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**Affinity Purification, Physico-chemical Characterization and Primary
Structure of an α -D-galactose Specific Jacalin-related Lectin from the
Latex of Mulberry (*Morus indica*)**

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Abstract

An α -D-galactose specific lectin belonging to the family of jacalin-related lectins (JRL) has been purified by affinity chromatography on cross-linked guar-gum. NanoESI Q-ToF mass spectrometric studies revealed that the protein has two chains like all the members of gJRL; the heavier chain consists of 133 amino acids and the lighter chain consists of 20-21 amino acids. The heavier chain is glycosylated on Asn21 with two types of oligosaccharide chains; one of the glycans is pauci-mannose type GlcNAc₂(Fuc)Man₃(Xyl) and the other one is complex type GlcNAc₂(Fuc)Man₃(Xyl)GlcNAc(Fuc)Gal. Circular dichroism spectroscopy indicated that the secondary structure of the lectin is made up of predominantly β -sheets, whereas the thermal denaturation temperature was found to be 76.6 °C by differential scanning calorimetry. MTT cell viability assays on MCF-7 and MDCK cells showed that the lectin is highly cytotoxic towards both the cell lines when dosed at micromolar concentrations, suggesting that it may play a role in the defence mechanism of the plant.

Introduction

Lectins are a special class of proteins which recognize mono or oligosaccharides in a highly specific way using various non-covalent interactions but are devoid of catalytic activity [Lis and Sharon, 1973]. These proteins are abundantly found in nature and show significant diversity in their structures as well as in carbohydrate specificity. Although in animals lectins are known to participate in many cellular communication processes [Kilpatrick, 2002] their functions in plants are poorly understood. Some lectins are known to be involved in the defence mechanism of plants most likely by interacting with the cell-surface carbohydrate structures of fungi or insect herbivores [Peumans and Van Damme, 1995], whereas some others accumulate in the vegetative storage organs e.g. seeds, bulb or bark and have been proposed to function as storage proteins [Van Damme et al., 2002]. Nevertheless, recognition

of a vast array of carbohydrate structures by these lectins and their abundance and ease of purification have made them important tools in the field of glycobiology [Wu et al., 2009; Ambrosi et al., 2005].

Jacalin-related lectins (JRLs) are one of the families of plant lectins which show a characteristic subunit fold, known as type-1 β -prism fold. This type of subunit fold has three four-stranded β -sheets parallel to each other arranged in a prism [Sankaranarayanan et al., 1996]. There can be two types of JRLs based on the difference in carbohydrate specificity; they are either galactose-specific (gJRLs) or mannose-specific (mJRLs). Both mJRLs and gJRLs exhibit similar subunit folds and share high sequence identity but differ from each other in their structures of protomers and subcellular localization apart from carbohydrate specificity [Raval et al., 2004]. This difference originates due to different post-translational modifications. While gJRLs are two-chained protomers located in the cytoplasm, mJRLs are vacuolar proteins containing single-chain protomers [Van Damme et al., 2002]. gJRLs show a number of interesting properties owing to their carbohydrate specificity. Specific binding to the α -anomeric form of the tumour associated T-antigenic disaccharide, Gal β 1-3GalNAc is one of them [Mahanta et al., 1990]. The Thomsen-Friedenreich antigen (T-antigen) is expressed on the outer cell surfaces of most human carcinomas [Glinsky et al., 2001]. This explains the potential of gJRLs as targeting agents in the detection and development of therapeutics for the treatment of cancer. Apart from that, jacalin the first gJRL identified and the crystal structure of which was resolved first is known to have other interesting properties as well. For instances, it binds to immunoglobulin A1 selectively in human serum, stimulates T-cell lymphocytes and also binds to a region of HIV virus [Kabir, 1998]. This particular family of plant lectins is known to a lesser extent compared to other families. Over the years the number of known JRLs has increased, but very few of them are characterized well regarding their structures, carbohydrate specificity and prospective biological function.

Therefore, in order to shed light on all these aspects it is important to purify and characterize new JRLs.

The present study was carried out to purify a gJRL from mulberry (*Morus indica*) latex which we shall henceforth refer to as MLGL (Mulberry Latex Galactose-specific Lectin) by affinity chromatography and to investigate its structural properties using high resolution mass spectrometry, circular dichroism spectroscopy and differential scanning calorimetry. The carbohydrate specificity of the lectin was determined by performing hemagglutination-inhibition assays. The cytotoxic effect of the lectin was assessed towards MCF-7 and MDCK cells in order to find out its plausible function in the latex.

Results

Purification and carbohydrate specificity

MLGL was purified by a simple one-step affinity chromatographic method. Crude latex was collected in ice-cold 10 mM Tris-HCl (pH 8.0) buffer containing 0.15 M NaCl (TBS), frozen at -20° C for 24 h and then thawed at room temperature. The sample was subjected to centrifugation and the supernatant was collected and loaded onto a guar-gum column, washed extensively with TBS and then the lectin was eluted with 0.2 M galactose. Approximately 2 mg of protein was obtained from a 60 mg of crude soluble protein fraction (Fig. S1, chromatogram). SDS-PAGE of MLGL revealed a single band at ~ 16 kDa both under reducing (β -mercaptoethanol was employed as reducing agent) and non-reducing conditions (Fig. 1) indicating that no intermolecular disulfide linkages are present in the protein. In gel filtration experiment the elution volume of native MLGL was comparable to that of bovine serum albumin ($M_r \sim 66$ kDa), suggesting that the lectin is most likely a homotetramer. This is consistent with the observation that jacalin and MornigaG, two other members of the gJRL

family that exhibit high sequence homology with MLGL (see below) are also homotetramers..

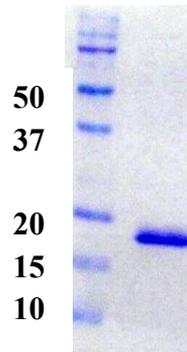


Figure 1: SDS-PAGE under reducing condition. Lane 1: molecular weight marker; lane 2: MLGL purified by guar-gum column.

The isoelectric point (IEP) of MLGL was determined as 4.35 ± 0.07 by measurement of the electrophoretic mobility of the protein evaluating the zeta potential at different pH values (cf. Fig. 2).

MLGL could agglutinate human ABO (+) and rabbit erythrocytes with equal efficiency up to concentrations as low as 625 ng/mL. Galactose was a poor inhibitor of the hemagglutination activity of MLGL, whereas methyl- α -D-galactopyranoside (Me α Gal, 31 mM) could readily inhibit the activity of MLGL. In contrast, methyl- β -D-galactopyranoside and lactose could not inhibit the hemagglutination activity of MLGL even at 250 mM and 125 mM, respectively, suggesting that the lectin does not recognize the β -anomer of galactose. The preferred stereospecific recognition of galactose in the α -anomeric configuration is further corroborated by the finding that other α -D-galactose derivatives were also able to inhibit the hemagglutination activity of MLGL as shown in Table 1.

