Glycoconj J (2010) 27:309–320
DOI 10.1007/s10719-010-9279-0

Purification, characterization and molecular cloning of a monocot mannose-binding lectin from *Remusatia vivipara* with nematicidal activity

Ganapati G. Bhat · Kartika N. Shetty · Nagaraja N. Nagre · Vivek V. Neekhra · S. Lingaraju · Ramesh S. Bhat · Shashikala R. Inamdar · K. Suguna · Bale M. Swamy

Received: 20 November 2009 / Revised: 24 January 2010 / Accepted: 24 January 2010 / Published online: 6 March 2010

Abstract A mannose-binding lectin (RVL) was purified from the tubers of *Remusatia vivipara*, a monocot plant by single-step affinity chromatography on asialofetuin-Sepharose 4B. RVL agglutinated only rabbit erythrocytes and was inhibited by mucin, asialomucin, asialofetuin and thyroglobulin. Lectin activity was stable up to 80°C and under wide range of pH (2.0–9.3). SDS-PAGE and gel filtration results showed the lectin is a homotetramer of Mr 49.5 kDa, but MALDI analysis showed two distinct peaks corresponding to subunit mass of 12 kDa and 12.7 kDa. Also the N-terminal sequencing gave two different sequences indicating presence of two polypeptide chains. Cloning of RVL gene indicated posttranslational cleavage of RVL precursor into two mature polypeptides of 116 and 117 amino-acid residues. Dynamic light scattering (DLS) and gel filtration studies together confirmed the homogeneity of the purified lectin and supported RVL as a dimer with Mr 49.5 kDa derived from single polypeptide precursor of 233 amino acids. Purified RVL exerts potent nematicidal activity on *Meloidogyne incognita,* a root knot nematode. Fluorescent confocal microscopic studies demonstrated the binding of RVL to specific regions of the alimentary-tract and exhibited a potent toxic effect on *M. incognita*. RVL-mucin complex failed to interact with the gut confirming the receptor mediated lectin interaction. Very high mortality (88%) rate was observed at lectin concentration as low as 30 μg/ml, suggesting its potential application in the development of nematode resistant transgenic-crops.

Keywords Monocot mannose binding lectins · High mannose N-glycans · MALDI-MS · DLS · Nematicidal-activity

G. G. Bhat · N. N. Nagre · S. R. Inamdar · B. M. Swamy (✉)
P.G. Department of Studies in Biochemistry,
Karnatak University Dharwad,
Dharwad, India
e-mail: sawmy_bm@yahoo.co.in

K. N. Shetty · K. Suguna
Molecular Biophysics Unit, Indian Institute of Science Bangalore,
Bangalore, India

S. Lingaraju
Department of Pathology,
University of Agricultural Sciences Dharwad,
Dharwad, India

V. V. Neekhra · R. S. Bhat
Institute of Agriculture Biotechnology,
University of Agricultural Sciences Dharwad,
Dharwad, India

Introduction

Lectins represent a heterogeneous group of carbohydrate binding oligomeric proteins that vary widely in size, structure, molecular organization and in their carbohydrate binding domains. They belong to distinct protein families yet many have very similar sequences and structural features [1]. Based on the sequence data obtained through molecular cloning and protein sequencing, plant lectins reported till now are classified in to four major groups namely legume lectins [2], chitin binding lectins [3], type 2 ribosome inactivating proteins [4] and monocot mannose-binding lectins [5]. In the recent past monocot mannose-binding lectins have received greater attention, because of their interesting molecular and biological properties [1, 6]. First monocot mannose specific lectin to be reported was *Galanthus nivalis* agglutinin (GNA), purified from the bulbs of snowdrop [7]. Since then several structurally and evolutionarily related lectins are reported from different

monocot families, *viz.*, Amaryllidaceae [7, 8], Alliaceae [9], Araceae [1, 10], Orchidaceae [11] and Liliaceae [12]. Because of their exclusive carbohydrate specificity towards mannose, they are referred to as monocot mannose-binding lectins (MBLs) and recently as GNA related lectins [13]. Many of them exhibited weak affinity towards mannose, but strong affinity towards oligomannosides and high mannose containing N-glycans [14]. All the reported MBLs to date consist of highly homologous sequences and of similar tertiary structures, suggesting these proteins comprise a single super family of mannose-binding proteins [6]. Although all monocot mannose-binding lectins are very similar at the protein level there are important differences in the processing and posttranslational modifications [15].

In general they are either dimers or tetramers containing subunits of M_r 12–14 kDa stable to heat and a wide range of pH. In some but not all cases the protomers are post-translationally cleaved into polypeptides of nearly equal size [15]. Because of their novel sugar binding properties monocot mannose-binding lectins are being exploited for biochemical and biomedical applications. Based on the potent inhibitory effect of some monocot mannose-binding lectins on human and animal retroviruses including HIV [16, 17] and also on their ability to block the adhesion receptors of man-fimbriated *E-coli* in the small intestine [18], they can have potential applications in biomedical research. Recently some of the monocot mannose-binding lectins are reported to have antiproliferative and apoptosis inducing effects on different tumor cells [19–23]. Monocot mannose-binding lectins are being explored extensively for crop protection in plant biotechnology as many of them are reported to exhibit potent toxic effects on sucking pests and nematodes.

Very few lectins are characterized from Araceae species as compared to several lectins reported from other monocot families [1, 10, 24]. *Remusatia vivipara* is an epiphyte found in Western Ghats of South India and all its parts *viz*., tubers, leaves and the stalks are edible. Tubers are traditionally used as folk medicine for treating inflammation and arthritis. In this paper we report purification, physicochemical characterization and cloning of a lectin with potent nematicidal activity named RVL from edible tubers of *R. vivipara*, a member of the Araceae family.

Materials and methods

Materials

The tubers of *R. vivipara* were collected during early winter (October–November) from the Western Ghat region of Southern India (Uttara Kannada District, Karnataka state) and stored at−20°C for further use in airtight containers.

Mucin (porcine stomach, type III), fetuin (fetal calf serum), thyroglobulin (bovine thyroid) and various sugars and sugar derivatives used for hapten inhibition studies were purchased from Sigma Chemical Co., St. Louis, USA. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, asialofetuin-Sepharose 4B, affinity matrix was prepared by coupling asialofetuin to cyanogen bromide activated Sepharose 4B according to the method of March *et al*. [25]. Rabbit blood samples were obtained from local animal house. All other chemicals used were of analytical reagent grade and the reagents were prepared in double distilled water.

