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(54) Benævnelse: **CANCERCELLEBINDENDE, REKOMBINANTE LECTINER MED ANTITUMORAKTIVITET OG FREMSTILLINGSFREMGANGSMÅDE**

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DESCRIPTION

Description

FIELD OF INVENTION

[0001] The invention relates to the field of carbohydrate binding proteins, and more specifically to a protein which binds specifically to particular oligosaccharides associated with particular medical significance. The invention specifically relates to lectins, more particularly to recombinant fungal lectins having ability to recognize glycans of clinical importance and their antiproliferative effect on cancer cells finding applications in cancer diagnosis, therapy and research.

BACKGROUND OF INVENTION

[0002] Lectins are proteins, or glycoproteins that agglutinate erythrocytes of some or all blood groups in vitro. They are important group of bioactive proteins found in most organisms. Lectins are used as tools for diagnostic and therapeutic purpose in health care areas. Lectins are also used in the purification of glycoproteins, oligosaccharide analysis and in cell selection processes. Lectins can bind reversibly with monosaccharides or the sugar moiety of, glycoproteins or glycolipids or polysaccharides.

[0003] Changes in carbohydrate structures on the cell surfaces are a hallmark during cancer. Such changes results in exposure of tumor associated carbohydrate structures. Amongst them, is the increased occurrence of Thomsen-Friedenreich antigen, a disaccharide Galβ1 -> 3GalNac-O-Ser/Thr. In normal cells, this antigen is masked by certain sugars, sialic acid or sulphates. Some of the lectins have ability to specifically recognize and bind to these altered structures and exerts various physiological effects such as apoptosis, cytotoxicity, proliferative/antiproliferative activity, metastasis, inhibition of cell adhesion, etc., Hence such lectins are currently being considered for use as cancer diagnostic and therapeutic agents and also for anti tumor activity.

[0004] Several lectins have been isolated and characterized from variety of sources. They however differ in their physico-chemical properties such as molecular size and sugar specificities. There is also characterization of a few recombinant proteins. The recombinant mushroom lectin from *Marasmius oreades* has a molecular size of 33 kDa and shows high affinity for Galα1, 3Gal and Galαl, 3Galβ1, 4G1cNAc. (R.P.Kruger et al., J.Biol.Chem., 277, 15002-15005, 2002; H.C.Winter et al. J.Biol.Chem ,277, 14996-15001, 2002., I.J. Goldstein, et

al. US Patent :6958321)

[0005] Peanut lectin that shows affinity to Thomsen-Friedenreich antigen has been cloned (V.Sharma and A.Surolia, Gene (Amst), 148, 299-304, 1994) and subsequently some mutants of this protein with a subunit molecular mass of ~28kDa has also been expressed in E.coli (V.Sharma, M.Vijayan and A.Surolia, J. Biol. Chem., 271, 21200-21213,1996) with some difference in its preference for sugar specificity. A much smaller lectin of molecular mass of 11.73 kDa with high binding affinity to L-fucose from Pseudomonas aeruginosa has also been expressed in E.coli as a Yellow fluorescent Protein-lectin fusion (BioTechniques, 41, 327-332, 2006).

[0006] A mushroom lectin (ABL) from Agaricus bisporus, which has antiproliferative activity on human colon cancer cells and its curative effect in psoriasis is reported by Jonathan M. Rhodes; US patent # 5607679.

[0007] *Sclerotium rolfsii* lectin (SRL) purified from the sclerotial bodies of the phytopathogenic fungus *Sclerotium rolfsii* exhibit complex carbohydrate specificity and exhibit specificity towards TF antigen. TF antigen is an oncofetal antigen, a disaccharide Galβ1, 3GaNAc α ser/thr and is known to expressed on cell surfaces in different cancers. SRL apart from binding to TF antigen, it also bind to sialylated and sulfated disaccharide. The crystal structure of this lectin and its complete amino acid sequence is determined (Demetres 2007 and Satisha 2008). Binding studies indicated that SRL binds to different human cancer tissues and also to cultured cells. Further binding of SRL to cultured human leukemic cells and colon cells results in their antiproliferation and in colon cells effect is shown to be mediated by inducing apoptosis. However the purified native lectin has solubility problems for its use in various applications, hence there is need of forms of this lectin with improved stability for practical applications.

[0008] Lectins thus have enormous potential in the area of medical assays. The instant application proceeds to provide certain lectins with novel characteristics so as to be useful in the medical field and improved properties.

SUMMARY OF THE INVENTION

[0009] There is described herein recombinant lectins having specificity to certain sugar chains uniquely found on certain cancer cells. In one embodiment of the invention, there is provided a method for preparing two recombinant lectins (designated as UC-SS/CSR-1803 and UC-SS/CSR-1.805 or simply Rec-2 and Rec-3 respectively) expressed in *E.coli.* The method comprises the synthesis of lectin genes whose sequences are derived by deliberate modifications made to the original amino acid sequence of a lectin from *Sclerotium rolfsii* (Sathisha et al., Amino Acids; 2008, 35, 309-320), a soil borne phytopathogenic fungus, and its cloning in *E.coli.* The recombinant lectins expressed as soluble proteins and are purified by ion exchange and gel filtration chromatographic techniques.