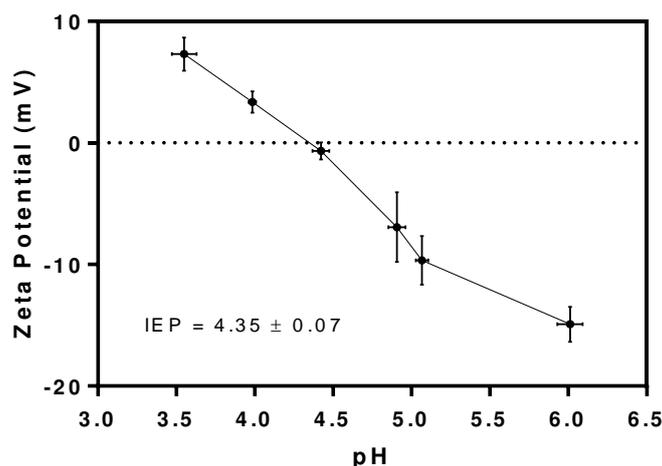


Figure 2: Determination of isoelectric point (IEP) of MLGL (0.1 mg/ml in PBS) using zeta potential measurements (titrated with 0.1 M HCl from pH- 6.0 to 3.0 with pH change steps of 0.5 ± 0.2 at 25 °C).

Table 1: Inhibition of hemagglutination activity of MLGL by various sugars. Final concentration of MLGL used was 4 μ g/ml.

Sugar ¹	Minimum Inhibitory Concentration (mM)
4-Methyl-umbellyeryl- α -D galactopyranoside	0.045
4-Nitrophenyl- α -D-galactopyranoside	1.2
Methyl- α -D-galactopyranoside	8
Melibiose	16
N-Acetyl-D-galactosamine	32
Galactose	63

¹The following sugars did not inhibit the activity of MLG up to the resultant concentrations indicated in the parentheses: Glucose, mannose, galactose, fructose, sucrose, lactulose, fucose, 2-deoxy-galactose, N-acetyl-glucosamine, methyl- α -D-glucopyranoside, methyl- α -D-mannopyranoside, raffinose (all 250 mM), lactose (125 mM).

Primary structure

The mass spectrum of MLGL obtained under denaturing nanoelectrospray ionization (nanoESI) conditions (50% acetonitrile and 2% formic acid) indicates the presence of two smaller components with molecular masses of ~2163 Da and ~2475 Da and a larger one with ~16 kDa (Fig. 6, *vide infra*). This observation along with the results of *de novo* sequencing suggests that MLGL comprises two non-covalently linked components, *viz.* one large polypeptide chain of ~16 kDa and a smaller subunit. *De novo* sequencing of peptides obtained from *in-solution* as well as *in-gel* proteolysis by use of various proteolytic enzymes (trypsin, chymotrypsin, elastase, thermolysin, endoproteinase Glu-C) yielded the complete amino acid sequence of MLGL. Each amino acid has been determined from at least two independent proteolytic peptides. Fragmentation of the doubly charged tryptic precursor ion at m/z 679.32⁺² revealed the N-terminal sequence of the heavier chain of MLGL shown as a representative example in Supplementary Fig. S2. The primary structures of the small subunit was derived by evaluation of the CID spectrum (Fig. 3) obtained from the doubly charged precursor ions at m/z 1082.04+2 (calc. 1082.04+2) representing the most abundant ionic species (*cf.* Fig. 6, *vide infra*).

Additionally, an isoform of the smaller subunit elongated by three amino acids gave rise to signals of low intensity. However, collisional activation allowed for identification of the first 3 N-terminal amino acids (*cf.* Fig. 4). The finally obtained sequences for both chains of MLGL and their alignment with two other jacalin-related lectins *viz.* MornigaG and jacalin is shown in Fig. 4. Evaluation of peptide sequences revealed some microheterogeneity present in MLGL as a result of some amino acid substitutions (*cf.* Fig. 4).

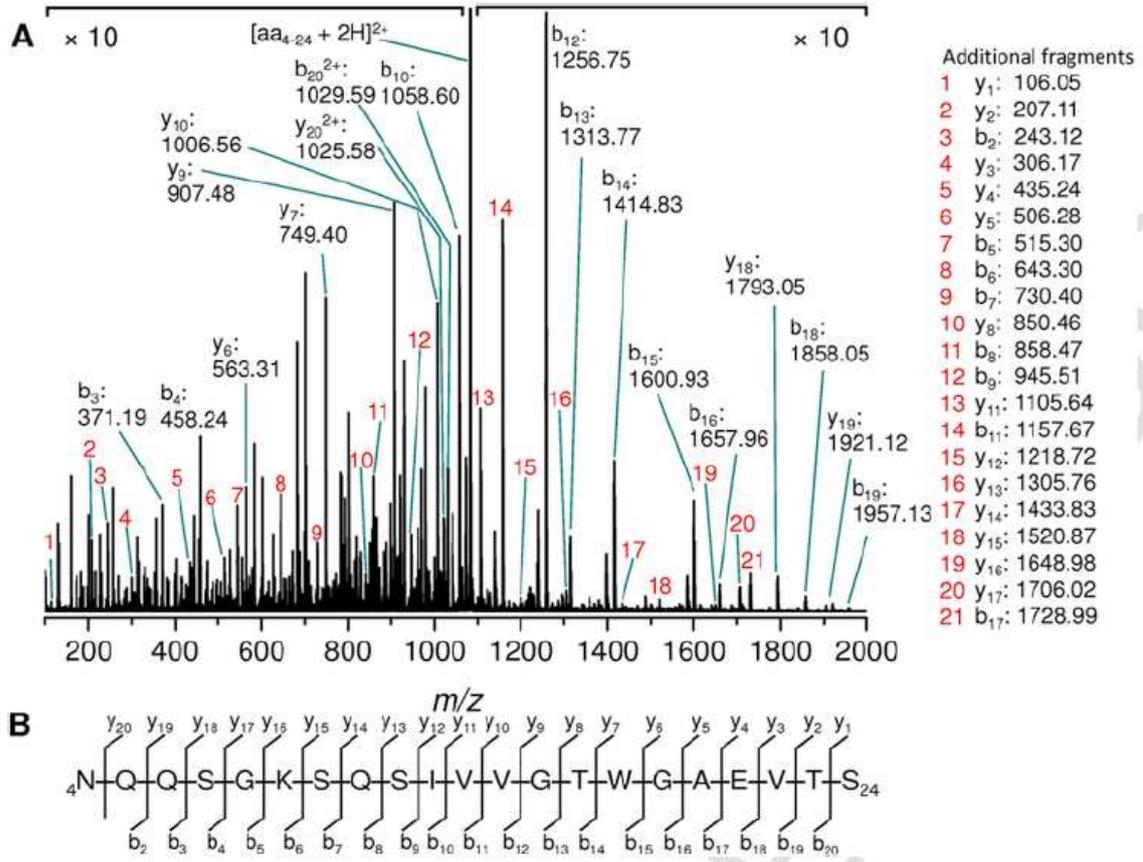


Figure 3: (A) NanoESI Q-TOF fragment ion spectrum obtained from the doubly charged peptide precursor ions at m/z 1082.04+2. The analyte comprises amino acids 4–24 of the smaller subunit of MLGL. **(B)** Corresponding fragmentation scheme.