Isolation and purification of *R. vivipara* lectin (RVL)

Fresh tubers of *R. vivipara* (10 g) were cut to small pieces and homogenized in a waring blender and the lectin was extracted with 200 ml 50 mM sodium phosphate buffer containing 154 mM NaCl, pH 7.2 (PBS), overnight at 4°C. The extract was filtered by passing through muslin cloth and the filtrate was clarified by centrifugation (4000 *g* for 20 min). Resulting clear supernatant was heated to 50°C in a water bath for 20 min, cooled and the precipitate formed was removed by centrifugation (4500 *g* for 20 min). Clear supernatant was extensively dialyzed first against PBS finally with water and the dialyzate was lyophilized and stored at 4°C.

Affinity purification

Lyophilized powder (30 mg) suspended in 5 ml of PBS was passed through to an asialofetuin-Sepharose 4B affinity column (1.5×10 cm), that had been equilibrated with PBS, at a flow rate of 15 ml/h and the eluting fractions of 3.0 ml were collected using FRAC-100 fraction collector (Pharmacia). Unbound proteins were eluted by washing the column with PBS until the absorbance of the eluting fractions read zero at 280 nm. Affinity bound lectin was eluted using 0.1 M glycine-HCl buffer containing 500 mM NaCl, pH 2.0. All the affinity column operations were carried out at 4°C. Lectin activity in the eluting fractions was determined by hemagglutination assay using trypsinized rabbit erythrocytes and the lectin peak fractions were pooled, dialyzed against PBS followed by water and freeze dried. Homogeneity of the affinity purified lectin preparation was confirmed by SDS-PAGE on 15% gel. Fluorescein isothiocyanate-conjugated RVL (FITC-RVL) required for binding studies was prepared as described by Goldman [26].

Hemagglutinating activity and carbohydrate specificity

Hemagglutinating activity of the lectin was routinely determined at various stages of purification by serial two

fold dilution technique in 96 well microtitre assay plates using trypsinized rabbit erythrocytes. The highest dilution of the extract causing visible agglutination was regarded as the titre and the minimum concentration of the protein required for agglutination (MCA) as one unit of hemagglutinating activity. The specific hemagglutinating activity is expressed as activity unit mg^{-1} protein. Carbohydrate binding specificity of the purified lectin was determined by hapten inhibition assay.

Inhibition assays were carried out by incubating the lectin sample with serially diluted sugar/glycoprotein in a total volume of 50 µl, prior to the addition of erythrocytes and the hemagglutination was visually observed. The lowest concentration of the sugar/glycoprotein, which inhibited the agglutination, was taken as the inhibitory titre of the hapten.

Protein concentration was determined by the method of Lowry [27] using crystalline bovine serum albumin as standard. The sugar content of protein was determined by the phenol sulfuric acid method [28].

Determination of molecular weight by gel filtration chromatography

The native molecular mass (M_r) of purified RVL was estimated by gel filtration chromatography on a calibrated column of Superdex G-75 equilibrated in 25 mM Tris-HCl containing 154 mM NaCl, pH 7.2. The column was earlier calibrated using standard molecular weight markers *viz*., lysozyme (14.3 kDa), lactoglobulin (18.4 kDa), chymotrypsinogen (25.6 kDa), pepsin (34.7 kDa), ovalbumin (45.0 kDa) and bovine serum albumin (BSA, 66.0 kDa).

SDS-PAGE

Purified RVL was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis in 15% (w/v) acrylamide gel of 0.75 mm thickness according to Laemmli [29] for determining homogeneity and the subunit molecular mass of the lectin. Subunit molecular mass (M_r) of RVL was estimated from the calibration curve obtained using standard protein markers.

MALDI-TOF-MS

MALDI-TOF-MS analysis was performed on Ultraflex TOF/TOF (Bruker Daltonics, Germany) mass spectrometer, equipped with a UV nitrogen laser of 337 nm. Protein solution (1 µl) was mixed with 1 µl matrix solution (saturated solution of sinapinic acid in acetonitrile / 0.1% aqueous trifluroacetic acid) and a total volume of 1 µl of this mixture was deposited on the probe plate. The spectra were recorded in the reflectron positive ion mode after the evaporation of the solvent and spectra were acquired and analyzed by Bruker Daltonics FLEX control software.

Effect of temperature and pH

To determine the thermal stability, freeze dried RVL (50 µg/ml) was suspended in PBS and the sample aliquots were heated in a water bath maintained at different temperatures (20–80°C) with an increment of 20°C for 20 min. At each temperature an aliquot was drawn and cooled to room temperature for estimating the lectin activity by hemagglutination assay.

Stability of the purified lectin at different pH was determined by determining the hemagglutination activity of the lectin samples suspended in different buffers of pH 2.0 (100 mM glycine-HCl buffer), pH 4.3 (25 mM sodium acetate buffer), pH 7.2 (50 mM sodium phosphate buffer) and pH 8.5 (50 mM Tris-HCl 25 mM) and pH 9.3 (50 mM sodium carbonate buffer), left overnight at 4°C. The pH of the lectin samples was adjusted to pH 7.2 by adding 0.1 N NaOH before determining the lectin activity by hemagglutination assay.

Determination of N-terminal sequence

N-terminal sequencing of the purified RVL was determined from the PVDF electro blots after SDS-PAGE in 15% gel. The sequence was determined on automated Edman degradation protein sequencer, PROCISE Protein Sequencing System (Applied Biosystems). The Procise Protein Sequencing System sequentially cleaves N-terminal amino acids from protein/peptide chains and analyzes the resulting phenylthiohydantoin (PTH) amino-acid residues.

Dynamic light scattering (DLS)

In order to investigate the oligomeric properties of purified RVL, dynamic light scattering studies were carried out. Polydispersity (pd) and the hydrodynamic radius (R_H) of the molecule were estimated by DLS. The data was collected with DYNAPRO instrument (USA) for purified RVL (0.1 mg/ml) suspended in water at room temperature and the correlation function was analyzed using Dynamics V6 software.