[0010] In some embodiments of the invention, there is provided two recombinant lectins (Rec-2 and Rec-3) having approximate subunit masses (M, 16.1 kDa), which are specific to certain O-glycosidically linked glycans occurring in glycoproteins and on cell surfaces, but does not show specificity to N-linked glycans. The property of recognizing O-glycans but not N-glycans and showing no specificity to human blood groups makes them different from the other recombinant lectin disclosed in copending patent application 30/MUM/2008. It is also found that the recombinant lectins Rec-2 & Rec-3 does not bind to fetuin but exhibit very strong affinity for asialofetuin, thereby making it different from that of said recombinant lectin (patent application 30/MUM/2008) and also the native lectin. By virtue of this recognition property they bind to certain cell surface molecules expressed during malignancy (Fig. 1) and thus bind to human Breast cancer, colon cancer, ovarian cancer tissues (Fig .2) and also cultured cells and leukemic cell lines (table ₁1). Apart from binding to cultured human cancer cells, recombinant lectins exert *in vitro* antiproliferative activity on the cells (Table 2) contemplating their diagnostic and therapeutic properties.

[0011] In another embodiment of the invention, there is provided DNA sequences for **Rec-2** and **Rec-3** coding for 141 amino acids long lectins in each case similar to the native lectin (S. *rolfsii* lectin), but with few deliberate modifications synthesized chemically and cloned into *E. coli* (SEQ ID NO: 1.1 and SEQ ID NO: 2.1). The cloned lectins were expressed after induction with the inducer IPTG. The expressed protein appeared as soluble and in active form in each case. The recombinant lectins were purified to essential homogeneity by resorting to anion exchange and molecular sieve chromatography. The mass spectrometric and SDS PAGE estimate of molecular weights show a value of approximate 16.1 kDa. The isoelectric focusing experiment shows the PI value of 6.614 whereas the native lectin has 6.219, the property markedly helped in improving the solubility and stability of recombinant proteins. Improved solubility, stability and the binding to desialylated TF antigen of the recombinant lectins have greater implications of their applications in various fields as reagents apart from ease of production.

[0012] Also disclosed herein is a method for preparing a recombinant lectin expressed in a host cell such as *E.coli or yeast.* This method comprises the synthesis of a lectin gene based on amino acid sequence derived from the MALDI MS/MS of the *Sclerotium rolfsii* lectin (SEQ ID NO: 1), a soil borne phytopathogenic fungus, and its cloning in the host cell.

[0013] In order to obtain desired properties the amino acid sequence of the native lectin was modified deliberately and recombinant lectins were obtained expressing in *E-coli.* Also described herein are two recombinant lectins with modified amino acid sequences but have close resemblances with native lectin with respect to their binding properties towards cancer tissues, in vitro cultured cells, and their apoptotic activity to cultured cells. However these two lectins have better solubility and stability and desired sugar specificity properties for applications as compared to native lectin.

[0014] The recombinant lectin expressed as a soluble protein is purified by ion exchange and gel filtration chromatographic techniques.

[0015] The amino acid sequence SEQ ID NO: 2 and 3 represents such lectin having all the above characteristics. The nucleic acid molecule encoding the amino acid for the protein of the above characteristics is also within the scope of the invention. The DNA sequence of SEQ ID NO: 1.1 and 2.1 represents such nucleic acid encoding the lectin having the above characteristics. The sequences also envisage any change in the nucleotide or amino-acid sequences including but not limited to any substitution, deletion, addition or modification such as acetylation, nitration, glycation, sulphonation etc. in any of the positions of the entire sequence and also to include truncations at 5'or 3' end of the sequences which do not alter the properties disclosed in the instant application.

[0016] In one aspect, there is a lectin protein comprising the amino acid sequence of SEQ ID NO: 1, characterised by having an amino acid modification/ substitution at one or more of the positions selected from 1, 14, 34, 113 and 123 of SEQ ID NO: 1; and having better solubility and stability properties as compared to the lectin of SEQ ID NO: 1, and wherein said lectin binds to human breast cancer, colon cancer, ovarian cancer tissues and also cultured cells and leukemic cell lines; and exerts an in vitro antiproliferative activity on the cancer cells.

[0017] In one aspect there is a use of this recombinant lectin for *in vitro* detection of a cancer cell. Also described herein is where this recombinant lectin in a biotinylated form or modified with a fluorogenic chromophore can be used for the detection of cancer cells or cancer associated specific antigens.

[0018] The growth modulatory effect of the recombinant lectin can have potential application in cancer therapy as anti tumor agents.

[0019] In another aspect the recombinant lectin can be used in cancer or therapy. Also described herein, the recombinant lectin can be used in research.

[0020] In one embodiment of the invention, there is provided a DNA sequence (SEQ ID NO: 1.1 and 2.1) coding for a 141 amino acids long lectin similar to the lectin *(S. rolfsii),* but with a few modifications was chemically synthesized and cloned into *E.coli.*

[0021] The method for producing said recombinant lectin has also been disclosed. The cloned lectin is expressed after induction with the inducer IPTG.

[0022] In one embodiment, there is provided a lectin protein comprising an amino acid sequence of SEQ ID NO: 2 or 3. The lectin protein of the invention has better solubility and stability properties as compared to lectin of SEQ ID NO: 1.

[0023] The present invention also provides the lectin protein of the present invention for use in cancer therapy and the use of the lectin protein of the present invention in in vitro detection of cancer cells.