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1JAC_A ----- 0
mlgl ----- 0
Q8LGR4 MASSSFLSLSFLVLLFSISSANTRKWSLSNGLDQKPISIIIEAAIGVSEDLLNNGMEAKN 60
Q8LLC9 MASSSFLSLSFLILLFSISSANTRKWSLSNGLDQKPISIIIEAAIGVSEDLLNNGMEAKN 60

1JAC_A -----GKAFDDGAFTGIREINLSYNKETAIGDFQVVYDLNGS 37
mlgl -----GVAFDGGSYTGIREINFEYNNETALGSLQVTYDVDGT 37
Q8LGR4 NQQSGKSQTIIVVGTWGAQVTSSNGVAFDDGAYTGIREINFEYNNETAIGSIQVTYDVNGT 120
Q8LLC9 NQQSGKSQTIIVVGTWGAQVTSSNGVAFDDGAYTGIREINFEYNNETAIGSIQVTYDVNGT 120
          *  *****:*****:.*:*:*:*:*:*:*:*:*:*:*:

1JAC_A PYVGQNHKSFITGFTPVKISLDFPSEYIMEVSGYTGNSVSGYVVVRSLSLTFKTNKKTYPYG 97
mlgl PFEAKKHASFLTGFTPVKLSLDFPSEYLVESVSGYTGKVSGLVVRSLTFKTDKETYPYG 97
Q8LGR4 PFEAKKHASFIKGFQVKISLDFPNEYIVEVSGYTGKLSGYTVVRSLSLTFKTNKETYPYG 180
Q8LLC9 PFEAKKHASFITGFTQVKIGLDFPSEYIVEVSGYTGKVSGLTVRSLTFKTNKETYPYG 180
          *: .:* **:.*** **:.*****.**:*****:*** :*****.*:******

1JAC_A VTSGTPFNLPIENGLIVGFKGSIQYWLDFSMYLSL 133
mlgl VTSGTYFKLPLQDGLIVGFKGSVGYWLDYLGFLHLSL 133
Q8LGR4 VTSGTHFKLPIQNGLIVGFKGSVGYWLDYIGFLHLSL 216
Q8LLC9 VTSDTHFKLPIQNGLIVGFKGSVGYWLDYIGFLHLSL 216
          *** * *:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*

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Figure 4: Amino acid sequence of the heavier polypeptide chain of MLGL derived from mass spectrometry and its alignment with the known jacalin related lectins, mornigaG (accession number-Q8LGR4 and Q8LLC9) and jacalin (accession number – JAC1_A). Sequence alignment was established using the online software Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clusalo/>)

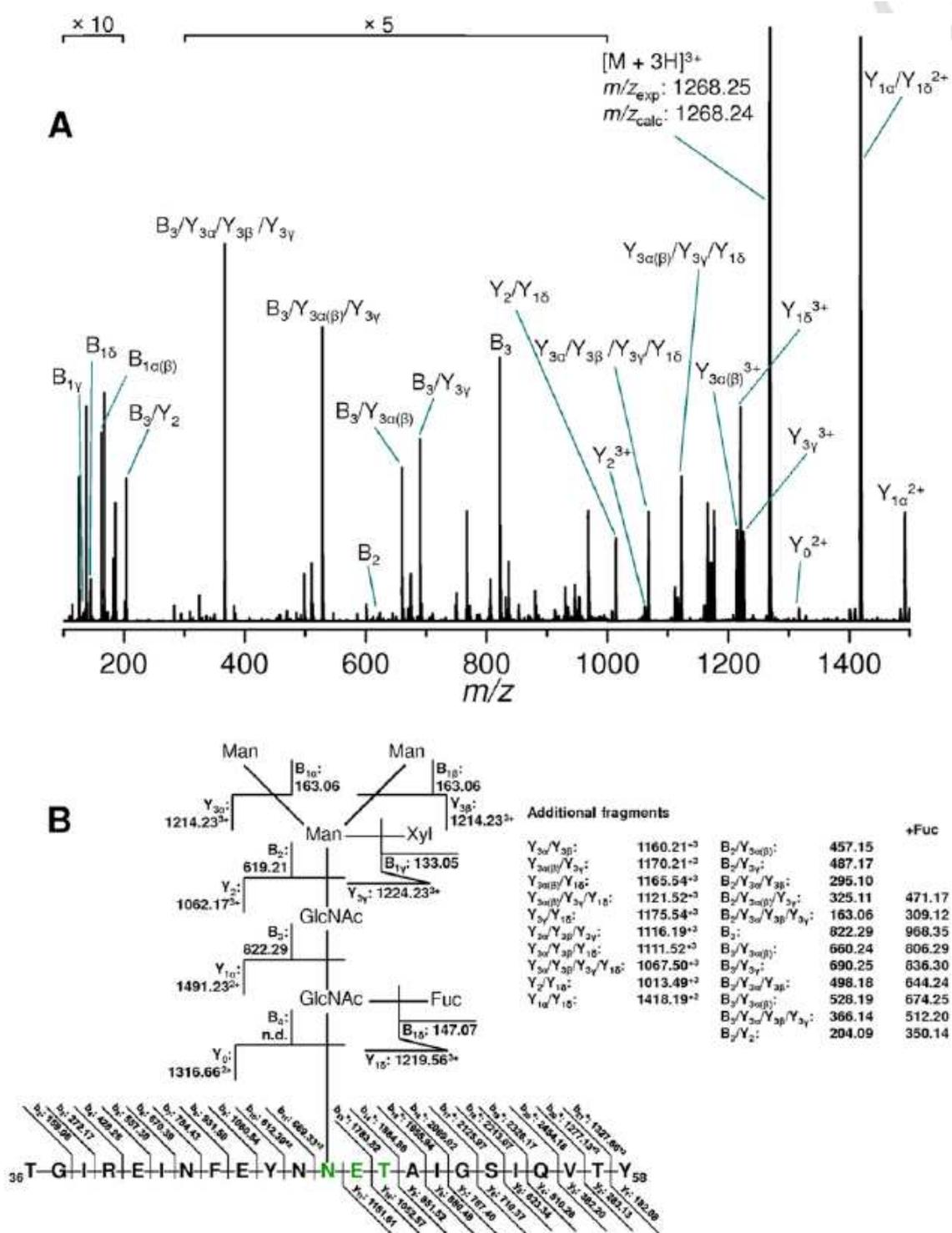
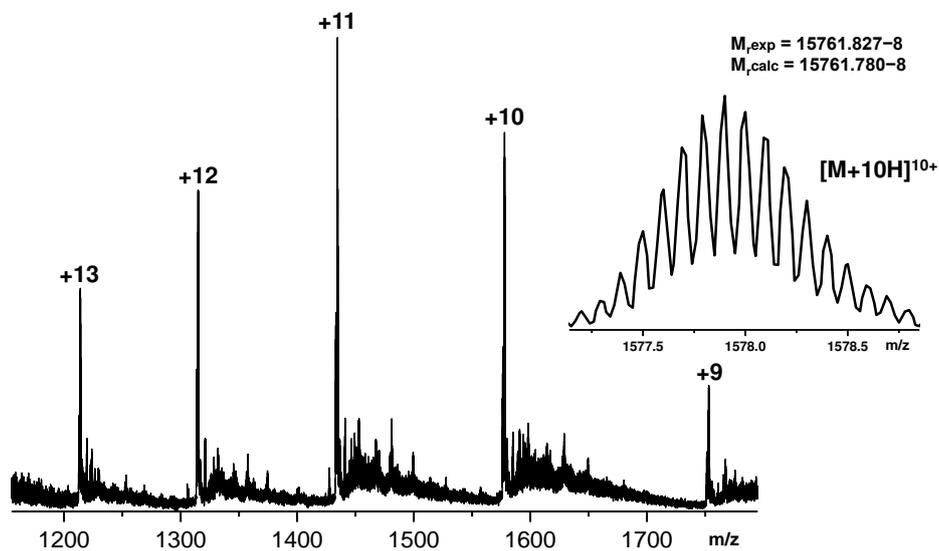