Cloning full length cDNA of RVL

Total mRNA from the leaves of *R. Vivipara* was extracted using RNA isolation kit (Fermentas, USA) as per the description provided by the manufacturer. PolyA^+ mRNA (0.4 ng/µl) was used to synthesize the first strand cDNA (3′-ready cDNA) using SMART™ RACE cDNA amplification kit (Conetech), and 3′ RACE PCR was carried out

using forward primer RBP44_RACE_F:5′-ATGCAG(C/G/T)A(G/C) GAC(T/G)GCAACCTG-3′ designed based on the conserved regions of previously reported mannose-binding monocot lectins. The amplified product was purified and cloned into pTZ57R/T, T/A cloning vector (InsT/A clone™, PCR product cloning kit from MBI Fermentas, USA), and transformed into *Escherichia coli* DH5α. The positive clones were picked up and sequenced. After the synthesis of first strand cDNA (5′-readycDNA), 5′ RACEPCR was carried out using a reverse primer RBP44_RVL_RACE_R:5′-GGGCCCAAGACGACCAGC-3′. The product was purified and cloned into the pTZ57R/T. Full-length cDNA sequence of RVL was deduced by assembling the sequences of the 3′ RACE and 5′ RACE products. Full length sequence of RVL was amplified using specific forward primer RVL_F:5′TTGGCCATGGC CAAGCTGCTCCTC3′ and reverse primer RVL_R:5′GGC GAATTC TACGACGCAGCAA3′, designed containing restriction enzyme site (NcoI and EcoRI). Complete coding sequence of RVL was subsequently amplified by genomic DNA also using same specific primers. PCR amplification was performed under following conditions: cDNA denatured at 94°C for 5 min, followed by 30 cycles of amplification (94°C for 1 min, 57°C for 30 s, and 72°C for 2 min) and extension at 72°C for 10 min. The same program was used for performing 3′ and 5′ RACE and also for genomic DNA amplification mentioned earlier. The amplified full-length sequence of RVL was cloned into pTZ57R/T, and transferred to *E. coli* DH5α. The clone was confirmed by restriction digestion, RVL-specific PCR (Data not shown) and sequencing.

Nematicidal activity of lectin

Nematicidal activity of purified RVL on *Meloidogyne incognita* was assayed similar to the method described for pinewood nematodes by Gaofu *et al* [30]. Fifty freshly hatched nematodes *(Meloidogyne incognita*) after washing with sterile PBS were transferred to eppendorf tubes containing different concentrations of RVL (30 µg, 15 µg, 10 µg, 7.5 µg and 6 µg ml^{-1}) in PBS 50 mM, pH 7.2 and incubated at 28°C. Negative control without lectin was used for comparison. Number of live and dead nematodes from each lectin concentration set was counted at different time intervals (3, 6, 12, 24 and 48 h) under the microscope. Percentage mortality of nematodes was calculated from the average of triplicate experiments for each concentration.

Interaction of lectin with *M. incognita*

Interaction of RVL with *M. incognita* was investigated using FITC-RVL by fluorescent microscopy. Nematodes (15) were suspended in 1.0 ml solution of FITC-RVL (30 µg/ml PBS) and incubated at 28°C in the dark. At different intervals of time (3,6,12,24 and 48 h), 200 µl aliquots were drawn and washed thrice by centrifugation (1000 rpm for 2 min) with PBS to remove excess FITC-RVL. Nematodes were finally collected on a membrane sieve (25 µm), mounted on glass slides and observed under a fluorescent microscope (Zeiss, FL-40) with an excitation wavelength of 450–490 nm and a barrier filter of 515 nm. Also the 3D-image of nematodes was observed under a confocal microscope (Olympus DP 71).

Receptor-mediated RVL binding to nematode was confirmed by using FITC-RVL complexed with mucin. FITC-RVL (30 µg) was incubated with mucin (125 µg) in 1.0 ml of PBS for 1 h at room temperature. To this lectin-sugar complex solution, 15 nematodes were added and incubated as described earlier. After 48 h nematodes were observed for the fluorescence label under the florescent microscope.

Results and discussion

Purification of *R. vivipara* lectin

Crude extracts of *R. vivipara* tubers showed 100 fold higher hemagglutination activity towards trypsinized rabbit erythrocytes as compared to untrypsinized cells. However no activity was found with either trypsinized or untrypsinized human erythrocytes of A, B and O groups a property exhibited by many of the monocot mannose-binding lectins [13, 31].

The crude extract was highly viscous because of high polysaccharide content, which was eliminated by heat treatment and lectin purification was achieved in a single step by affinity chromatography on asialofetuin-Sepharose 4B column. The elution profile of the lectin on the affinity column is presented in Fig. 1a and its homogeneity was established by SDS-PAGE in 15% gel (Fig. 1b). Starting from 100 g tubers a total of 390.0 mg of the purified lectin was recovered with overall 3.7 fold purification. The lectin content in *R. vivipara* tubers constituted approximately 26.70% of the total protein content, which is in correspondence to other Araceae family lectins previously reported from *Arum maculatum, Arisaema flavum, Arisaema consanguineum, Saurom atumvenosum* [19, 32].

The results of hapten inhibition studies are presented in Table 1. Results demonstrated strong inhibition of hemagglutinating activity of RVL by glycoproteins mucin, asialomucin, asialofetuin and thyroglobulin but not by fetuin and any of the simple sugars tested including mannose indicating complex sugar specificity. Amongst all the glycoproteins tested, mucin was the most effective hapten for RVL. Similar carbohydrate binding properties are reported for several monocot lectins like *Colocasia esculenta* lectin, *Xanthosoma sagittifolium* lectin, *Arum*