[0024] The present invention further provides a nucleic acid molecule encoding a lectin protein of the present invention, wherein said nucleic acid molecule is DNA or RNA, preferably wherein the nucleic acid molecule is SEQ ID NO: 1.1 or 2.1.

[0025] The present invention also provides a recombinant vector comprising operatively linked in the 5' to 3' direction: a promoter which functions in a host cell; a structural nucleic acid sequence of the present invention wherein said nucleic acid sequence encodes a lectin protein; and a termination signal.

[0026] In addition, the present invention provides a transformed host cell containing the vector of the present invention.

[0027] Still further the present invention provides a process for producing a recombinant Sclerotium rolfsii lectin protein comprising: culturing a host cell containing the recombinant vector of the present invention coding for the lectin protein; expressing the recombinant lectin protein; and isolating said lectin protein from the culture.

BRIEF DESCRIPTION OF FIGURES

[0028]

Figure 1: Comparative profiles of native SRL receptors isolated from the membranes of human normal resting PBMCs (1), PHA-activated PBMCs (2), Molt-4 (3) and Jurkat cells (4). Membrane proteins (40 µg) were resolved by SDS-PAGE (10% gel) and blotted on to nitrocellulose membrane. SRL interacting proteins were probed with biotinylated lectin and compared with standard molecular weight protein markers (M) after visualization with streptavidin-HRP reaction.

Figure 2:
Binding pattern of Rec 2 and Rec 3 and native lectin to human cancerous tissues of breast and ovary in comparison with H & E staining. Biotinylated lectins were used for the immunohistochemistry

Figure 3:
The Amino acid sequence of lectin protein isolated from *S. rolfsii* (SEQ ID NO: 1);

Protein Sequence of the recombinant lectin Rec 2 of the instant application. The number of amino acid coding for this lectin is 141 and is represented by SEQ ID NO: 2. The altered/modified positions have been highlighted in the sequence.

Protein Sequence of the recombinant lectin Rec 3 of the instant application. The number of amino acid coding for this lectin is 141 and is represented by SEQ ID NO: 3. The altered/modified positions have been highlighted in the sequence.

Multiple sequence alignment of the SEQ ID NO: 1 -3.

Figure 4: A DNA sequence encoding the lectin of the instant application, SEQ ID NO: I.I and 2.1 The length of the optimized sequence is ----- nucleotides which includes the 5' end Nde I (CATATG) and 3' end BamHI (GGATCC) restriction sites. SEQ ID NO: 1.1; GENE NAME; UC-SS/CSR-1803 (Rec-2); SEQ ID: NO: 2.1; GENE NAME: UC-SS/CSR-1805 (Rec-3)

DETAILED DESCRIPTION OF THE INVENTION

[0029] There is described herein cloned genes for two new variants, designated here as Rec-2 and Rec-3, in *E-coli* that are more close to the native S. rolfsii lectin in amino acid sequence, but still differ in surface charges. Surface charge was altered deliberately in order to enhance the better solubility and stability of the proteins as compared to native lectin. Two lectin genes coding *S. rolfsii* lectin with few alterations are chemically synthesized and expressed in *E-coli.* Recombinant lectins are purified by conventional chromatographic methods and are shown to have molecular mass approximate 16,000 Da. The two recombinant lectins described here are different in blood group specificity, recognize all the human blood groups and have exclusive ability to recognize TF antigen and its cryptic forms. The two blood group non specific recombinant lectins are more closely resemble with native *S. rolfsii* lectin in sugar binding, cell binding and in bringing apoptotic properties. However the recombinant lectins have better stability and solubility properties compared to native lectin making them ideal for various applications.

[0030] Also described herein, the various applications to the use of the recombinant lectins may be in the following:
Considering the property of cancer cell binding, in one part of the disclosure R1 and R2 can be used as drug delivery agents for cancer treatment and may also find application as diagnostic tools in the detection of microbial / viral pathogens.

[0031] In one part of the disclosure, these recombinant lectins can be used for the detection of certain glycoproteins and glycoconjugates present in biological fluids.

[0032] In one part of the disclosure, the lectins exhibits substrate specificity towards cancer associated cell surface carbohydrate such as desialylated TF antigen, they can have application as affinity matrices for the purification of glycoproteins and glycoconjugates of physiological and biochemical significance that bind to these recombinant lectins.

[0033] In one part of the disclosure, recombinant lectins can be used as fusion partners for the purification of recombinant lectins.

Lectin Gene construct and cloning:

[0034] The amino acid sequence derived from the MALDI MS/MS and x-ray crystallography data of the lectin purified from the soil fungus *Sclerotium rolfsii* formed the basis for the gene construct. The gene corresponding to this sequence SEQ ID NO: 1 was chemically synthesized by following the standard protocols. The assembled gene was first inserted into a pUC57 vector. The insert was released by digesting the plasmid with NdeI & BamHI and then recloned in to pET20b previously digested NdeI and BamHI. After ligation the plasmid was used to transform E.coli DE3 (GOLD) host strain for expression. The recombinant clones were analyzed for release of insert after digestion with Nde I and Bain HI. The SDS-PAGE analysis of recombinant E.coli upon induction with IPTG showed the expected ~161cDa protein.

Cell growth

[0035] A single colony of the recombinant E.coli was inoculated into 5m1 LB-Amp allowed to grow at 37^0C overnight with shaking. The overnight grown culture was inoculated into the Fermentation Media and grown at 37° C till an OD of ~2.0 was reached, the cultures were then induced with 250µM IPTG (fmal concentration) and grown overnight at 20^0C.

