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Purification and molecular characterization of a sialic acid specific lectin from the phytopathogenic fungus *Macrophomina phaseolina*

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Abstract—A lectin was isolated and purified from the culture filtrate of the plant pathogenic fungus *Macrophomina phaseolina* by a combination of ammonium sulfate precipitation, affinity chromatography on fetuin-Sepharose 4B and ion-exchange chromatography on DEAE-A 50. The lectin designated MPL was homogeneous by PAGE and HPLC and a monomeric protein with a molecular weight of ~34 kDa as demonstrated by SDS-PAGE. It is a glycoprotein and agglutinated human erythrocytes regardless of the human blood type. Neuraminidase treatment of erythrocytes reduced the agglutination activity of the lectin. It is thermally stable and exhibits maximum activity between pH 6 and 7.2. Its carbohydrate binding specificity was investigated both by hapten inhibition of hemagglutination and by enzyme-conjugated lectin inhibition assay. Although, *M. phaseolina* lectin bound sialic acid, it exhibited binding affinity towards neuraminyl oligosaccharides of N-linked glycoproteins, α-Neu5Ac-(2→3)-β-Gal-(1→4)-GlcNAc being maximum.

Keywords: *Macrophomina phaseolina*; Lectin; Hemagglutination-inhibition; Carbohydrate specificity

1. Introduction

Lectins, multivalent cell-agglutinating proteins of non-immune origin, by virtue of their unique sugar-binding property are being widely used in the studies of biochemistry, cell biology, immunology, glycobiology and have widespread applications in biomedical researches.[1–3] During the last decade, fungal lectins have gained much attention in view of their antitumor, antiproliferative and immunomodulatory activities.[4–8] Sialic acid binding lectins are of current interest since sialic acids play important roles in many biological processes including in the physiology of normal and transformed cells. However, lectins of such binding specificity isolated from fungi are rare[9–12] and apart from few,[11,13,14] the precise carbohydrate specificities of fungal lectins have not been investigated in detail. Keeping in view, the importance of sialoglycoconjugates[15] in biological processes it would be useful to study a number of lectins, which can bind specifically to sialic acid or sialooligosaccharides. We report herein the purification and molecular characterization of *Macrophomina phaseolina* lectin and describe its recognition of α-Neu5Ac-(2→3)/(2→6)-β-Gal-(1→4)-GlcNAc by ECLIA using various sialic acid derivatives and sialoglycoproteins.

2. Results and discussion

2.1. Purification of *M. phaseolina* lectin

Since 80% ammonium sulfate fraction of the culture filtrate of *M. phaseolina* exhibited binding affinity towards fetuin and the binding was enhanced in presence of Ca^{2+} ions, this material was loaded on fetuin-Sepharose 4B column and eluted with citrate buffer (50 mM, pH 5) devoid of Ca^{2+} ions (Fig. 1a). The eluted protein was

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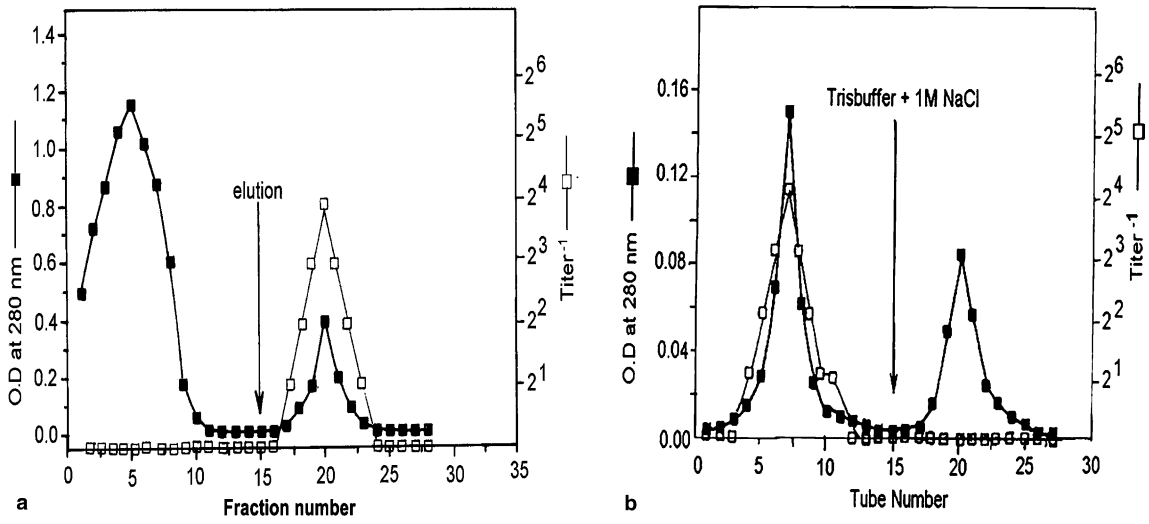