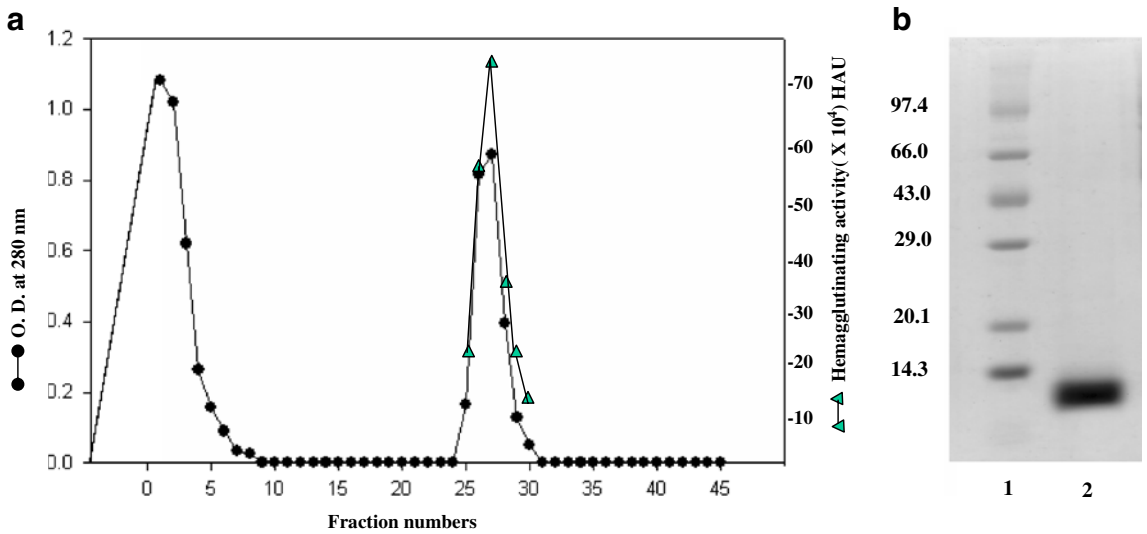

Fig. 1 **a**. Purification of *Remusatia vivipara* lectin (RVL) by affinity chromatography using asialofetuin Sepharose-4B column (1.5× 10 cm), equilibrated in PBS and the affinity bound lectin was eluted with 100 mM Glycine-HCl buffer, pH 2.0 containing 500 mM NaCl. Fractions of 3.0 ml were collected at a flow rate of 15 ml /h. ●—● Absorbance at 280 nm; △—△ Hemagglutinating activity. **b**. SDS-PAGE of affinity purified RVL in 15% gel. Lane 1 and lane 2 contain standard molecular weight markers and purified RVL (20 µg) respectively

Table 1 Carbohydrate binding specificity of RVL

Haptens tested for inhibition	Minimum concentration required for inhibition in µg (MIC)
Monosaccharides and derivatives	
1. D-Glucose	NI
2. D-Galactose	NI
3. D-Mannose	NI
4. L-Fucose	NI
5. D-Lactose	NI
6. Glucosamine	NI
7. N-Acetyl D-galactosamine	NI
8. N-Acetyl D-mannosamine	NI
9. Methyl α-D-Manno Pyranoside	NI
10. Sialic Acid	NI
Glocoproteins	
1. Transferrin	NI
2. Ovalbumin	NI
3. Fetuin	NI
4. Asialofetuin	3.125
5. Mucin	1.56
6. Asialomucin	3.125
7. Thyroglobulin	3.125
8. Gum Guar	NI

NI No Inhibition

The carbohydrate binding specificity of RVL was determined by hemagglutination assay using simple sugars, sugar derivatives and some glycoproteins. The final concentration of the sugars and sugar derivatives was 200 mM and the final concentration of glycoprotein was 1 mg/ml.

maculatum lectin and *Typhonium divaricatum* lectin [10, 13, 22]. These lectins do not recognize mannose but strongly bind to asialofetuin and thyroglobulin, which are known to contain high mannose N-glycans and are referred to as Monocot mannose-binding lectins [13]. Recent report on the carbohydrate binding specificities of *Galanthus nivalis* agglutinin (GNA) and GNA like the lectin from *Zea mays* (GNA $_{maize}$) was determined by glycan array analysis which demonstrated that GNA recognizes mannose weakly, but strongly binds to high mannose type glycans. GNA $_{maize}$, unlike GNA binds exclusively to high mannose type and complex N-glycans [33]. Considering the exclusive carbohydrate specificity of these lectins towards high mannose type N-glycans commonly occurring in animal glycoproteins, it is speculated that these plant proteins are evolved for defense purpose [13].

Temperature and pH stability

Purified RVL was stable in neutral pH up to 80°C for 20 min, also the lectin exhibited tolerance under wide ranging pH of 2.0 to 9.3. Observed stability of RVL to heat and in wide ranging pH is similar to *G. nivalis* agglutinin (GNA), *Aspidistra elatior* lectin and some lectins from *Araceae* family [13, 24, 34].

Molecular weight by gel filtration, SDS-PAGE and MALDI-TOF-MS

Purified RVL eluted as a single symmetrical peak (Fig. 2) on superdex G-75 column and the molecular mass was

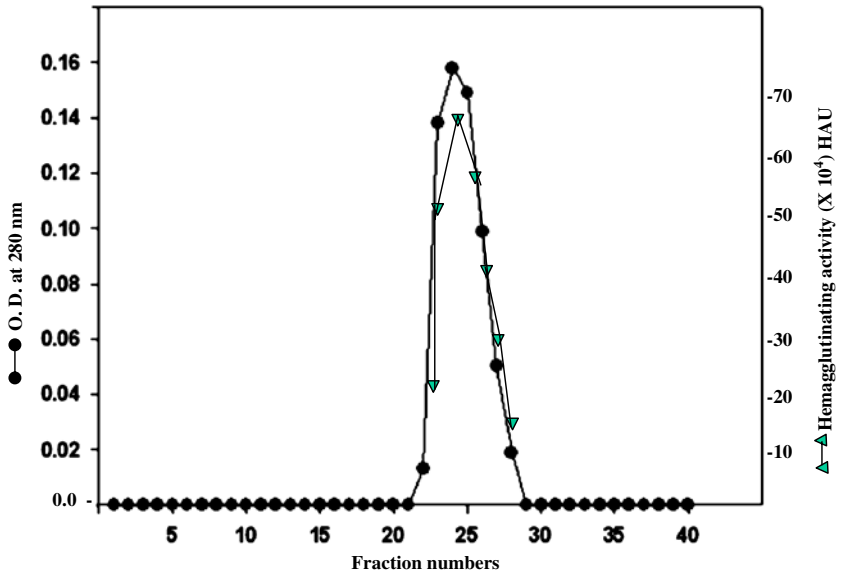

Fig. 2 Molecular mass determination of *RVL* by gel filtration chromatography on superdex-75 column (1.5×80 cm) equilibrated in PBS. Purified lectin (2 mg) in 2 ml of PBS was applied and eluted from the column at a flow rate of 18 ml/ h and the fractions of 3 ml were collected; the column was precalibrated using molecular weight marker proteins; Bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa), chymotrypsinogen (25.6 kDa), lysozyme (14.3 kDa). (●—●) Absorbance at 280 nm; (△—△) Hemagglutinating activity