Cell extract preparation

[0036] The overnight grown cultures were centrifuged at 8000rpm for 10min and the pelleted cells (4gms) were suspended in 40m1 of 50mM Tris-HC1 pH8.0 containing 1mM PMSF and 1mM EDTA. The cell suspension was sonicated for 20min using the sonicator. The cells were centrifuged at 12000rpm, 10min at 40C. The supernatant was used for further purification.

Purification of the protein

DEAE-cellulose chromatography:

[0037] The supernatant (400mg of protein) in 50 mM Tris-HC1 buffer pH 8.0 was loaded on 25ml DEAF (BIORAD) column equilibrated in 50mM Tris-HC1 buffer pH 8.0. The column was washed with 2 column volumes of buffer followed by step-wise elution of the column with buffer b containing 75mM NaCl and 200mM NaCl. The lectin was eluted with buffer containing 300mM NaCl. This fraction contained 216 mg of protein.

PEI chromatography:

[0038] The DEAE elute (216mg) was loaded on to the NUCLEOSII, 4000-7-PEI (250 x 10mm) column, equilibrated with 50 mM Tris-acetate buffer pH 8.0. The bound protein was eluted by applying a linear gradient of NaCl from 0-100% in 15min at 2ml/min flow rate using the FPLC system and the protein eluted at 98%B (60mg). The eluted protein was dialyzed against water and repurified on the same PEI column using ammonium bicarbonate buffer.

[0039] The PEI column was equilibrated with 20mM ammonium bicarbonate buffer pH 8.0 and the protein was loaded on the column. The bound protein was eluted using buffer gradient from 20-500mM ammonium bicarbonate buffer. The eluted protein (30mg) was dialyzed against water, lyophilized and stored at -200C.

[0040] Gel filtration chromatography: The final purification of the recombinant protein was achieved by gel filtration chromatography on Superdex G-75 equilibrated with 25 mM TBS, pH 7.2 on AKTA Prime plus purification system.

[0041] The expression of the protein and the purification of the protein were checked on 15% SDS PAGE and the results are given here. There was a major Coomassie blue stainable protein band corresponding to a molecular size of 16kDa.

EXAMPLES

Solubility studies:

[0042] Purified recombinant lectins were dialyzed extensively against water and lyophilized. Lyophyllized samples were suspended in buffers of different pH, the protein content and the hemagglutination activity were determined in the clear supernatant after centrifugation. Solubility and the hemagglutinating activities were compared with native lectin.

Histochemical studies:

[0043] Binding of the lectins to tissue sections of confirmed cases of human colon epithelial cancer were performed using biotinylated lectins and the binding was observed by light microscopy. Tissue sections were stained routinely using hematoxylin and eosin and lectin histochemical studies were carried out by standard protocols using five pm tissue sections of cancerous tissues. Sections of colon cancer tissues each of 5 µm thickness used for histochemical studies were obtained using rotary microtome semi-automatic (Leica RM 2145) and deparaffinised by heating on a slide warmer at 60 °C for 1 h. The tissue sections were rehydrated by keeping them in two changes of xylene for five minutes each which were then transferred to absolute and 95% alcohol for three minutes each. As a routine procedure to confirm the state of tissues being in normal or cancerous condition, tissue sections were

stained with hematoxylin and eosin (H and E) stains. Tissue sections, after rehydration were treated with basophilic hematoxylin stain for 20-25 min, followed by 1% HCl, acid-alcohol dip and the tissue sections were gently washed under running tap water. Then, sections were counterstained with eosin for 2 minutes, washed with water, dried and dipped in xylene and mounted with DPX (Distrene 80 Dibutyl Phthalate Xylene), a nonaqueous permanent mounting medium and observed using light microscope (Carl zeiss Jenalumar) and photographed.

[0044] Histochemical staining was carried out, using biotinylated lectins as described by Danguy and Gabius, in Gabius and Gabius (1993). All the reagents were equilibrated to room temperature prior to the staining. At no time the tissue sections were allowed to dry during the staining procedure. Study also included control slides prepared by excluding the biotinylated lectin. To block the endogenous peroxidase activity, tissue sections after rehydration were incubated with methanol containing 0.3% hydrogen peroxide for 30 minutes at room temperature. Sections were then rinsed with water for 5 minutes and then with TBST (pH 7.2) for 5 minutes. After the tissue sections were rinsed with PBS pH 7.2, sections were incubated with 1% periodate treated BSA in 0.1 M PBS solution for 15 min to block nonspecific binding sites by BSA. Sections were then rinsed with TBST for 5 min. Biotinylated lectins (20µg/ml in PBS) were carefully applied to cover the sections completely and incubated for 1 h, so as to facilitate the complete binding. Sections were then rinsed with TBST, for 5 minutes. These tissue sections were then incubated with streptavidin-HRP (1->1000 dilution) in PBS, pH 7.2, for 30 minutes at room temperature. Sections were then rinsed with two changes of TBST, pH 7.2, for 5 minutes. After streptavidin-HRP conjugation, tissue sections were developed in DAB Chromogen/H202 substrate in buffered solution (pH 7.5), which was prepared according to the guidelines of manufactures. Tissue sections were incubated for 10 minutes and then gently rinsed with water for 5 minutes. The slides were allowed to dry and dipped in xylene and mounted in DPX. Sections were then examined under light microscope (Carl zeiss Jenalumar) to observe and study the staining intensity patterns by the normal and cancer tissues and photographed.

