56
Fetuin	12.5
Asialofetuin	12.5

D-galactose, D-glucose, D-mannose, D-arabinose, D-fucose, L-(-)-fucose, β-D(+)-glucose, 2-deoxy-D-glucose, rhamnose, 1-amino-1-deoxy-β-D-glucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine monohydrate, 1-*O*-methyl-galactose, methyl-α-D-mannopyranoside, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-glucopyranoside, p-aminophenyl-β-D-galactopyranoside, p-aminophenyl-β-D-glucopyranoside, sialic acid, lactulose, β-lactose, cellobiose, melibiose and raffinose did not inhibit the lectin activity up to concentrations of 200 mM. Also, ovalbumin (2 mg/ml), and the plant polysaccharides guar gum (1 mg/ml), gum acacia (10 mg/ml) and arabinogalactan (10 mg/ml) were not inhibitory

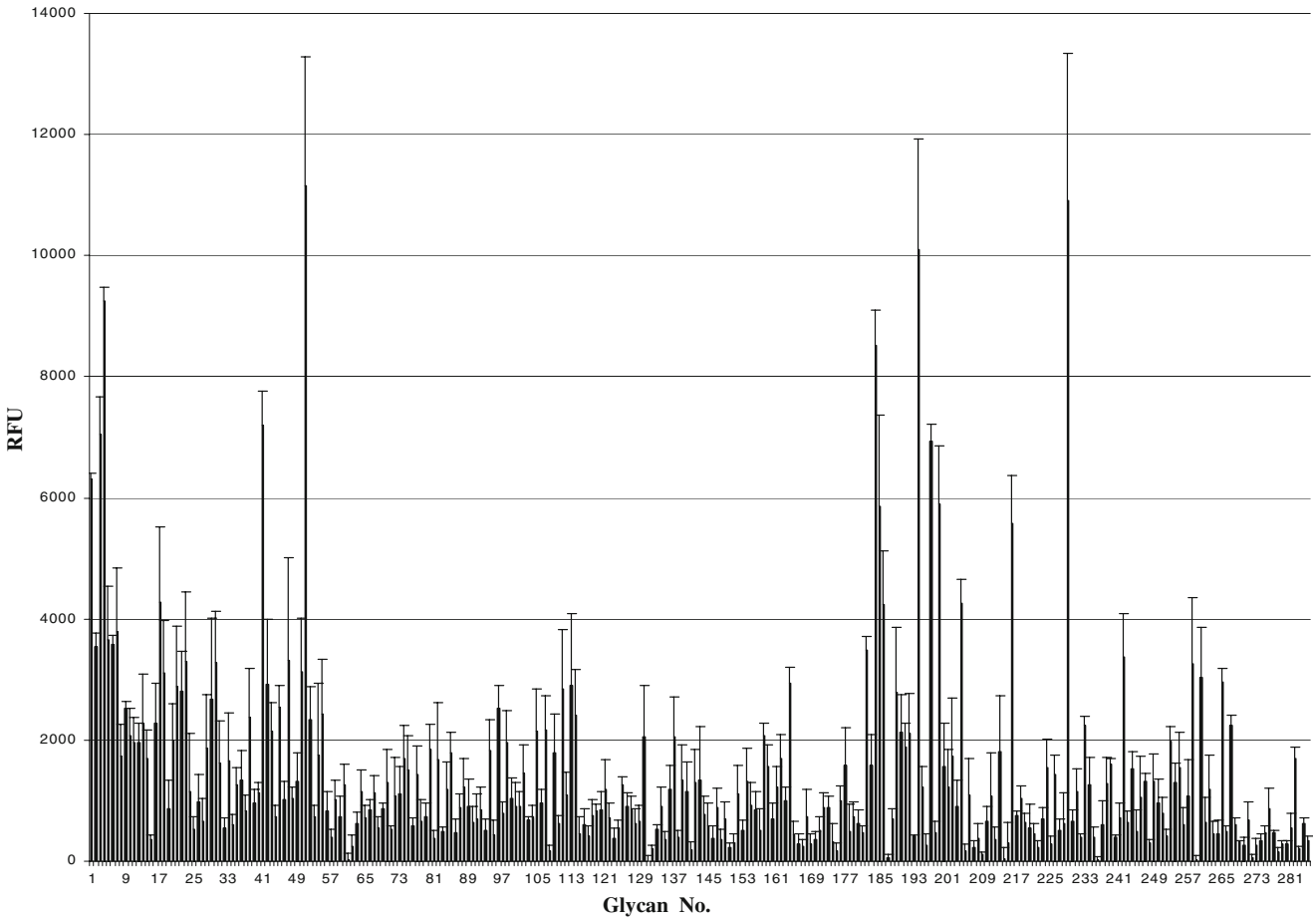

Fig. 2 Glycan array analysis of RBL (50 μg/ml): A total of 285 glycans were screened for binding along with positive and negative controls, as described under "Experimental procedures." *Error bars* represents the mean ± SD of replicates from a single experiment

Table 4 Fine sugar specificity of RBL determined by glycan array analysis

Glycan no	Glycan structure-spacer arm	RFU[a]	% CV[b]
51	GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Gly	11,152	38
229	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3) GlcNAcβ–Sp0	10,906	45
194	Manα1-2Manα1-2Manα1-3(Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6)Manβ1-4GlcNAcβ 1-4GlcNAcβ-*N*	10,102	36
4	Ceruloplasmine	9,255	5
184	GlcAβ-Sp8	8,525	14
41	6-H2PO3Manα–Sp8	7,206	15
3	AGP-β1	7,041	18
197	Manα1-6(Manα1-3)Manα1-6(Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-*N*	6,943	8
1	AGP	6,320	3