estimated to be 49.54±1 kDa from the calibration curve (Fig. 3). Whereas the subunit molecular mass of 11±1 kDa was obtained by SDS-PAGE (Fig. 1b). The molecular weight determined by MALDI-MS gave two distinct peaks (Fig. 4) corresponding to 12.02 and 12.76 kDa indicating that the RVL has two non-identical subunits, which were not detected by either gel filtration or SDS- PAGE. These results were not surprising as some of the earlier observations reported that some lectins composed of GNA type domains have nearly identical subunits and in some two domain protomers are cleaved into two polypeptides of nearly equal size [1, 13].

Determination of N-terminal sequence

N-terminal sequence was determined from the lectin blots on the PVDF membrane after SDS-PAGE by automated Edman degradation protein sequencer and the PTH derivatives were identified. N-terminal sequence analysis gave two amino acid sequences of eight residues each, sequence A-LGTNYLLS and sequence B-NIPFTNNL. These results supported the presence of two different subunits in RVL shown by MALDI analysis. This property was similar with lectins from *Arum maculatum* and *Colocasia esculenta* [1].

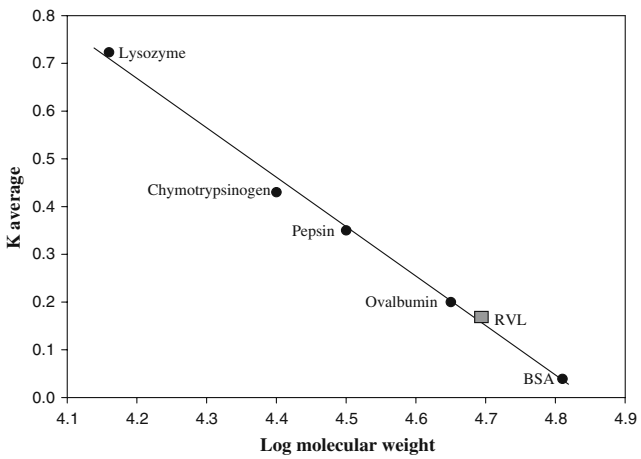

Fig. 3 Calibration curve for the estimation of molecular weight of RVL: Log molecular weight plotted against K average, marker proteins; BSA (66 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa), chymotrypsinogen (25.6 kDa), lysozyme (14.3 kDa)

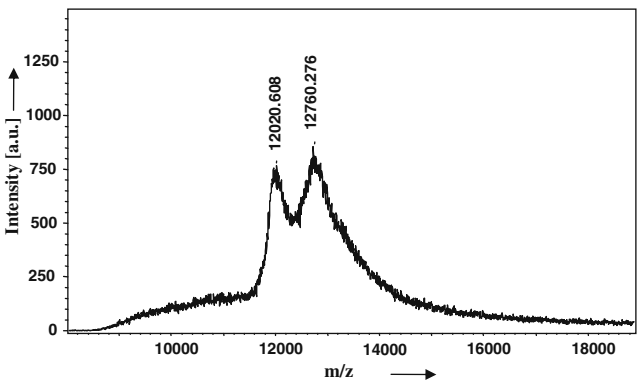

Fig. 4 Molecular weight determination by MALDI analysis showing two prominent peaks at 12.02 and 12.77 kDa respectively

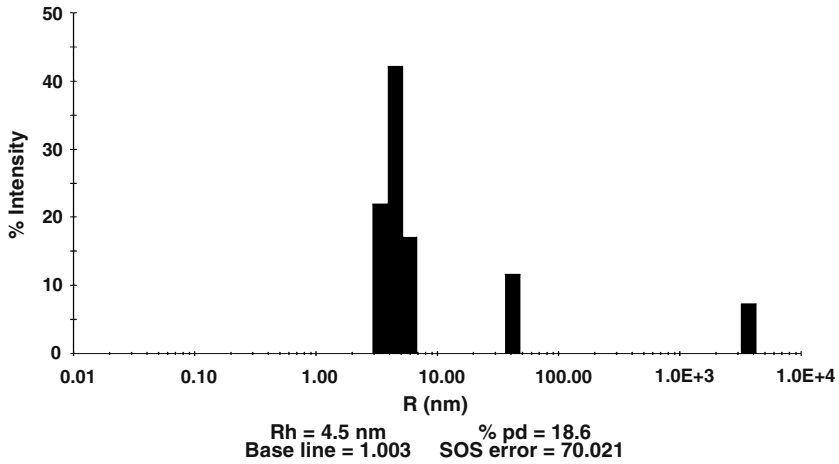

Fig. 5 Hydrodynamic radius of purified RVL determined by dynamic light scattering studies, showing the plot of % intensity *vs.* hydrodynamic radius, using concentration of 0.1 mg/ml of RVL in PBS

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 1 atggccaagc tgctcctctt cctcctcccg gccattctcg gcctactcgt tcctcggtca
    M  A  K   L  L  L  F   L  L  P   A  I  L   G  L  L  V   P  R  S
61 gccgtggcac tgggcaccaa ctacctactg tccggccaaa ccctagacac agaaggccat
    A  V  A ↓ L  G  T  N   Y  L  L   S  G  Q   T  L  D  T   E  G  H
121 ctcaagaacg gcgacttcga cttggtcatg caggatgact gcaacctcgt cctgtacaat
    L  K  N   G  D  F  D   L  V  M   Q  D  D   C  N  L  V   L  Y  N
181 ggcaattggc aatccaacac ggccaacaac ggacgagact gcaagctcac cctgaccgac
    G  N  W   Q  S  N  T   A  N  N   G  R  D   C  K  L  T   L  T  D
241 tacggcgagc tcgtcatcaa aaacggcgac ggatccacgg tgtggaagag cggcgcccag
    Y  G  E   L  V  I  K   N  G  D   G  S  T   V  W  K  S   G  A  Q
301 tccgtcaagg gcaactacgc cgccgtcgtc catccggatg ggaggctggt cgtcttgggc
    S  V  K   G  N  Y  A   A  V  V   H  P  D   G  R  L  V   V  L  G
361 ccatccgtct ttaagattga cccttgggtc cgtggcctga acagcctgcg cttccgcaac
    P  S  V   F  K  I  D   P  W  V   R  G  L   N  S  L  R   F  R ↓ N
421 atccctttca ccaacaactt gctcttctcc ggccaagtcc tctacggcga cggcaggcta
    I  P  F   T  N  N  L   L  F  S   G  Q  V   L  Y  G  D   G  R  L
481 accgcgaaga accatcagct cgtcatgcag ggcgactgca acctggtcct atacggtggc
    T  A  K   N  H  Q  L   V  M  Q   G  D  C   N  L  V  L   Y  G  G
541 aaatacggct ggcagtccaa cacccacggc aacggcgagc actgcttcct caggctgaac
    K  Y  G   W  Q  S  N   T  H  G   N  G  E   H  C  F  L   R  L  N