Figure 1. (a) Elution profile of the 80% ammonium sulfate fraction of *M. phaseolina* culture filtrate on fetuin-Sepharose 4B column. (b) Elution profile of the fetuin-Sepharose 4B purified fraction on DEAE Sephadex A-50.

further applied on DEAE Sephadex A-50. The active protein was collected in break-through fraction with Tris buffer as eluent and no more lectin was eluted with 1 M NaCl (Fig. 1b). The specific activity of the lectin was increased nearly to 2000 with a 62-fold purification compared to the culture filtrate. The protein recovered was 3.25%. Table 1 shows the purification scheme of *M. phaseolina* lectin (MPL).

2.2. Homogeneity and molecular weight

The purified lectin was apparently homogeneous as it showed a single band in 12.5% non-denaturing gel. This was confirmed by production of a single symmetrical peak in HPLC (Fig. 2a). Upon SDS-PAGE in the presence and absence of 2-mercaptoethanol, MPL produced a single band with an apparent molecular mass of 34 kDa (Fig. 2b). However, its molecular weight as determined by HPLC was ~31 kDa (Fig. 2c).

2.3. Hemagglutination assay

MPL agglutinated human erythrocytes of O, A, B and AB blood groups equally well, as well as erythrocytes of rat, hamster, mice, guinea pig, rabbit, chicken, goat, cow and horse (Table 2). Pronase treatment of human and animal erythrocytes enhanced the agglutination titer, which indicates that there is a cryptantigen which is de novo exposed by removing the (glyco)protein coat from the erythrocyte surfaces. However, the agglutination titer of human and animal erythrocytes (rat, rabbit, goat and chicken) was reduced by neuraminidase treatment. This preludes to occurrence of neuraminic acid at the terminal of the erythrocyte surface glycoprotein, which is a ligand of the receptor, MPL. Thus removal of ligand by neuraminidase decreased MPL titer. Neuraminidase treated chicken erythrocytes were feebly agglutinated by MPL. The reason may be that the chicken erythrocytes mainly contain *N*-acetyl neuraminic acid on their surfaces,[15] which could be well removed by neuraminidase, whereas human as well as other animal erythrocytes besides Neu5Ac also contain di-, tri- and tetraacetylated Neu5Ac as well as glycolyl neuraminic acid (Neu5Gc). These are not completely removed by neuraminidase as observed in the present investigation. For example, cow erythrocytes contain a large number of different kinds of Neu5Ac,[15] which are to some extent resistant to the action of neuraminidase resulting in almost the same titer value as that of untreated erythrocytes. Increase in the hemagglutination

Table 1. Purification of extracellular lectin from *M. phaseolina*

Fraction	Protein (mg/mL)[a]	Titer^{-1}	Specific activity[b]	Purification fold	Protein recovery (%)
Culture filtrate (0–80%)	2	64	32	1	
$(NH_4)_2SO_4$ fraction	0.7	64	91.43	2.86	35
Fetuin-Sepharose adsorbed fraction	0.075	128	1706.7	53.33	3.75
DEAE Sephadex A-50 purified fraction	0.065	128	1969.2	61.53	3.25

[a] Minimum amount of protein giving agglutination.
[b] Expressed as titer/mg-protein/mL.