199	Man5_9mix *N*	5,903	32

Glycans with the highest affinities for RBL are listed. Micro array slides were incubated with RBL at a concentration of 50 μg/ml. A complete list of glycans and designations of spacer arms are available at http://www.functionalglycomics.org

[a-] Relative fluorescence units represent degree of binding RBL towards various glycans

[b-] Percentage of coefficient of determined as standard deviation/mean × 100

The data are presented as the mean ± SE of six independent experiments done in triplicate

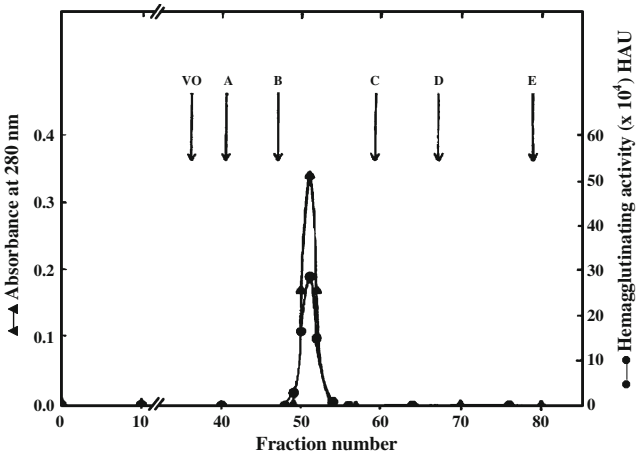

Fig. 3 Molecular weight determination of *R. bataticola* lectin by gel filtration chromatography on Bio Gel P-100 column: Purified lectin sample (1 mg) in 2 ml of 50 mM PBS, pH 7.2 containing 150 mM NaCl, was applied onto the column (150×1.6 cm) and eluted at a flow rate of 12 ml/h. Fractions of 3 ml were collected. The column was calibrated using the molecular weight marker proteins; (**a**) bovine serum albumin (66 kDa), (**b**) ovalbumin (45 kDa), (**c**) carbonic anhydrase (29 kDa), (**d**) chymotrypsinogen-A (21 kDa), (**e**) Cytochrome-C (12.4 kDa). V_o Blue dextran; ▲—▲ Absorbance at 280 nm; ● ● Hemagglutinating activity

(Fig. 5a). The receptor-mediated lectin binding to PBMCs was confirmed by studying the binding of RBL after pre-incubation of the lectin with different competing glycoconjugates or haptens. Flow cytometry histograms of RBL binding to PBMCs after blocking with asialofetuin, mucin and fetuin are presented in Fig. 5b,c,d respectively. Mucin, fetuin and asialofetuin (100 μg/ml) equally and effectively inhibited the binding of RBL to PBMCs (Table 5).