Figure 5: (A) NanoESI Q-TOF fragment ion spectrum obtained from a CID experiment on the triply charged precursor glycopeptide ions with m/z 1268.25⁺³ derived from a chymotryptic in-solution digest. (B) Corresponding fragmentation scheme showing paucimannose type N-glycan and the peptide backbone.

Apart from the products of proteolysis, the CID experiments performed on intact species of MLGL also helped in deducing the amino acid sequence. The exact mass of each subunit of MLGL was also determined from the MS experiments performed on the intact species of MLGL. These experiments revealed that MLGL consists of two polypeptide chains in its single subunit. The smaller chain exhibits some variety in the length as well as in the sequence. However, the most abundant species is NQQSGKSQSIVVGTWGAEVTS which is derived from the doubly charged peptide ion at m/z 1082.037⁺². The larger polypeptide chain is glycosylated and can have a few variants in its sequence.



1 **G**VAFDDG **S**YTGLREINF **E**YNNETAIGS **I**QVTYD**V**DT **P**F**E**AKK**H**ASF **L**T**G**F**T**PVKLS
LDFPSEYLVE **V**SGY**T**GKVSG **Y**LVVRS**L**TFK **T**N**K**ET**Y**GPY**G** **V**T**S**G**T**H**F**KL**P**
LQ**D**GLIV**G**FK **G**SVGY**W**LD**Y**L **G**F**H**L**A**L₁₃₃

Figure 6: Mass spectrum of multiply charged peptide ions obtained from the intact species of MLGL. The most abundant species fits perfectly to the sequence depicted in the figure carrying a GlcNAc₂(Fuc)Man₃(Xyl)glycan ($\Delta=3.0$ ppm)

Elucidation of N-glycosylation

To this end, N-glycopeptide ions derived from in-solution proteolytic digests were subjected to CID analysis and their glycan structure as well as their respective glycosylation site were deduced from the observed fragmentation pattern. As a representative example, Fig. 5A depicts the fragment ion spectrum of CID experiments on triply charged N-glycopeptide precursor ions with m/z 1268.25+3 obtained from an in-solution chymotryptic digest. The spectrum reveals the typical fragmentation pattern of a pauci-mannose type N-glycan [25]. Neutral loss of either a pentose (xylose) and/or a deoxyhexose (fucose) from the intact precursor ions as well as from fragment ions arising from subsequent cleavage of two hexoses (mannoses) was observed. Elimination of a third hexose from the $Y3\alpha/Y3\beta$ fragment ions is not observed, in fact this fragmentation process is preceded by loss of a pentose. This observation clearly pinpoints the presence of a xylose linked to the innermost mannose. Likewise, cleavage of the proximal N-acetylhexosamine (N-acetylglucosamine, GlcNAc) giving rise to Y0 fragment ions appears solely following the elimination of the deoxyhexose thus substantiating the expected core fucosylation. Besides characterization of the glycan structure the detection of an almost complete b-type fragment ion series along with several y-type ions derived from the peptide backbone allowed deduction of the amino acid sequence ${}_{36}\text{TGIREINF EYNN}_{47}\text{ETAIGSIQV TY}_{58}$ proving Asn₄₇ to be glycosylated (Fig. 5B). An additional glycopeptide species giving rise to triply charged ions at m/z 1438.61⁺³ was shown to harbor the corresponding complex-type congener attached to the same peptide (cf. Supplementary Fig. S3). In addition to proven glycosylation at Asn₄₇, less abundant peptides harboring another potential N-glycosylation site at aa-position 61 have been identified. However, no evidence for a modification by glycan attachment has been found. Furthermore, sequencing of most peptide ions comprising the respective peptide stretch revealed a deamidation of Asn₆₁ due to the vicinity to Gly.

Besides information on the lighter chain of MLGL, molecular weight (M_r) of the intact larger polypeptide glycoforms was obtained from the high resolution mass spectrum of purified MLGL ionized under denaturing nanoESI conditions (Fig. 6). M_r was determined by mass deconvolution of the centroid distribution in a nearly Gaussian shaped envelope of isotopically resolved multiprotonated species (cf. Fig. 6 insets B and C). The resulting values are in excellent agreement with the theoretical values calculated from the amino acid sequence displayed in Fig. 4 assuming post-translational modification by either a paucimannose (GlcNAc₂(Fuc)Man₃(Xyl)) or a complex-type (GlcNAc₂(Fuc)Man₃(Xyl)GlcNAc(Fuc)Gal) glycan.