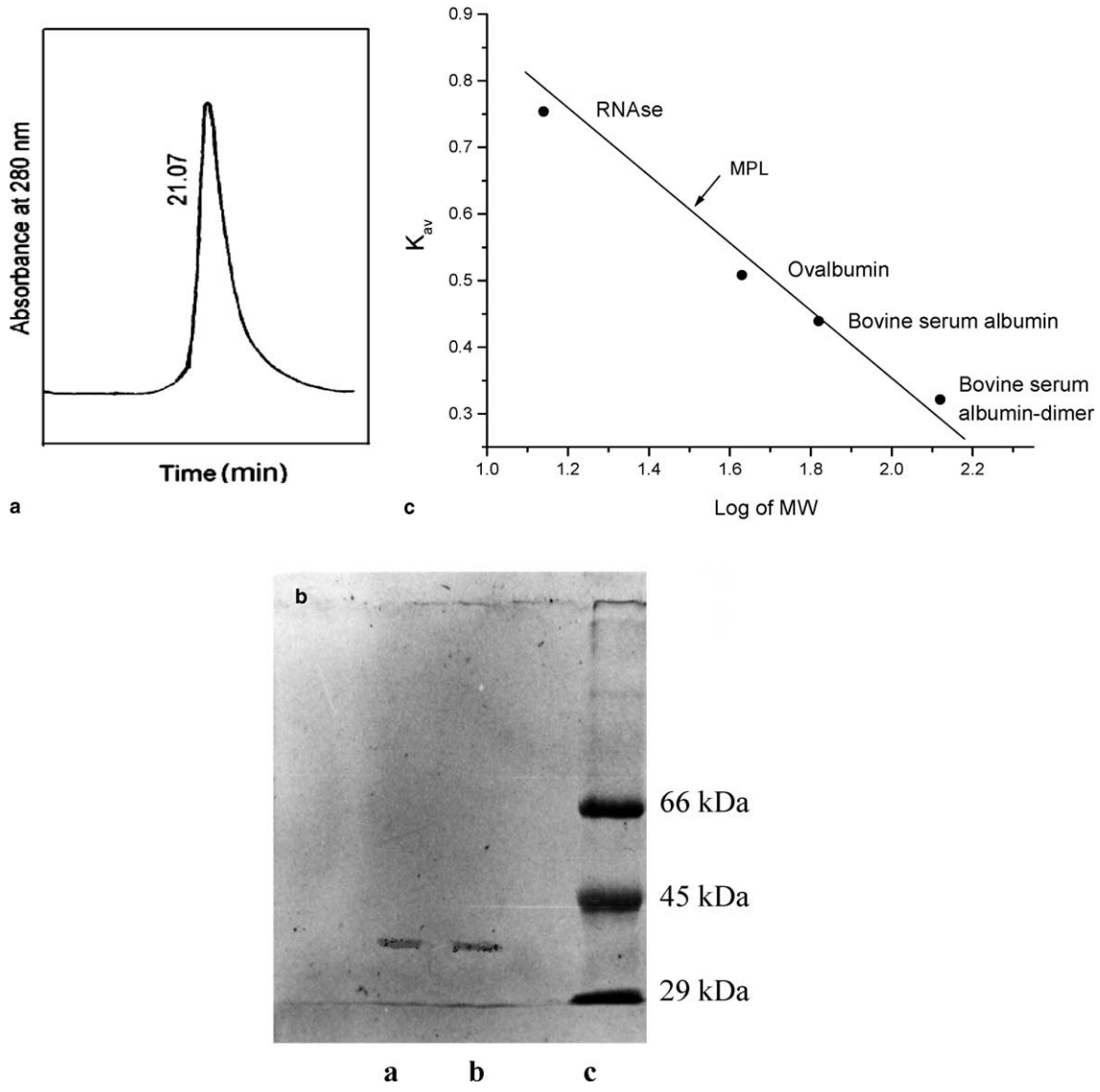

Figure 2. (a) Gel filtration chromatography of MPL on protein PAC 300 SW column (7.5 × 300 mm) connected to a precolumn Ultra PAC TSK SWP column (7.5 × 75 mm, LKB, Sweden) in HPLC. (b) SDS-PAGE of *M. phaseolina* lectin in the presence (lane a) and absence (lane b) of 2-mercaptoethanol; lane c contains protein markers; BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa). (c) Determination of molecular weight of MPL by gel filtration. The molecular mass standards were bovine serum albumin-dimer (132 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and RNAse (13.7 kDa).

activity of lectins was also observed from the fungi *Phaelepiota aurea*[16] and *Pleurotus tuber-regium*[17] with pronase and trypsin treated erythrocytes.

2.4. Physico-chemical properties

MPL was active between pH 4 and 10, being maximum in the range of pH 6–7.2. The hemagglutinating activity was nearly unaffected between 10 and 30 °C. Thereafter, its activity gradually decreased with increase in temperature and became inactive after 90 °C. The hemagglutination activity of MPL was partially Ca^{2+} dependent since its removal by EDTA reduced markedly the activity of MPL but was not abolished. However, on addition of Ca^{2+}, its activity was fully restored whereas Mg^{2+}, Mn^{2+} and Zn^{2+} were less effective.