Identification of cell surface antigens:

[0045] Identification of neoglycans expressed on activated and transformed lymphocytes in comparison with normal lymphocytes by the recombinant lectin;
In order to identify the neoglycoproteins expressed on lymphocytes upon activation by PHA and transformed cells, the interaction studies were carried out with respective cell membrane proteins with biotinylated lectin.

Isolation of Cell Membrane Proteins:

[0046] Human peripheral lymphocytes were prepared from the blood collected from a healthy donor. The blood sample (20ml) collected in to glass centrifuge tube containing EDTA (12.5

mM final concentration) and the normal lymphocytes were separated by centrifugation in Ficoll-Hypaque (Pharmacia) gradient. A portion of normal lymphocytes were activated by incubating with PHA (2.5 µg/ml PHA) in RPMI-1640 medium supplemented with 10% FCS for 72 hours in CO2 incubator. Activated cells were harvested by centrifugation and the activation was confirmed by checking for the expression of CD25 receptors.

[0047] Normal lymphocytes, activated lymphocytes and leukemic cell lines; Molt-4 and Jurkat were finally maintained in RPMI 1640 culture medium, supplemented with glutamine (2 mM), heat inactivated FCS (10%), penicillin (100U/ml) and streptomycin (100-ug/ml). The cultures were maintained at 37°C in humidified atmosphere (95% air and 5% CO2). Membrane proteins from these cultured cells were isolated by using "Pierce Membrane Protein Extraction Reagent kit" method (Mem-PER, Prod #89826) and the protein contents were estimated using Bio-Rad's DC-Protein estimation Kit. Isolated membrane proteins were finally concentrated by chloroform-methanol precipitation, and the precipitated proteins were air dried and stored at 40C till further use.

Electrophoresis & western Blotting:

[0048] Polyacrylamide gel electrophoresis was performed in the presence of SDS using a minigel system (Hoefer Scientific Instruments, USA) in 10% gel for 60 mins at 120 volts. The proteins were transferred from polyacrylamide gels to Nitro Cellulose membranes in a semidry blotting equipment using transfer buffer (240mM Glycine, 25mM Tris, 0.1% SDS) containing 20% methanol at 75mA for 3hr at 4 OC. Membranes were saturated after transfer with blocking solution (3% p-BSA) and washed with TBST. Lectin binding was carried out at room temperature for 4 hours with biotin-coupled lectin at the final concentration of 20µg/ml (prepared in TBST). After thorough washing, blot was incubated with Streptavidin-HRP (1:1000 in TBST) at room temperature for 1 hr and excess Streptavidin-HRP was washed with TBST. Finally the lectin-glycoproteins bands were visualized by developing with DAB chromogenic system.

[0049] The results of these findings show that the present recombinant lectin has the ability to bind to some of the uniquely expressed neoglycans on the leukemic cells and also activated lymphocytes.

SDS PAGE and lectin blotting

[0050] Proteins from the ovarian cyst fluids samples were separated by SDS PAGE in 10 % gels as described by Laemmli et al using Hoefer Scientific Instruments, San Francisco, USA. Electrophoresis was carried out for 70 min.1 using 110 volts. Immediately after the electrophoresis fractionated protein bands were resolved were blotted onto nitro cellulose membrane using semidry blot assembly. Blotted membrane was washed with distilled water

and stained with Ponceau reagent to confirm the efficiency of blotting and to mark the location of standard proteins.

[0051] Finally the blot was washed with TBS containing 0.1 % Tween -20 (TBST) and treated with 3 % P-BSA in TBST for overnight to prevent non-specific binding. After washing with TBST, blot was incubated with biotinylated lectins (20 µg/ ml in TBST) at 37^0 C for 4-6 hours. Excess and unbound lectin was removed by washing the membrane with TBST and then the membranes were incubated with Streptavidin-HRP (1µg/m1 in TBST) for one hour. Unbound Streptavidin-HRP was removed by washing with TBST. The lectin binding glycoproteins were visualized by staining for peroxidnse activity using DAB system.

Proliferative and Antiproliferative activity on PBMCs and cultured leukemic and colon cancer cells

[0052] Proliferative/antiproliferative activity of SRL on human peripheral blood mononuclear cells (PBMCs) was determined by tritiated thymidine incorporation assay, using freshly isolated human PBMCs as described by Wang et al., [1992] with some modifications. PBMCs were isolated from the freshly collected blood samples of healthy donors and the cells were maintained in RPMI 1640 complete medium as described previously. The cells were diluted with RPMI medium containing 10% FCS and then seeded (1x105 cells/50 µl/well) in 96-well microplates (NUNC Denmark). Various concentrations (10, 5, 2.5, 1.25 and 0.625 µg/ml) of SRL in RPMI 1640 medium was added to each well in a final volume of 100 µl and incubated at 37°C in a humidified, 5% CO2 atmosphere for 72 h. Eighteen hours prior to the total incubation period, 1 µCi of tritiated thymidine was added to each well in a final volume of 10 µl and the incubation was continued for another 18 h. Finally, cells were harvested onto a glass filter paper (Amersham Biosciences) using cell harvester (NUNC, Denmark), washed thoroughly with distilled water and dried in micro-oven. Filter discs were transferred to scintillation vials (Tarsons, India) containing 600 µl of toluene-based scintillation cocktail. Finally the incorporated tritiated thymidine was measured by β-scintillation counter (Packard Bioscience Ltd.) as counts per minute (CPM). In order to confirm the lectin-receptor mediated proliferative activity, assays were performed using SRL preincubated with mucin. SRL (10 µg/ml) was preincubated with mucin (12.5 µg in 100 µl) for 1 h at 37°C and used for cell proliferation assay. Appropriate controls containing cells without lectin and cells with mucin alone were included. Results are representative of three independent experiments done in triplicate.