Secondary structure

The far-UV CD spectrum of the native protein (Figure 7) shows a negative peak around 220 nm and a positive peak around 200 nm indicating high β -sheet content and low α -helical content in the secondary structure of the protein. This interpretation was further confirmed by analysing the CD spectral data on the online software Dichroweb (www.cryst.bbk.ac.uk/cdweb/html/) using the programme CDSSTR. The content of various secondary structural elements in MIL were obtained by this analysis as: 2% α -helix (0% regular and 2% distorted), 34% β -sheet (21% regular and 13% distorted), 23% β -turns and 39% unordered structures. The calculated fit obtained from this analysis is also shown in Figure 7 (dotted line), from which it can be seen that the calculated fit is in good agreement with the experimentally obtained CD spectrum. The far-UV CD spectrum in presence of 20 mM Me α Gal showed only marginal changes indicating that sugar binding does not significantly alter the secondary structure of the protein (Figure 7).

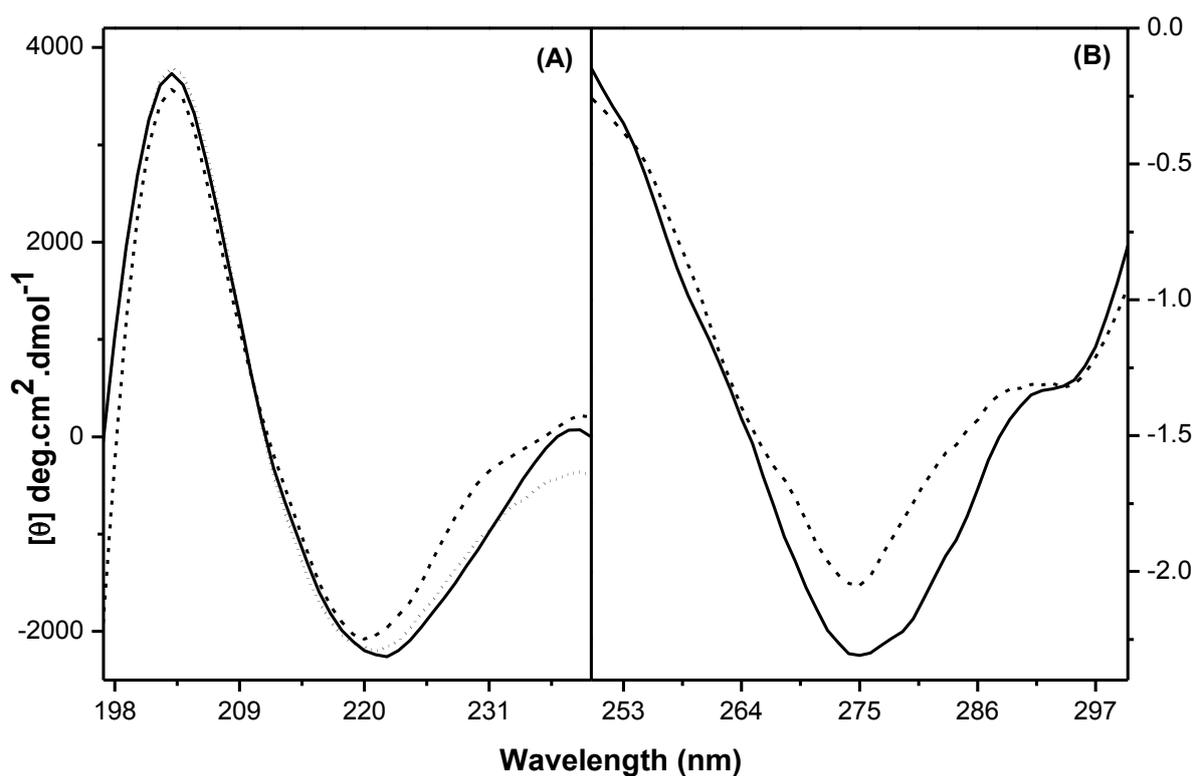


Figure 7: CD spectra of MLGL and analysis by CDSSTR method in the (A) far UV region and (B) near UV region are shown. Solid lines (-) represent experimental spectra, dotted line (...) represents the calculated fit and dashed lines (- - -) represent spectra obtained in presence of 20 mM Me α Gal.

The near-UV CD spectrum of MLGL is characterized by a minimum at ca. 275 nm and a shoulder at 294 nm (Figure 6). In presence of 20 mM Me α Gal, the CD signal decreases a little around 275 nm, but around 294 nm it retained its intensity as the native protein.

Thermal stability

Thermal stability of MLGL was investigated as a function of pH by differential scanning calorimetry at a scan rate of 60 °C /hour. A relatively higher scan rate was used in order to avoid sample precipitation after transition temperature, which would preclude accurate

determination of the transition enthalpy. At pH 7.4, the transition temperature of MLGL was observed to be 77.6 °C and the thermogram obtained could be fit to a non-two state model containing a single peak as shown in Figure 8 (thermogram 1). The occurrence of a single peak implies that the protein undergoes denaturation in a single step from its tetrameric form to completely unfolded form. DSC of MLGL in presence of 100 mM Me α Gal resulted in an increased thermal stability with an unfolding temperature at 79.1 °C (Figure 8, thermogram 2).