2.5. Chemical analysis

Carbohydrate analysis showed that MPL is a glycoprotein. Its neutral sugar content was estimated to be 16.4%. The glycan part of MPL as identified by GLC was composed of mannose (1.5%), galactose (8.74%), glucose (5.84%) and trace amounts of arabinose (0.17%) and fucose (0.11%). High content of sugar in MPL renders its thermostability as has been observed in other fungal lectins.[18,19] The amino acid contents of MPL are summarized in Table 3. MPL has markedly

Table 2. Hemagglutination profile of normal and enzyme treated human and animal erythrocytes by MPL

Erythrocytes	Hemagglutination titer^{-1}[a]		
	Normal	Pronase treated	Neuraminidase treated
Human A	32	128	16
B	16	128	8
O	16	128	16
AB	16	128	16
Rat	16	64	8
Hamster	16	64	16
Mice	16	64	8
Guinea pig	16	32	16
Rabbit	8	32	8
Chicken	32	64	4
Goat	16	64	8
Cow	16	128	16
Horse	8	32	8

[a] At a protein concentration of 65 μg/mL.

Table 3. Amino acid composition of *M. phaseolina* lectin

Amino acid	g/100 g	mol/mol
Asp	4.4	11.22
Glu	3.74	8.64
Ser	36	116.5
Gly	0.5	2.3
Lys	7.42	17.3
Arg	11.56	22.6
His	—	—
Thr	1.2	3.4
Pro	1.11	3.3
Tyr	3.8	7.04
Met	—	—
Cys	—	—
Ala	0.3	1.14
Val	2.63	7.64
Leu	2.42	6.3
Ile	1.1	2.8
Phe	2.7	5.51
Trp	4.5	7.5

high content of serine. Like many other fungal lectins such as those from *Flammulina velutipes*, *Volvariella volvacea* and *Pleurotus cornucopiae*,[20–22] MPL lacks cysteine, methionine and histidine residues.

MPL is a metalloprotein. It contains magnesium in highest amount (2.33 mol/mol of lectin), followed by calcium (1.73 mol/mol of lectin). Manganese and copper are present in very small amounts.

2.6. Chemical modification of amino acids and periodate oxidation

Table 4 summarizes the effects of chemical reagents on the hemagglutination activity of MPL. Acetic anhydride modified Lys including terminal α-amino group of Lys and phenolic hydroxy group of Tyr, which showed a marked decrease in the hemagglutinating activity suggesting that either Lys or Tyr or both are responsible for the carbohydrate binding property of lectin. To discriminate between Lys and Tyr, de-*O*-acetylation was performed with 1 M hydroxylamine, which removed *O*-acyl of Tyr but not *N*-acyl of Lys. The activity of Lys modified lectin made significant change in the HA activity.

The Arg residues were specifically modified by cyclohexane-1,2-dione and the resulting complex was stabilized by borate ions. This modification retained almost its activity and thus confirmed that Arg did not play any important role in the lectin activity.

MPL was sensitive to EDC followed by α-amino butyric acid methyl ester as it resulted in significant loss of hemagglutinating activity indicating the presence of glutamic acid and aspartic acids in the active site. The presence of the acidic amino acids may play an indirect effect in the activity of lectin probably involving the binding site for metal ions.

Spectrophotometric monitoring revealed that controlled addition of 10 mM NBS to MPL led to a decrease in the absorption at 280 nm with a sequential oxidation of Trp residues to their oxindole derivatives. Compared to the native MPL, the hemagglutination titer of the Trp modified MPL decreased with an increase in the oxidation of Trp residues. The addition of 30 μL NBS to MPL led to a drastic loss of activity. Chemical modification studies on MPL revealed the involvement of lysine, tryptophan and carboxylic acid residues in the activity of the lectin.

Hemagglutinating activity of MPL was lost up to 50% and 75% when it was treated with 25 mM periodate at 4 °C for 2 and 4 h, respectively. After 24 h of oxidation, hemagglutinating activity was almost abolished. MPL

Table 4. Effect of modifying agents on HA activity of MPL

Chemical treatment	Type of groups modified	Hemagglutination activity (%)[a]
None (native MPL)		100
Acetic anhydride	Lys ε-amino, N-terminal α-amino, Tyr hydroxy	25
Acetic anhydride followed by hydroxylamine	Lys ε-amino, N-terminal α-amino	30
Cyclohexane-1,2-dione	Arg guanido	90
Carbodiimide, α-amino butyric acid methyl ester	Glu γ-carboxy and Asp β-carboxy	35
N-Bromosuccinimide	Trp indole ring	15

[a] The percentage hemagglutinating activity of the modified lectin was determined by comparing its hemagglutination titer with that of the native one.

lost its activity by sodium periodate, which indicates that the carbohydrate moiety of MPL may involve in the activity of the lectin.