Flow cytometry analysis also demonstrated significant binding of RBL to the human ovarian cancer cell line, PA-1, with 80.38% cells positive for RBL binding with a MFI of 370.72. Mucin, fetuin and asialofetuin abolished the RBL binding to these cells (Fig. 6).

Mitogenic activity of RBL

Mitogenic activity of RBL on human PBMCs was determined by measuring the uptake of tritiated thymidine. RBL exerted a 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 a maximum proliferative effect at 1.25 μg/ml concentration (Fig. 7a). There was a time-dependent increase in proliferation in both RBL and PHA-L stimulated PBMCs. However, the kinetics of the response elicited by RBL was different compared to PHA-L with respect to fold-increase in proliferation at 48 h. In the case of PHA-L, no further significant increase occurred at 72 h, but with RBL, there was a significant increase in proliferation from 48 to 72 h post stimulation (Fig. 7b).

Cytotoxic effect of RBL

RBL had a strong cytotoxic effect on PA-1 cells as revealed by the MTT assay. RBL mediated the cytotoxic effect in a

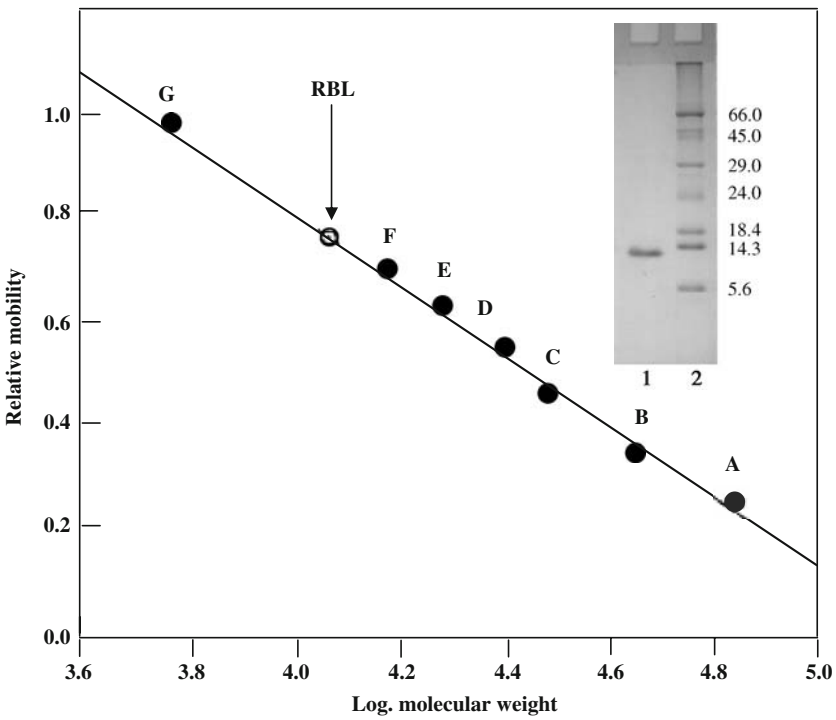

Fig. 4 Calibration curve for the estimation of the molecular weight of RBL by SDS- PAGE: Log_{10} molecular weight plotted against relative mobilities of marker proteins; (**a**) BSA (66 kDa), (**b**) ovalbumin (45 kDa), (**c**) carbonic anhydrase (29 kDa), (**d**) trypsinogen (24 kDa), (**e**) lactoglobulin (18.4 kDa), (**f**) lysozyme (14 kDa), (**g**) insulin (5.6 kDa). Inset: SDS-PAGE of affinity purified RBL in a 15% gel. Lane 1: purified lectin (20 μg). Lane 2: standard molecular weight markers. The gel was stained with Coomassie brilliant blue