[0053] Proliferative/antiproliferative effect of SRL on Molt-4 leukemic cell line was determined by tritiated thymidine incorporation assay as described previously. Proliferative/antiproliferative effect of SRL on Molt-4 leukemic cell line was determined by tritiated thymidine incorporation assay as described previously. Cells were seeded (1x105 / well) in a 96-well tissue culture plate and allowed to acclimatize for 3h before lectin treatment Serial concentrations of SRL from 6.25 µg/ml to 100 µg/ml in 50 µl of RPMI medium was added to each well and incubated for 72 h. All other steps of assay remained same as described earlier. For lectin blocking

assay, SRL (100 µg/ml) was preincubated with 12.5 µg of mucin in a total volume of 100 µl for 1 h at 37°C and used for assay.

Recombinant Sequences

[0054] The gene sequences and the proteins having the following biological activity: recognize all the human blood groups and have exclusive ability to recognize TF antigen and its cryptic forms, resemble with native *S. rolfsii* lectin in sugar binding, cell binding and in bringing apoptotic properties, have better stability and solubility properties compared to native lectin are described in the application. The nucleotide molecules encoding the recombinant molecules may be DNA or RNA. Binding of recombinant lectins to different cultured cells are presented in table 1.

Table; 1, Binding of Recombinant lectins to Cultured leukemic (Molt 4 & Jurkat), Ovarian (PA 1) and colon (COLO 205) cells and the inhibition of binding by mucin

Cell lines		% positive cells		
		Rec 2	Rec 3	Native
Molt 4	Lectin	84.03	99.87	88.45
	Lectin+Mucin	0.24	0.30	0.24
Jurkat	Lectin	81.35	97.85	33.84
	Lectin+Mucin	0.89	1.02	0.50
PA-1	Lectin	97.97	97.58	96.72
	Lectin+Mucin	2.5	4.28	1.63
COLO 205	Lectin	66.53	90.29	91.66
	Lectin+Mucin	0.87	1.08	0.99

[0055] In each case binding of R2 and R3 with Molt 4, Jurkat (leukemic cells), PA-1 (ovarian cells) and COLO 205 (colon cells) is greater than 90 % and the binding can be abolished by mucin.

[0056] Also described herein, is the immunomodulatory effect on these cultured cells. Both R2 and R3 exerted anti proliferative activity on the cultured cells, highest effect (60%) on leukemic cells as compared to ovarian cells (50%) and colon cells (40%). their growth modulatory effect can have potential application in cancer therapy. (table 2).

Table; 2, Anti proliferative activity of recombinant lectins on cultured cells by MTT assay

	Decrease in cell viability (%)		
Cell lines	Rec 2 (25 µg/ml)	Rec 3 (25 µg/ml)	Native (25 µg/ml)
Molt 4	60.68	61.10	57.93

	Decrease in cell viability (%)		
Cell lines	Rec 2 (25 µg/ml)	Rec 3 (25 µg/ml)	Native (25 µg/ml)
PA-1	46.56	50.60	53.85
COLO-205	26.67	40.43	23.44

[0057] In one aspect, the expressed lectin protein (SEQ ID NO: 2 and 3) differs from the native lectin protein in terms of at least one amino acid modification/substitution at positions. Of these, positions changes are indicated in Table 3.

Table: 3: Amino acid positions changed for Rec 2 and Rec 3 compared to native lectin sequence

Lectin	Amino acid positions changed				
	1	**14**	**34**	**113**	**123**
Native	Ac-T	N	T	E	E
S1	T	D	T	Q	Q
S2	V	D	S	Q	Q

[0058] Described herein, the recombinant lectin sequences can be biotinylated or attached with fluorogenic chromophore by using commonly known methods to be used for the detection of cancer cells or cancer associated specific antigens. The growth modulatory effect of the recombinant lectin finds its application in cancer therapy. Also described herein is the use of recombinant lectin as a drug delivery agent for cancer treatment.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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CANCERCELLEBINDENDE, REKOMBINANTE LECTINER MED ANTITUMORAKTIVITET OG FREMSTILLINGSFREMGANGSMÅDE

PATENTKRAV

1. Lectinprotein omfattende aminosyresekvensen ifølge SEQ ID NO: 1, kendetegnet ved at have en aminosyremodifikation/substitution ved én eller flere af positionerne valgt blandt 1, 14, 34, 113 og 123 i SEQ ID NO: 1; og have bedre opløsnings- og stabilitetsegenskaber sammenlignet med lectinet i SEQ ID NO: 1; og hvor lectinet binder til human brystcancer, coloncancer, ovariecancervæv og også dyrkede celler og leukæmiske cellelinjer; og udøver en antiproliferativ aktivitet in vitro på cancercellerne.