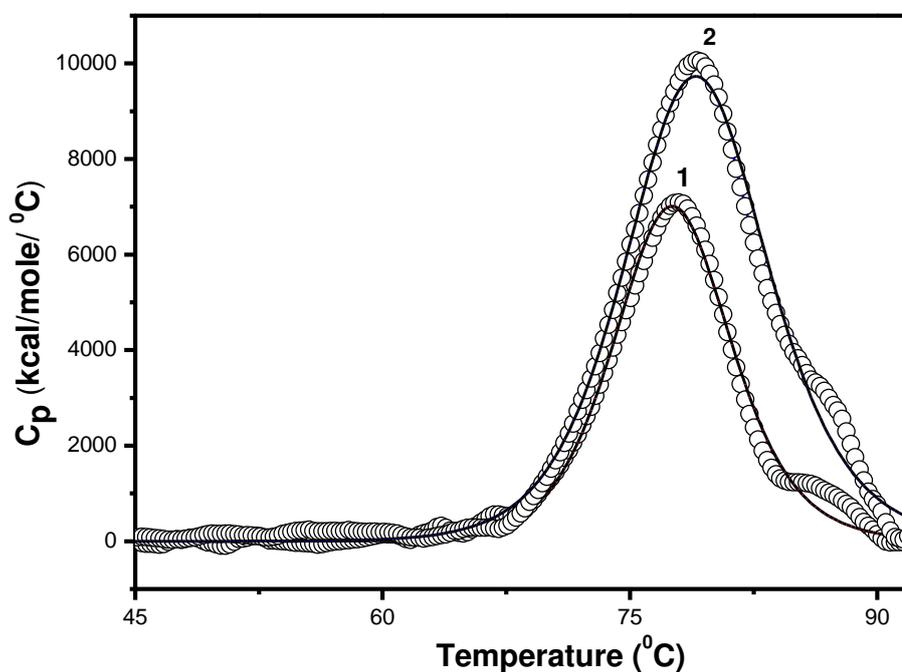


Figure 8: DSC thermograms of MLGL in the absence and in presence of 100 mM methyl- α -D-galactopyranoside. Thermogram 1 corresponds to MLGL alone and thermogram 2 corresponds to MLGL + Me α Gal. Data points are shown as open circles and the solid bold line represents the best fit of DSC data to a non-two state transition model. Concentration of MLGL was 31.2 μ M.

Cytotoxicity

Cytotoxicity of MLGL towards two different cell lines, namely MDCK and MCF-7 cells, was evaluated colorimetrically by measuring the colour intensity formed due to the reduction of MTT to purple-coloured formazan by the cellular oxidoreductase enzymes. Two different concentrations (1.25 and 0.625 μM) of the lectins were used. Triton X100 (10% w/v) was used as the positive control. In addition to MLGL, the cytotoxicity of two well-known lectins, WGA and jacalin, was also evaluated to compare their effect with that of MLGL on the two different cell lines.

Wheat germ agglutinin (WGA) is an N-acetyl-D-glucosamine and sialic acid specific lectin which is known for its strong binding ability to cell surface glycoproteins and hence it is used as a molecular probe in conjugation with a fluorophore in fluorescence microscopy [Strathmann et al., 2002; Kostrominova, 2011]. Jacalin, on the other hand, is a gJRL which can be expected to have similar properties as MLGL. The cytotoxic effect on both cell lines was determined after 72 h of incubation and the relative cell viabilities are shown in Figure 9.

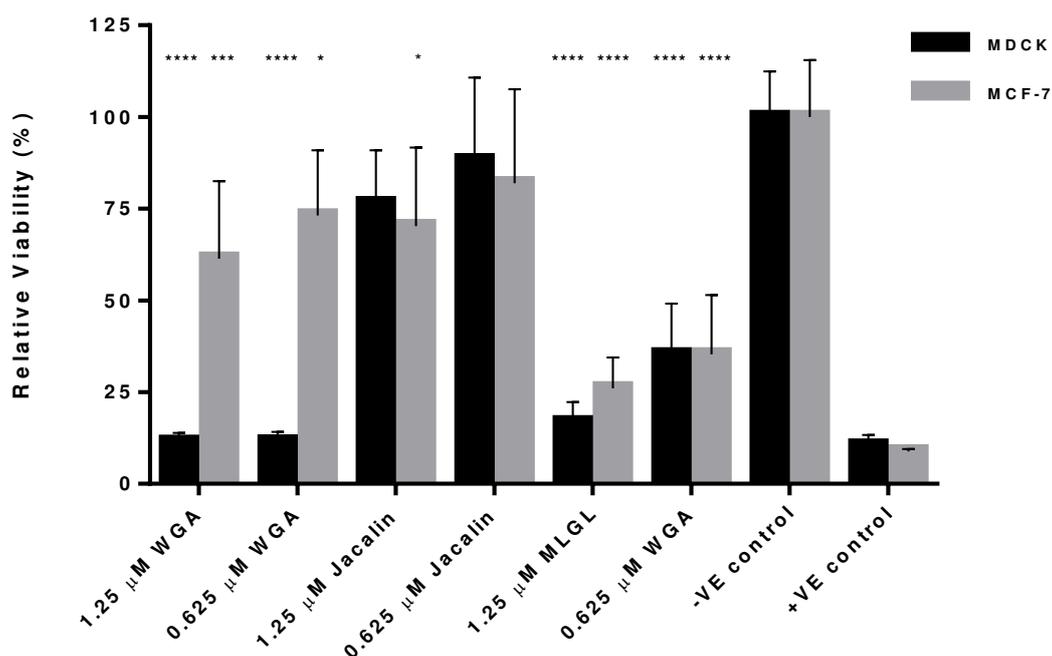


Figure 9: Relative viability of MDCK and MCF7 cells after treatment for 72 hours with two different concentrations of MLGL, WGA and jacalin as determined by the MTT assay (n = 3). WGA (Wheat germ agglutinin) and jacalin have been used as control lectins. Statistical significance: Kruskal-Wallis test (* p < 0.05, *** p < 0.001, **** p < 0.0001). In each case, the difference in relative viability has been compared with the untreated control (negative control).

From the plot, it is clear that after treatment with the lower concentration (0.625 μ M) of MLGL the viability for both cell lines reached ~35%, whereas the greater dose reduced the viability for MDCK cells even more (~17% viability) than for MCF-7 cells (~26% viability). In both cases, the differences are statistically significant compared to the untreated control (p < 0.0001, Kruskal-Wallis test). The effect of WGA on the cell viability of MDCK cells is very pronounced (~ 12% viability) at both concentrations (p < 0.0001, Kruskal-Wallis test), whereas for MCF-7 cells this effect is much less marked (~ 50% viability) (p < 0.001 for 1.25 μ M WGA and p < 0.05 for 0.625 μ M WGA). In case of jacalin, by contrast, only a slight reduction in relative cell viability was observed on the two cell lines at both tested concentrations. A significant reduction (~ 75% viability) though, was observed in MCF-7 cells at the highest tested dose of the lectin (p < 0.05 for 1.25 μ M).

Discussion

The unique ability of lectins to recognize specific carbohydrates in a noncatalytic manner is consistent with their involvement in many biological phenomena that are mediated by protein-carbohydrate interactions [Lis and Sharon, 1986]. In this study, an α -D-galactose specific lectin has been purified to homogeneity from the latex of mulberry (*Morus indica*). The lectin has an isoelectric point of 4.35, implying that at physiological pH MLGL exists as

a negatively charged entity. Hemagglutination-inhibition assays performed with different sugars showed that the lectin has rather weak affinity towards simple galactose, but its affinity towards methyl- α -D-galactopyranoside is very high. This observation is similar to jacalin which shows a binding affinity almost 50 times higher for Me α Gal than simple galactose due to formation of favourable interactions between the methyl group and a tyrosine residue in the binding site of the lectin [Sankaranarayanan et al., 1996].