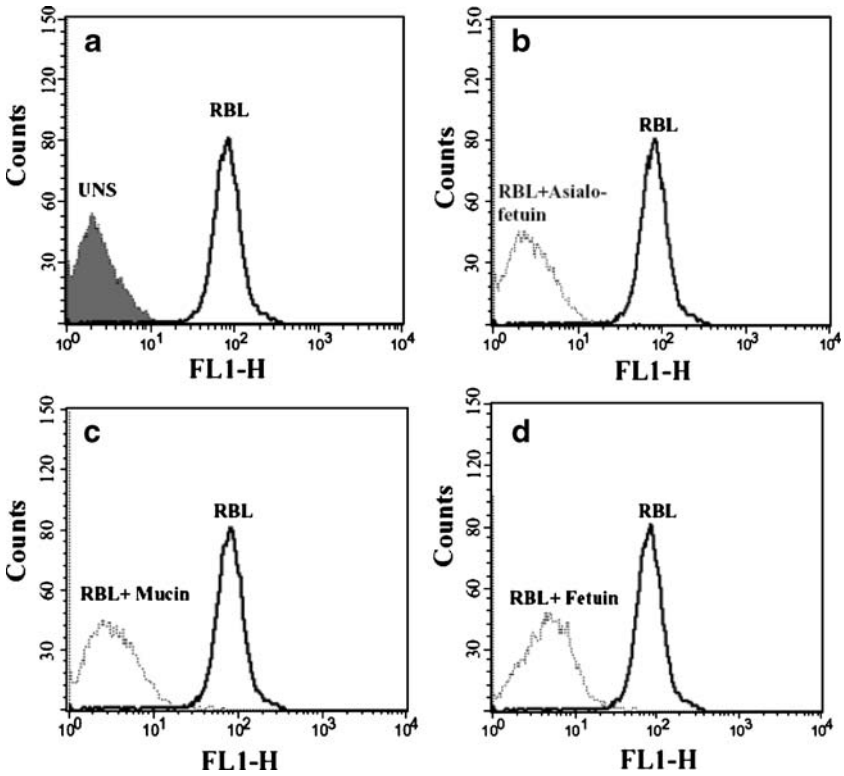

Fig. 5 Binding of RBL to human PBMCs and inhibition with glycoconjugates: PBMCs were stained with FITC-labeled RBL and subjected to flow cytometric analysis. *X-axis, FL-1H on a log scale* represents the fluorescence intensity of cells stained with FITC labeled RBL. *Y-axis* represents cell number. (**a**) 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 glycoconjugates: (10 μg/ml) (**b**) mucin, (**c**) asialofetuin and (**d**) fetuin are represented by dotted lines *The plots* are representative data of three similar experiments

dose-dependent manner, and 50% of cell death was observed at an RBL concentration of 6.25 μg/ml (Fig. 8). Percentage viability of RBL treated cells was compared with that of untreated cells (100% growth).

Table 5 Binding of RBL to human PBMCs and inhibition of binding with competing glycoconjugates

	% Positive cells	Fluorescence intensity-MFI (%)
RBL alone	99	100[a]
RBL + Asialo-fetuin (0.1 μg/100 μl)	99	90
RBL + Asialo-fetuin (10 μg/100 μl)	4	17
RBL + Mucin (0.1 μg/100 μl)	99	86
RBL + Mucin (10 μg/100 μl)	6	17
RBL + Fetuin (0.1 μg/100 μl)	99	86
RBL + Fetuin (10 μg/100 μl)	16	17

The table shows the percent positive cells and the percent fluorescence intensity in cells stained with FITC-labeled RBL in the absence and presence of competing glycoconjugates. 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%. [a] The fluorescent intensity with FITC-labeled RBL was considered as 100% for calculating the inhibition of binding in presence of competing glycoconjugates. Mucin from porcine stomach is used for these studies

Discussion

In the present study, a lectin from *R. bataticola* (RBL) was purified to homogeneity by ion exchange chromatography