2.7. Fluorescence quenching

The quenching of relative fluorescence intensity of MPL after incubation with Neu5Ac was measured at the wavelength of 335 nm at which it was maximal (Fig. 3a). It showed that the number of sugar binding sites for MPL is 2 ($n = 1.97$) and the association constant, K_a was found to be $6.72 \times 10^5\ M^{-1}$ as determined from the Schatchard plot (Fig. 3b). The fluorescence emission maxima at 335 nm is typical of Trp emission in proteins, indicating that the fluorescence is solely due to Trp exposed on the protein surfaces.[23] Tyrosine did not contribute to the fluorescence, as there was no emission in the region between 300 and 310 nm of the spectrum.

2.8. Equilibrium dialysis

Equilibrium dialysis (Fig. 4) also showed two sugar-binding sites/mole of lectin with an association constant $6.3 \times 10^5\ M^{-1}$.

2.9. Carbohydrate specificity

To determine the binding specificity of MPL, hemagglutination-inhibition experiments were performed with several glycoproteins as shown in Table 5. Of them, fetuin was the most potent inhibitor. Fetuin has both *O*-glycosidically and N-glycosidically linked sugar chains. The former is of two types, viz, α-Neu5Ac-(2→3)-β-Gal-(1→3)-GalNAc and α-Neu5Ac-(2→3)-β-Gal-(1→3)-[α-Neu5Ac-(2→6)]-GalNAc. The second one is a complex type triantennary carbohydrate chain containing three α-Neu5Ac-[(2→3)/(2→6)]-β-Gal-(1→4)-GlcNAc sequences, the portion of (2→3) and (2→6) linkage is almost equal. The desialylated fetuin was non-inhibitory up to a concentration of 10 mg/mL. This observation suggests that Neu5Ac probably plays a very important role in the interaction of fetuin with MPL. Human ceruloplasmin having one biantennary and one triantennary asparagine-linked structure of α-Neu5Ac-(2→3)/(2→6)-β-Gal-(1→4)-GlcNAc sequence was found to be a good inhibitor. BNG exhibited less inhibitory potency (1.25 mg/mL) compared to fetuin. This may be due to the presence of *O*-linked α-Neu5Ac-(2→3)-β-Gal-(1→3)-GalNAc and *O*-linked

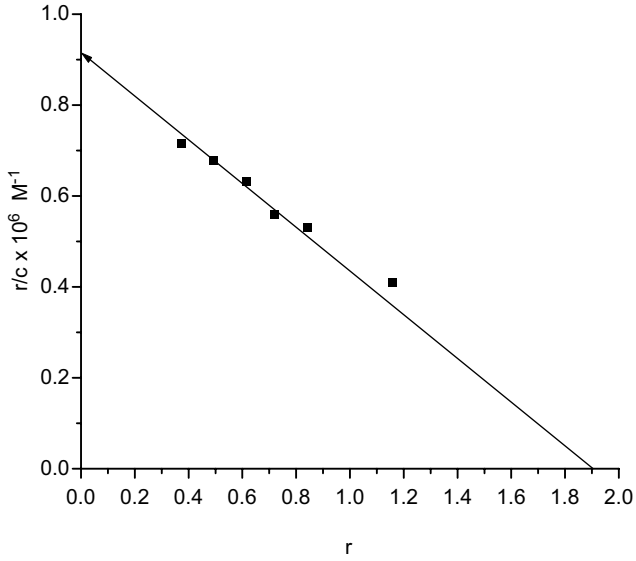

Figure 4. Equilibrium dialysis of *M. phaseolina* lectin with Neu5Ac. *r* is ratio of mole of sugar bound/mol of lectin and *c* is concentration of free sugar.