De novo sequencing of the lectin yielded a sequence which exhibits high homology to mornigaG, a well-characterized lectin, isolated from the bark of black mulberry (*Morus nigra*). In mulberry bark, the lectin (mornigaG) remains as a vacuolar storage protein which helps in the growth and development of the plant. Homology search on NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) has shown that the leaves of white mulberry (*Morus alba*) also expresses similar type of lectin (accession number - AEE92792). MLGL contains two polypeptide chains, the larger one consisting of 133 amino acids and the smaller one has 20 to 21 amino acids. Crystal structure analysis of two gJRLs, jacalin and *Maclura pomifera* lectin (MPA) has shown that the smaller chain of a gJRL does not have any contribution to the ligand binding [Lee et al., 1998; Sankaranarayan et al., 1996], but the N-terminal glycine of the larger chain is important for it. Here it is worth mentioning that although initially we thought the lectin purified by us is the same protein purified previously from the latex of *Morus indica* (*Morus indica* lectin, MIL) using a combination of ammonium sulphate precipitation and conventional chromatographic methods [Patel et al., 2011]. However, comparison of the partial sequence information obtained for MIL showed significant differences with the sequence of MLGL. It was observed that in the two peptides of MIL that were sequenced covering 29 amino acid residues there were 9 differences with respect to MLGL, suggesting that MLGL is a different protein from MIL. Further the sequence comparison shown in Figure 3 clearly indicates that MLGL resembles the bark lectin,

mornigaG, in its amino acid sequence although it differs slightly from MornigaG in terms of carbohydrate specificity. MornigaG was shown to bind D-galactose and Me α Gal with similar affinity from hapten inhibition assays [Rouge et al., 2003]. Thus MLGL shows exclusive preference for the α -anomer of galactose and hence it can be used for the selective detection of this sugar.

MLGL is a glycoprotein and contains two types of glycan structures; pauci-mannose type and complex type. The site of glycosylation for these two types of glycans is the same residue (N-21) which indicates the presence of heterogeneity in the protein in terms of its carbohydrate structures. The presence of glycosylation might be an indication for the lectin's dual role which is either to act as a lectin or as a receptor for terminal galactose or mannose binding lectins.

The secondary structure of the protein was found to consist of mainly β -sheets, with very little α -helical content. This is one of the characteristics of the type-I β -prism fold proteins. Ligand binding does not alter the secondary structure of the protein much. The near-UV CD spectrum is characterized by two minima; one at ca. 275 nm and the other at ca. 294 nm which are probably due to absorption of Tyr and Trp, respectively. Upon ligand binding, the CD signal decreases a little without destroying the character which has also been observed for jacalin and MPA [Young et al., 1989].

MLGL is stable up to 70 °C and undergoes a cooperative unfolding transition centred at 77.6 °C. The unfolding appears to be irreversible as a repeat scan did not show any transition up to 80 °C. In presence of the ligand, the lectin becomes more stable and its denaturation temperature increases by about 1.5 °C which might be due to increase of favourable interactions between the protein and the ligand.

Latex is the milky sap of plants which protects the plants by exuding under mechanical stress or while attacked by insect herbivores [Konno, 2011]. Earlier studies have reported that latex of white mulberry (*Morus alba*) accumulates a large amount of defense proteins related to biotic stress [Kitajima et al., 2013]. Lectins, on the other hand are proteins which are thought to play a role in the plant defense system. There are examples of lectins which are anti-fungal or anti-bacterial or possess anti-insect activities [Peumans and Van Damme, 1995]. It has been proposed that lectins can interact with the epithelial cells along the digestive tract of phytophagous insects or higher animals and exert their cytotoxicity as a part of the plant's defense mechanism. In our study the cell viability assays carried out for MLGL showed that the lectin is substantially cytotoxic against the epithelial (MDCK) as well as breast cancer cells (MCF-7). As gJRLs are able to bind to the tumor associated T-antigen which is also expressed on the cell surface of breast cancer cells [Cazet et al., 2010], the cytotoxicity effect might have been exerted due to an interaction between the T-antigen and the lectin. Similarly, MLGL shows cytotoxic effect towards MDCK cells possibly by interacting with the cell-surface glycoconjugates. The cytotoxic nature of the lectin towards epithelial cells might be an indication for its involvement in the defense system by protecting the plant from phytophagous insects or higher animals. These observations of the preliminary experiments are encouraging and provide impetus for further detailed studies in this direction.

Materials and Methods

Materials

Fresh latex was obtained from the mulberry plantation located in the campus of University of Hyderabad. Guar gum and all sugars were obtained from Sigma (St. Louis, MO, USA). Trypsin, chymotrypsin and endoproteinase Glu-C were obtained from Roche Diagnostics GmbH (Mannheim, Germany). Thermolysin was obtained from Sigma Aldrich Chemie

GmbH (Taufkirchen, Germany). Zip Tip pipette tips (C18 and C4) were purchased from Millipore (Billerica, USA). All the other chemicals used were of highest purity commercially available.

Purification of MLGL

Collection of latex was performed as described by Patel et al [Patel et al., 2011] with a little modification. Briefly, fresh latex of mulberry was directly collected by plucking the young leaves into ice cold 10mM Tris-HCl buffer containing 150mM NaCl. The collected latex was then kept at -20°C for one day and thawed at room temperature before further processing. Subsequently a cross-linked guar-gum column [Kumar et al., 1982] was equilibrated with Tris-HCl buffer (150mM NaCl) at 4°C. The crude latex was centrifuged at 10,000 rpm for 30 minutes at 4°C and the supernatant obtained was loaded onto the guar-gum column. The unbound proteins were washed with Tris buffer followed by elution of the lectin with 0.2 M galactose. The purity of the protein was checked by performing SDS-PAGE according to Laemmli [1970].

Zeta potential measurements

The isoelectric point of the lectin was determined by measuring the zeta potential at different pH by mixed laser Doppler velocimetry and phase analysis light scattering (M3-PALS). A Malvern Zetasizer NanoZS (Malvern Instruments Ltd., UK) fitted with a red laser light ($\lambda = 632.8$ nm) and equipped with a MPT-2 autotitrator was used for this method. A protein solution of 0.1 mg/ml in PBS was titrated with 0.1 M HCl from pH – 6.0 to 3.0 with pH change steps of 0.5 ± 0.2 at 25 °C. The measurements were done in triplicates using disposable folded capillary cell (DTS1070) from Malvern.