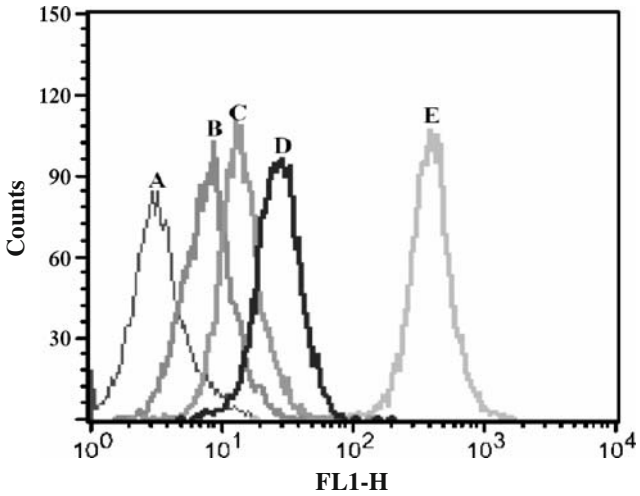

Fig. 6 Binding of RBL to the human ovarian cancer cell line, PA-1, and inhibition of binding with competing glycoproteins: PA-1 cells were stained with FITC-labeled RBL and subjected to flow cytometric analysis. *X-axis, FL-1H on log scale* represents the fluorescence intensity of cells stained with FITC labeled RBL. *Y-axis* represents cell number. The histoplot shows profiles of (**a**) Unstained cells. (**b**) Cells stained with FITC- RBL preincubated with mucin. (**c**) Cells stained with FITC- RBL preincubated with fetuin. (**d**) Cells stained with FITC- RBL preincubated with asialofetuin. (**e**) Cells stained with FITC-labeled RBL. *The plots* are representative data of three similar experiments

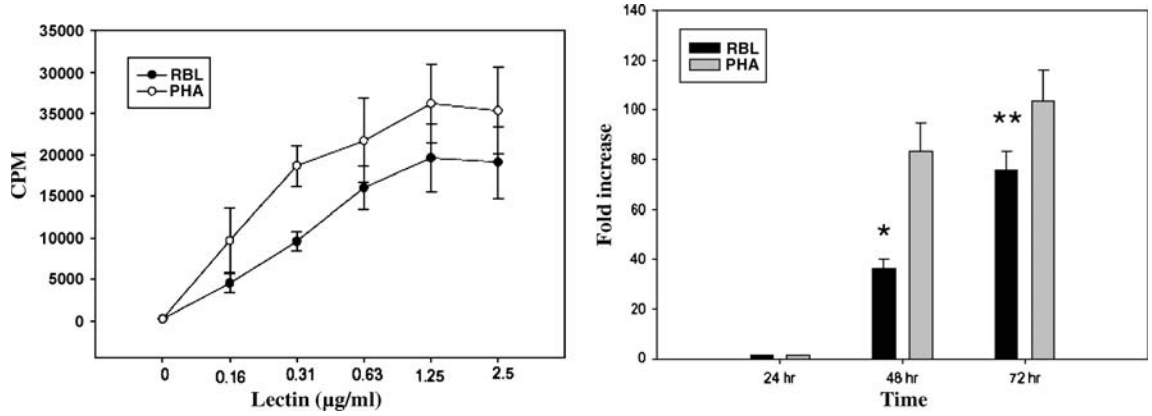

Fig. 7 Mitogenic activity of RBL**:** (**a**) PBMCs were isolated from blood of healthy donors and exposed to serial concentrations of RBL and PHA-L (0.16–2.5 µg/ml) for 72 h, and proliferation was measured by a tritiated thymidine incorporation assay. * difference ($p<0.05$) between unstimulated PBMC and PBMC stimulated with serial concentrations of RBL and PHA. (**b**). PBMCs were stimulated with RBL and PHA-L (1.25 µg/ml) for 24, 48 and 72 h. Fold increase in proliferation was calculated in comparison to the CPM of unstimulated cells at each time point. The data are presented as the mean ± SE of four independent experiments done in triplicate. * difference ($p<0.025$) between RBL and PHA-L at 48 h post stimulation. ** difference ($p<0.025$) between PBMCs stimulated for 48 h and 72 h with RBL

followed by affinity chromatography on asialofetuin-Sepharose 4B with an overall yield of 0.97 mg of purified lectin from 10 g of dry mycelia.