601 cacaagggcg agctcatcat caaggacgac gacttcaaga ccatatggag cagccgttcc
    H  K  G   E  L  I  I   K  D  D   D  F  K   T  I  W  S   S  R  S
661 agctccaagc agggtgagta cgtcctcatc ctccaggacg acggcttcgg cgtcatctac

    S  S  K   Q  G  E  Y   V  L  I   L  Q  D   D  G  F  G   V  I  Y
721 ggccctgcca tctttgagac cagctcaaag cgctccattg ctgcgtcgtag
    G  P  A   I  F  E  T   S  S  K   R  S  I   A  A  S
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Fig. 6 Full length DNA sequence and deduced amino acid sequence of RVL gene: Start and stop codons are underlined, arrows indicating the cleavage sites and conserved amino acid regions are shown in bold

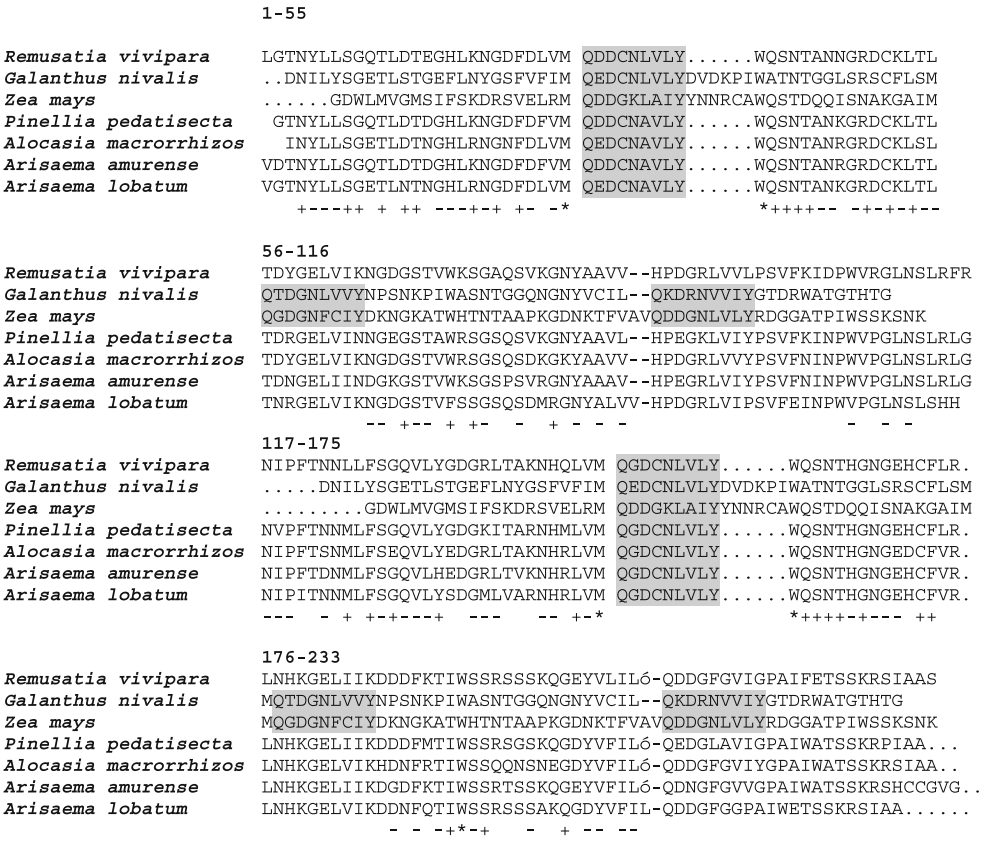

Fig. 7 Predicted amino acid sequence of RVL compared with the sequences of GNA, GNA_{maize}, and the lectins from Araceae family; *P. pedatisecta, A. macrorrhizos, A. amurense, A. lobatum*. Amino acid residues at the carbohydrate binding sites are shown in a grey box. (*) indicate the identical residues in all the seven lectins, (+) the identical residues in six of seven lectins and identical in five are shown by minus (−)

Dynamic light scattering

Dynamic light scattering studies were carried out to understand the oligomeric behaviour of the purified RVL. Hydrodynamic radius (R_H) of RVL obtained by DLS (Fig. 5) was 45 Å and showed 18.6% polydispersity indicating the existence of single dimer species. DLS studies carried out for peanut agglutinin [35] and soyabean lectin [36] also indicated similar properties. From these results, it was concluded that the native RVL is a dimeric molecule without indication for heterogeneity.