Hemagglutination and hemagglutination-inhibition assays

Hemagglutination and hemagglutination-inhibition assays were performed as described previously [Sultan et al., 2004]. In a nutshell, the lectin was serially two-fold diluted in a round bottomed 96-well ELISA plate and an equal amount of a 4% erythrocyte suspension of human B (+) blood prepared in 20 mM PBS was added. The mixture was incubated at 4°C for one hour and the hemagglutination titre was scored visually. Hemagglutination-inhibition assays were performed in a similar way. Briefly, the sugar solution of interest was serially two fold diluted making 40 µl as the final volume in each well. 10 µl of lectin solution was added to each well and the mixture was incubated at 4°C for at least 10 minutes. Then 40 µl of the erythrocyte suspension was added to each well which made the resultant volume of each well as 100 µl. After one hour of incubation the hemagglutination titre was scored visually.

Preparation of samples for subunit mass determination

40 µl of MLGL (~ 500 pmol) was reduced to a volume of 10 µl by use of a centrifugal evaporator. This type of samples were then processed in two ways. Some samples were heated at 95 °C for 5 minutes while some were left untreated. In both heated and not heated samples, 10% TFA was added to adjust the final concentration of TFA to 0.1%. Subsequently zip-tip procedure was followed using C18 and C4 zip-tip columns.

In-solution digestion

About 200 pmol of MLGL was redissolved in 25 mM ammonium bicarbonate buffer using Bio-gel P-6 spin columns prior to reduction and alkylation by 10 mM DTT and 55 mM iodoacetamide. The reduced and alkylated samples were desalted and reconstituted either in water or 25 mM ammonium bicarbonate buffer by using Bio-gel P-6 spin columns. The

samples were then evaporated in a centrifugal evaporator and reconstituted in 20 μ l water. This step was repeated twice.

In-gel digestion

SDS-PAGE of MLGL was performed under reducing condition as described earlier. The gel was stained using Coomassie brilliant blue R-250 and destaining was performed using a solution containing 40% methanol and 10% acetic acid. The destained gels were thoroughly washed with distilled water prior to performing in-gel digestion. The cut and excised bands representing MLGL were subjected to digestion by various proteases according to Shevchenko et. al.[Shevchenko et al., 2006] in the following way. Pure acetonitrile was added repeatedly to the gel pieces and left for 10 minutes until the gel pieces shrink. The protein was then reduced and alkylated using 10 mM DTT and 55 mM iodoacetamide, respectively. After reduction and alkylation the gel pieces were shrunk again with pure acetonitrile and dried. The dried gel pieces were covered with trypsin, chymotrypsin (each 0.1 μ g), elastase (0.2 μ g), thermolysin (0.5 μ g) and endoproteinase Glu-C (0.5 μ g). Thermolysin digest was incubated at 65 $^{\circ}$ C and all other protease digests were incubated at 37 $^{\circ}$ C overnight. The peptides were extracted using a combination of acetonitrile, formic acid and water. The samples were dried in vacuo.

Mass spectrometry

The products of proteolysis of in-gel and in-solution digests as well as the intact subunit mass samples were redissolved in a mixture containing 49 μ l acetonitrile, 49 μ l MS water and 2 μ l formic acid. All the mass spectrometric experiments were carried out on a quadrupole-time of flight (q-Tof) mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray atmospheric pressure ionization source in the positive ion mode (ESI+). Typical source parameters were: source temperature 80 $^{\circ}$ C, desolvation gas (N_2) flow rate of 75 l/h, a

capillary voltage of 1.1 kV, and a cone voltage of 30 V. For sample introduction a direct injection into the ionization source by use of home-made nanospray capillaries was performed. For low energy collision-induced dissociation (CID) experiments, the (glyco)peptide precursor ions were selected in the first quadrupole analyzer and fragmented in the collision cell using a collision gas (argon) pressure of 3.0×10^{-3} Pa and collision energies of 20-40 eV (E_{lab}).

Circular dichroism spectroscopy

CD spectra were recorded on an Aviv420 spectrometer (Lakewood, NJ US) equipped with a Peltier thermostat. For far-UV region a sample concentration of 1.56 μ M and for near-UV region a sample concentration of 14 μ M were used. All the measurements were performed using a rectangular cuvette of 2 mm path length and 700 μ l capacity. Buffer scans were recorded under similar conditions and subtracted from the sample spectra before further analysis.

Differential scanning calorimetry

DSC experiments were carried out on a MicroCal VP-DSC differential scanning calorimeter (MicroCalLLC, Northampton, MA, USA) equipped with two fixed cells, a reference cell and a sample cell. The protein samples were dialyzed extensively against the buffer of desired pH before being loaded in the cell. For the different pHs the following buffers were used; 20 mM phosphate buffer, pH 6-7.4, 20 mM sodium acetate buffer, pH 4-5, 20 mM citrate phosphate buffer, pH 3 and 20 mM KCl-HCl, pH 2. All the buffers contained 150 mM NaCl in order to maintain ionic strength. The thermograms obtained were analyzed using DSC Origin as provided by the manufacturer.

Cell culture

All the cell cultures were maintained in a 37°C incubator (Sanyo MCO-19AIC, Panasonic Biomedical Sales Europe BV, AZ Etten Leur, Netherlands) containing 5% CO₂ and humid atmosphere. Madine darby canine kidney (MDCK) cells were cultured in 75cm² cultured flasks using MEM supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM) and 1% penicillin-streptomycin (10000 units penicillin, 10000 units streptomycin in 0.9% NaCl). Human breast cancer cells (MCF-7) were cultured in the same way but instead of MEM, RPMI medium was used with the same supplements. Once the confluence was reached the cells were detached using 0.05% trypsin in EDTA. Number of cells per 1ml of medium was counted using a counting chamber from Improved Neubauer by diluting the cells 20-fold with trypan blue. The cells were subcultured by splitting at ratio of a 1:10 or seeded at a number of 5000/well in a 96-well tissue culture plate.

MTT cell viability assay

The cytotoxicity of MLGL towards MDCK and MCF-7 cell lines was assessed colorimetrically using the MTT assay. Briefly, 100 µl of cell suspension was transferred to one each well of 96-well tissue culture plate (5000 cells/well) and allowed to grow overnight. One hundred µl of the lectin diluted in supplemented medium was added in two desired concentrations (1.25 µM and 0.625 µM) and the plates were incubated at 37 °C for 72 hours. After incubation the sample was removed from the cells and 100 µl fresh medium was added followed by the addition of 25 µl of 5 mg/ml MTT dissolved in PBS. The plates were incubated for about 4 hours and then after removal of the samples, 100 µl of DMSO was added to each well to dissolve the dye. After 15 minutes of orbital shaking at 300 rpm the absorbance was measured at 570 nm using a microplate reader (Safire, Tecan AG, Salzburg, Austria). A 4% solution of Triton X100 was used as the positive control and respective mediums were used as the negative control.

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Supplementary Data

Supplementary data related to this article can be found at

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