The majority of lectins reported from fungi are either dimeric or tetrameric in nature. This is also the case with RBL, which is a tetramer with a subunit mass of 11 kDa. The lectin isolated from *Rhizoctonia crocorum* and the lectin from *Agaricus blazei* are both tetramers consisting of 11 kDa and 16 kDa subunits, respectively [22, 23]. Similarly, a lectin isolated from *Sclerotium rolfsii* is a homo dimer with subunit mass of 17 kDa, which shows pH dependent oligomerization [24, 25]. The N-terminal amino acid in RBL is blocked which was indicated by its failure to release dansyl derivative. Lectins from several *Rhizoctonia* species are reported to have blocked N-terminal amino acid [26]. Also, a well characterized lectin from *Sclerotium rolfsii*, a soil borne plant pathogen is shown to have acetylated N-terminal residue [27]. Another lectin purified from *Agrocybe aegerita,* an edible mushroom has pyroglutamyl residue at N-terminus [28]. Results of N-terminal amino acid sequence deduced for 10 amino acids of RBL revealed striking differences with the sequences reported for other fungal lectins [9] including *Rhizoctonia solani* [29].

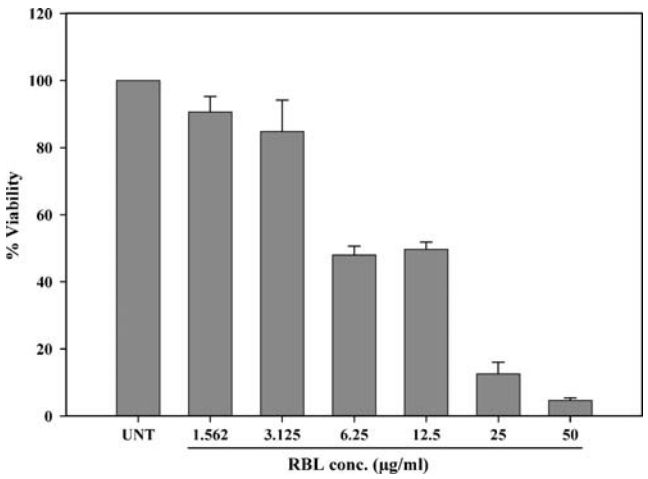

Fig. 8 Cytotoxic effect of RBL: The cytotoxic effect of RBL was tested on human ovarian cancer cell line, PA-1. The cells (5,000 cells/well) were exposed to different concentrations of RBL and the % viability was measured by MTT assay. The data are presented as mean ± SE of four independent experiments done in triplicate

Like most of the reported fungal lectins, RBL is a blood group nonspecific agglutinin, agglutinating blood types A, B, and O equally. However, a few exceptions to this have been found, for instance, a L-fucose-binding agglutinin associated with the hyphal surface of *R. solani*, agglutinates only type O cells, but not A and B type cells [30], whereas an intracellular lectin from the same fungus exhibits a clear preference for human type A over type B and O erythrocytes [31].

Results of hapten inhibition studies indicated the complex sugar specificity of RBL recognizing mucin, fetuin and asialofetuin strongly similar to other reported fungal lectins like lectins from *Fusarium solani* [32] and *Ganoderma lucidum* [33]. However, these results lead to ambiguities, since mucin and fetuin are known to contain both O-glycans and N-glycans, hence it was not clear whether the sugar specificity of RBL is directed towards *O*- or *N*- linked glycans. Also the glycan array analysis data of RBL demonstrated high binding towards ceruloplasmin and α 1-Acid glycoprotein (AGP) which are reported to contain high amounts of N-glycans. Asparagine-linked bi- and tri-antennary oligosaccharides of ceruloplasmin have been characterized [34], also AGP is reported to contain bi, tri