2. Lectinprotein ifølge krav 1 hvor lectinet:

(a) har specificitet for O-glycosidisk forbundne glycaner, der forekommer i glycoproteiner og på celleflader, men viser ikke specificitet for N-bundne glycaner;

(b) ikke binder til fetuin men udviser affinitet for asialofetuin.

3. Lectinprotein ifølge et hvilket som helst af krav 1 til 2, hvor ændringen/modifikationen af SEQ ID NO: 1 er en hvilken som helst af følgende: 14(N->D), 113(E->Q) og 123(E->Q).

4. Lectinprotein ifølge et hvilket som helst af krav 1 til 3, hvor ændringen/modifikationen af SEQ ID NO: 1 er en hvilken som helst af følgende: 1(Ac-T->V) og 34(T->S).

5. Lectinprotein ifølge et hvilket som helst af de foregående krav til anvendelse *in vivo* i cancerdiagnose eller cancerterapi.

6. Anvendelse af lectinprotein ifølge et hvilket som helst af krav 1 til 4 til påvisning *in vitro* af en cancercelle.

7. Nukleinsyremolekyle, der koder for et lectinprotein ifølge et hvilket som helst af krav 1 til 4.

8. Rekombinant vektor, der omfatter operativt bundet i 5'- til 3'-retningen:

(i) en promoter, der fungerer i en værtscelle;

(ii) en strukturel nukleinsyresekvens ifølge krav 7, hvor nukleinsyresekvensen koder for et lectinprotein; og

(iii) et termineringssignal.

9. Rekombinant vektor ifølge krav 8, hvor den rekombinante vektor kan replikeres, transskriberes, translateres og udtrykkes i en encellet organisme.

10. Transformeret værtscelle, der indeholder vektoren ifølge krav 8 eller krav 9.

11. Transformeret værtscelle ifølge krav 10 hvor værtscellen er Escherichia coli bacterium; eller en gærcelle.

12. Fremgangsmåde til frembringelse af et rekombinant Sclerotium rofsii lectinprotein omfattende:

- dyrkning af en værtscelle indeholdende den rekombinante vektor ifølge krav 8 eller krav 9, der koder for lectinproteinet;
- ekspression at det rekombinante lectinprotein;
- isolering af lectinproteinet fra kulturen.

13. Fremgangsmåde in vitro til diagnosticering af cancer, hvilken fremgangsmåde omfatter trinnet med at bringe en celleprøve fra en patient i kontakt med et lectin ifølge et hvilket som helst af krav 1 til 4.

DRAWINGS

Drawing

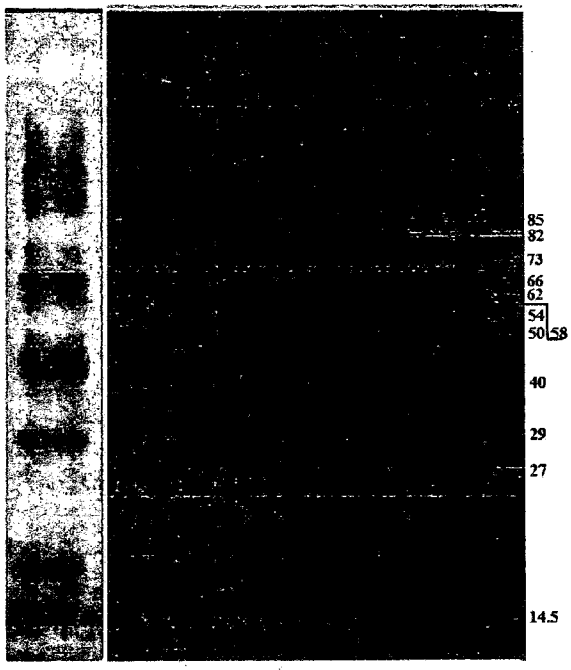
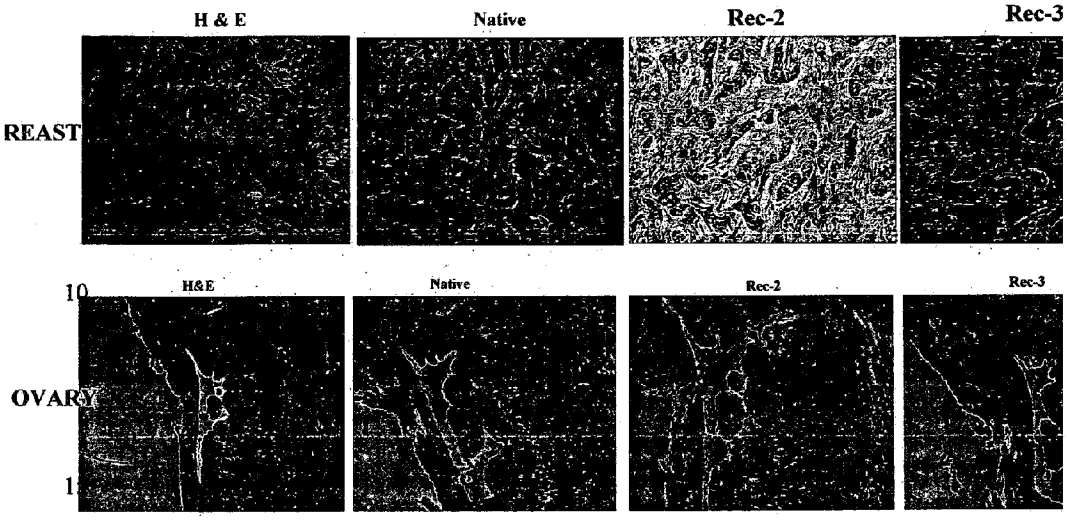