Cloning of full length cDNA of RVL and sequence analysis

Contig assembly using sequences of 3′ and 5′ RACE-PCR products gave an assembly of 771 bp coding sequence for RVL and the sequence analysis showed RVL gene was intronless. Hence the full length RVL gene was amplified from the genomic DNA of *R. vivipara* using the specific forward primer sequence RVL_F:5′TTGGCCATGGC-CAAGCTGCTCCTC3′ containing NcoI site and reverse primer sequence RVL_R:5′GGCGAATTCTACGACG-CAGCAA3′containing EcoRI site, which allowed the expression in *E. coli*. From the full length coding sequence of 771 bp, coding for a polypeptide chain corresponding to 256 amino-acids (Fig. 6) accounting to molecular mass of 28.2 kDa. The N-terminal sequence A-LGTNYLLS of RVL determined by Edman sequencing coincided with deduced amino acid sequence of the cDNA between L_{24} to S_{31}, whereas the sequence B-NIPFTNNL coincided between N_{140} to L_{147}. Considering the rules for predicting the signal peptide [37], recombinant RVL has got a signal peptide of

Table 2 Nematode bioassay with RVL; RVL is incubated with *Meloidogyne incognita* at different concentrations with a control (PBS) without lectin

Time in h	% Mortality at different concentrations					
	30 µg/ml	15 µg/ml	10 µg/ml	7.5 µg/ml	6 µg/ml	Control
3	0	0	0	0	0	0
6	6	0	0	0	0	0
12	30	22	18	10	4	0
24	60	56	48	32	22	0
48	88	82	74	58	40	0

23 amino acids accounting for the molecular mass of 2.5 kDa, and has a cleavage site between A_{23} and L_{24} residues. Therefore the lectin polypeptide having 233 residues with molecular mass of 25.7 kDa is derived from the precursor molecule of 256 amino-acid residues. Considering N terminal sequence-B, which aligned from 140–147 residues of the deduced amino-acid sequence, it becomes evident that the lectin polypeptide is synthesized on a single large precursor, which gets cleaved posttranslationally between residues R_{139} and N_{140}. These findings also indicated the presence of two putative domains in RVL, each containing one mannose binding site as reported for *Arum maculatum* lectin, *Colocasia esculanta* lectin, *Xanthosoma sagittifolium* lectin [1], *Pinellia pedatisecta* lectin [38–40] and *Typhonium divaricatum* lectin [22], all of them are defined as monocot mannose-binding lectins from Araceae family.

Mass spectrometric data of RVL showed two consistent peaks of masses 12.020 kDa and 12.760 kDa. Calculated mass of 12.715 kDa for the polypeptide chain of 116 amino acids (24–139) is in agreement with MALDI mass of 12.760 kDa. However, the mass of 13.00 kDa calculated for the polypeptide chain of 117 residues (140–256) did not agree with the MALDI estimate of 12.02 kDa. This discrepancy could be attributed to posttranslational processing of propeptide on the C-terminal side of the polypeptide chain (140–256) as suggested for other GNA related lectins by Van Damme *et al*., [13].

Data base retrieval with BLASTp (NCBI-blast) showed very high homology with many of the reported monocot mannose-binding lectins. Multiple sequence alignment of the deduced amino acid sequence of RVL is compared with sequences of lectins from *G. nivalis*, *Z. mays*, *P. pedatisecta*, *A. macrorrhizos*, *A. amurense* and *A. lobatum* (Fig. 7). It is evident from the sequences RVL showed 80–90% homology with several reported monocot mannose-binding lectins of Araceae family. However, the extent of sequence homology of RVL with GNA and GNA $_{\text{maize}}$ is only 41% and 38% respectively. Indeed RVL shared a common conserved motif of QXDXNXVXY at the carbohydrate binding site. Any changes occurring due to insertion or deletion of key amino acids in this conserved motif is determinant in conferring exclusive specificity for these lectins towards high mannose N-glycans [1, 41]. Thus RVL is closely related to other monocot mannose binding lectins of Araceae family, but is different from GNA with respect to mannose binding property and the number of binding domains.

Nematicidal activity of lectin and interaction of lectin with *M. incognita*

Nematicidal activity of purified RVL at different concentrations against *Meloidogyne incognita* a root knot nematode was evaluated and the results of survival of the nematodes as function of time are presented in Table 2.

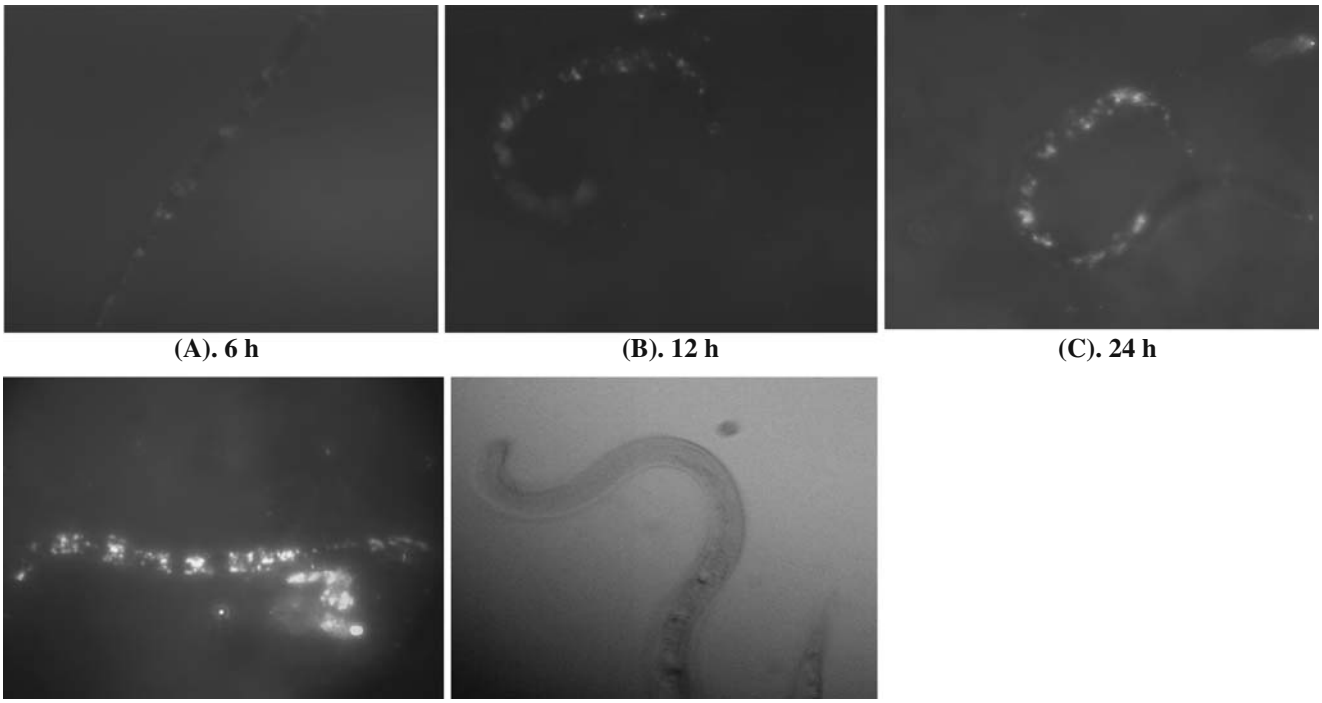

Fig. 8 Binding of FITC-RVL to the alimentary tract of *Meloidogyne incognita* and inhibition of binding in the presence of mucin (**e**). Nematodes were suspended in FITC-RVL (30 μg/ml) solution and incubated at room temperature; binding of the lectin was observed under the fluorescent microscope after 6 h, 12 h, 24 h and 48 h (**a**–**d**)