and tetra antennary complex N-glycans. All these glycan chains in both glycoproteins are fucosylated (Lewis type) and with terminal sialyl residues, but the extent of sialylation differs in AGP and ceruloplasmin [35, 36]. In contrast to high affinity of RBL towards these N-glycan rich glycoproteins, hemagglutination assay revealed high affinity binding to mucin, which is generally believed to contain only *O*-linked glycans. However, there are very few reports suggesting that mucins also contain *N*-linked glycans [37-39] although there are no details available on the nature of these glycans. It was clear from the glycan array analysis that the sugar specificity of RBL is directed to *N*-linked glycans as shown by its high affinity towards glycans; GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Gly [glycan # 51] a complex N-glycan, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ–Sp0 [glycan# 229], a tandem repeat Lewis glycan with sialyl terminus. In addition, RBL also showed higher binding to high mannose tri antennary N-glycans; Manα1-2Manα1-2Manα1-3(Manα1-2Manα1-3(Manα1-2Manα1-6)Manα1-6)Manβ1-GlcNAcβ1-4GlcNAcβ-*N* [glycan # 194] and Manα1-6(Manα1-3)Manα1-6(Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-*N* [glycan # 197]. Interestingly glycan # 194 and glycan # 197 recognized by RBL are part of CA125 antigen, an established diagnostic marker for epithelial ovarian cancer [40]. CA125 is a high molecular weight mucin containing both *O*- and *N*-linked glycans and is shown to contain high mannose and complex bisecting type N-linked glycans including Man_5-$Man_9GlcNAc_2$ [41]. Considering the significant sugar specificity of RBL it could serve as an important molecular tool for the characterization of N-glycans.

Flow cytometry analyses of RBL binding to PBMCs not only demonstrated that receptor mediated binding can be effectively blocked by glycoproteins, but also confirmed the preferential affinity of RBL toward mucin compared to fetuin or asialofetuin. Like PHA-L, RBL increased the proliferation of PBMCs in a dose-dependent manner. Mitogenic stimulation is one of the most dramatic effects of the interaction of lectins with cells. Some fungal lectins like the lectins from *V. volvacea* [42], *Boletus santanas* Lenz [4], *Flammulina velutipes* [43], *Ganoderma lucidium* [44], *Lentinus edodes* [45] and *Agrocybe cylindracea* [46] exhibit potent mitogenic activities towards lymphocytes from different species. A group of lectins including those from *V. volvacea*, *F. velutipes* and *G. lucidium,* collectively designated as fungal immunomodulatory proteins (FIPs), possess potent mitogenic activities towards human lymphocytes [47-49]. The present study revealed that RBL markedly stimulated the proliferation of human PBMCs. The proliferation was effectively inhibited by mucin, fetuin and asialofetuin, suggesting the involvement of lectin receptors. PBMCs stimulated by PHA-L showed a significant increase in proliferation compared to RBL at 48 h, but not after 72 h, indicating a delayed mitogenic response by RBL compared to PHA-L and suggesting that RBL might induce proliferation by a different mechanism. The signaling pathway involved in RBL mediated mitogenic effect is currently under investigation.

Interestingly, RBL also showed significant receptor mediated binding toward human ovarian cancer cell line, PA-1, that was blocked in the presence of competing glycoproteins. Upon binding, RBL had a potent cytotoxic effect in PA-1 cells, with an IC_{50} value 6.25 μg/ml.

Fungal lectins are gaining interest mainly due to their antitumor activities against different cell lines. For example, a lectin from *V. volvacea* shows antitumor activity against sarcoma S-180 cells [50], *Grifola frondosa* lectin is cytotoxic to HeLa cells [51], *Agaricus bisporus* lectin possesses antiproliferative activities against human colon cancer cell line HT29 and breast cancer cell line MCF-7 [52], and *Tricholoma mongolicum* lectin inhibits mouse mastocytoma P815 cells *in vitro* and sarcoma S-180 cells *in vivo* [53].

To date, only a few well known plant mitogenic lectins such as PHA-L, Con A and PWM (Pokeweed mitogen) are commercially available as tools to study lymphocyte differentiation, as models of antigen activation, initiation of cell division and growth and to determine the immune status of an individual suffering from immune disorders. For example, jacalin is strongly mitogenic for human $CD4^+$ T lymphocytes, and this property has made it a useful tool for the evaluation of the immune status of patients infected with HIV-1 [54]. Therefore, the potential of RBL as a novel mitogenic lectin is of great significance. In addition, considering the growing interest in fungal lectins due to their tumor suppressing activity, the cytotoxic effect of RBL suggests that this lectin has potential as a useful tool in cancer research in general and as an anticancer agent in particular. However, a detailed investigation is needed to understand the molecular mechanism of antitumor effect induced by RBL.

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