FIGURE 1


H & E
Native
Rec-2
Rec-3
REAST
10
H&E
Native
Rec-2
Rec-3
OVARY
1

SEQ ID: 1 Amino acid sequence of native lectin

Ac-TY-K-I-T-V-R-V-Y-Q-T-N-P-N-A-F-F-H-P-V-E-K-T-V-W-K-Y-A-N-G-G-T-W-T-I-T- D-D-Q-

H-V-L-T-M-G-G-S-G-T-S-G-T-L-R-F-H-A-D-N-G-E-S-F-T-A-T-F-G-V-H-N-Y-K-R-W-C-D-I-V-T-

N-L-A-A-D-E-T-G-M-V-I-N-Q-Q-Y-Y-S-Q-K-N-R-E-E-A-R-E-R-Q-L-S-N-Y-E-V-K-N-A-K-G-R-

N-F-E-I-V-Y-T-E-A-E-G-N-D-L-H-A-N-L-I-I-G-COOH

SEQ ID: 2 Amino acid sequence of Rec 2

TYKITVRVYQTNPDAFFHPVEKTVWKYANGGTWTITDDQHVLTMGGSGTSGTLRFHADNGE
SFTATFGVHNYKRWCDIVTNLAADETGMVINQQYYSQKNREEARERQLSNYQVKNAKGRNF
QIVYTEAEGNDLHANLIIG

SEQ ID:3 Amino acid sequence of Rec 3

VYKITVRVYQTNPDAFFHPVEKTVWKYANGGTWSITDDQHVLTMGGSGTSGTLRFHADNGE
SFTATFGVHNYKRWCDIVTNLAADETGMVINQQYYSQKNREEARERQLSNYQVKNAKGRNF
QIVYTEAEGNDLHANLIIG

Comparison of amino acid sequences of recombinant lectins (Rec-2 and Rec-3) with native lectin (Ntv)

Ac-TYKITVRVYQTNPNAFFHPVEKTVWKYANGGTWTITDDQHVLTM
Ntv
TYKITVRVYQTNPDAFFHPVEKTVWKYANGGTWTITDDQHVLTM Rec-2
VYKITVRVYQTNPDAFFHPVEKTVWKYANGGTWSITDDQHVLTM Rec-3

GGSGTSGTLRFHADNGESFTATFGVHNYKRWCDIVTNLAADETG Ntv
GGSGTSGTLRFHADNGESFTATFGVHNYKRWCDIVTNLAADETG
Rec-2
GGSGTSGTLRFHADNGESFTATFGVHNYKRWCDIVTNLAADETG Rec-3

MVINQQYYSQKNREEARERQLSNYEVKNAKGRNFEIVYTEAEGN Ntv
MVINQQYYSQKNREEARERQLSNYQVKNAKGRNFQIVYTEAEGN
Rec-2
MVINQQYYSQKNREEARERQLSNYQVKNAKGRNFQIVYTEAEGN
Rec-3

DLHANLIIG Ntv
DLHANLIIG Rec-2
DLHANLIIG Rec-3

SEQ ID: 1.1; GENE NAME; UC- SS/CSR—1803 (Rec-2)

CATATGACCTATAAAATTACCGTGCGCGTGTATCAGACCAACCCGGATGCCTTTTTCCAT
CCGGTGGAAAAAACCGTGTGGAAATATGCGAATGGCGGTACCTGGACGATTACGGATGAT
CAGCATGTGCTGACGATGGGTGGTAGCGGTACCAGCGGCACCCTGCGTTTTCACGCAGAT
AATGGCGAAAGCTTCACCGCCACCTTTGGTGTGCATAATTATAAACGCTGGTGTGATATT
GTGACCAACCTGGCAGCGGATGAAACCGGCATGGTTATTAATCAGCAGTATTATAGTCAG
AAAAACCGCGAAGAAGCGCGTGAACGCCAGCTGAGTAACTATCAGGTGAAAAATGCGAA
A
GGCCGTAACTTCCAGATTGTTTATACCGAAGCGGAAGGCAATGATCTGCATGCGAACCTG
ATTATCGGCTAAGGATCC

SEQ ID: 2.1; GENE NAME: UC-SS/CSR-1805 (Rec-3)

CATATGGTGTATAAAATTACCGTGCGCGTGTATCAGACCAACCCGGATGCCTTTTTCCATC
CGGTGGAAAAAACCGTGTGGAAATATGCGAATGGCGGTACCTGGTCTATTACGGATGATC
AGCATGTGCTGACGATGGGTGGTAGCGGTACCAGCGGCACCCTGCGTTTTCACGCAGATA
ATGGCGAAAGCTTCACCGCCACCTTTGGTGTGCATAATTATAAACGCTGGTGTGATATTGT
GACCAACCTGGCAGCGGATGAAACCGGCATGGTTATTAATCAGCAGTATTATAGTCAGAA
AAACCGCGAAGAAGCGCGTGAACGCCAGCTGAGTAACTATCAGGTGAAAAATGCGAAAG
GCCGTAACTTCCAGATTGTTTATACCGAAGCGGAAGGCAATGATCTGCATGCGAACCTGA
TTATCGGCTAAGGATCC

FIGURE 4