These results were the average values of triplicate sets; each contained 50 juveniles of second stage. RVL showed concentration dependent toxicity on *M. incognita*, even at a concentration of 7.5 µg/ml more than 50% mortality was observed after 48 h. Highest mortality greater than 80% was found at concentrations beyond 15 µg/ml demonstrating the potential nematicidal activity of RVL. In order to get an insight to the mode of the RVL toxicity effect on *M. incognita*, we determined the binding of RVL after incubating the nematodes in FITC-RVL solution. Binding of RVL in nematodes was observed by fluorescent microscopy at different intervals of time (Fig. 8 a–d). Lectin binding was found at a specific region mostly in the mid portion of the alimentary-tract, without any binding either at anterior or posterior regions of the gut. Binding was not observed in nematode gut incubated with FITC-RVL complexed with mucin (Fig. 8 e), which confirmed carbohydrate receptor mediated binding of RVL on the gut epithelial cells. The binding of RVL in the mid gut of the alimentary-tract was clearly established from the 3-D images acquired on fluorescent confocal microscopy (Fig. 9). Although nematicidal properties of few lectins are reported, there are no direct evidences available for the mechanisms of their toxic effects. In contrast to our present findings of RVL gut binding, earlier reports so far described showed cuticular binding of lectins specifically to the head region, and to the pores of excretion and reproduction of different nematodes by plant lectins [42–49]. Considering the cuticular surface binding, it is argued that the lectin binding inhibits the chemoreception of host signals disturbing the localization of hosts by nematodes. Whereas our results, for the first time demonstrated that the lectin ingested by the nematodes specifically interacts across the gut lining and the binding increased proportionally with time leading to death of the nematode in 48 h. However some lectins having insecticidal properties are known to bind to the midgut epithelium of insects causing disruption of microvilli leading to abnormalities in these epithelial cells [50, 51]. It is not clear from our findings how the observed lectin binding results in the killing of the nematode, it may be probably mediated by a complex processes involving signal transduction or by causing abnormalities in the epithelial cells as reported in insects.

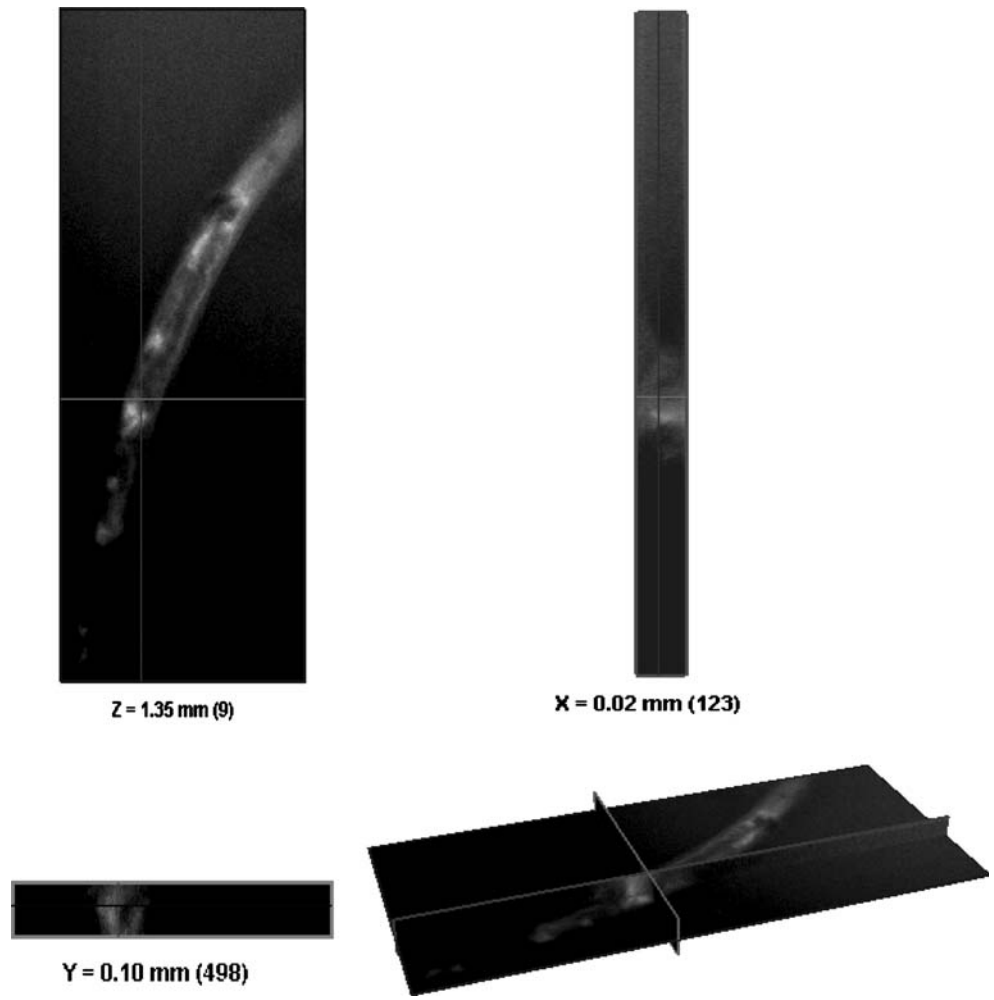

Fig. 9 Three dimensional images of binding of FITC-RVL to the alimentary tract of *Meloidogyne incognita*. Nematodes were suspended in FITC-RVL (30 µg/ml) solution and incubated at room temperature; 3D-image was observed under confocal microscope after 48 h

Acknowledgement Authors thank Dept. of Science and Technology (DST), Government. of India for the financial support.

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