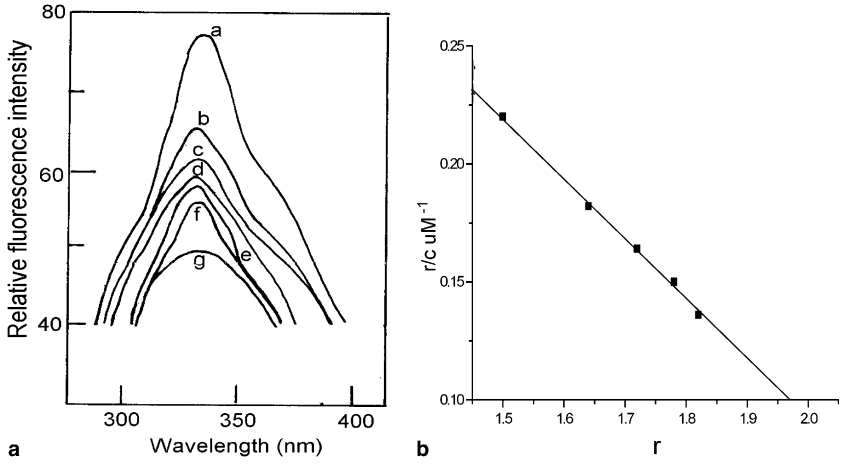

Figure 3. (**a**) Fluorescence quenching study of *M. phaseolina* lectin in presence of Neu5Ac: (a) native MPL, (b) MPL + 0.25×10^{-4} M Neu5Ac, (c) MPL + 0.5×10^{-4} M Neu5Ac, (d) MPL + 0.75×10^{-4} M Neu5A, (e) MPL + 1×10^{-4} M Neu5Ac, (f) MPL + 1.5×10^{-4} M Neu5Ac, (g) MPL + 2×10^{-4} M Neu5Ac. (**b**) Fluorescence quenching of *M. phaseolina* lectin with Neu5Ac: *r* is concentration of the bound ligand (ligand bound/protein) and *c* is free ligand concentration.

Table 5. Inhibition of *M. phaseolina* lectin induced hemagglutination by various sialoglycoproteins

Glycoproteins	Minimum inhibitory concentration[a] (mg/mL)
Fetuin	0.156
Asialofetuin	NI
BSM	NI
PTG	2.5
Asialo PTG	NI
Ceruloplasmin	0.625
BNG	1.25
Transferrin	5.0
Colominic acid	2.5

[a] Required for complete inhibition of two hemagglutinating doses of lectin against pronase treated human erythrocytes.

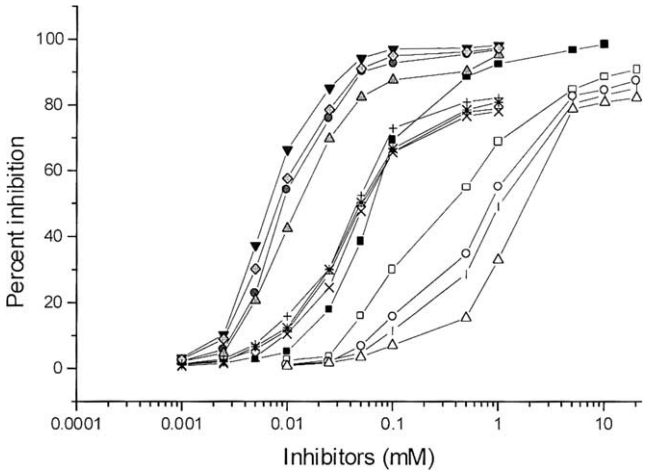

Figure 5. Representative curves for the binding-inhibition of MPL with fetuin in presence of neuraminic acid and different neuraminic acid containing oligosaccharides [–■– *N*-acetyl neuraminic acid, –●– 6′-*N*-acetylneuramin-*N*-acetyllactosamine, –▲– 6′-*N*-acetylneuraminyllactose, –▼– 3′-*N*-acetylneuraminyl-*N*-acetyllactosamine, –◆– 3′-*N*-acetylneuraminyl-*N*-acetyllactose. –+– 3′-*N*-acetylneuraminyl-3-fucosyllactose, –×– *N*-acetylneuramin-lacto-*N*-tetraose b, –∗– *N*-acetyl- neuraminyl-lacto-*N*-neo-tetraose c, ––– *N*-acetylneuraminyl-fucosyllacto-*N*-neo-tetraose, –|– Neu5, 7(8), 9 Ac_3, –□– *N*-acetyl neuraminic acid methyl ester, –○– *N*-glycolyl neuraminic acid, –△– periodate treated *N*-acetyl neuraminic acid].

α-Neu5Ac-(2→3)-β-Gal-(1→3)-GalNAc-(1→3)-GalNAc chains in BNG. PTG and transferrin contain mostly terminal α-Neu5Ac-(2→6) and a small amount of α-Neu5Ac-(2→3) linked to -β-Gal-(1→4)-GlcNAc in N-linked oligosaccharide chain. From the foregoing results, it could be suggested that the specificity of MPL is probably directed towards α-Neu5Ac, which is α-(2→3) or α-(2→6) glycosidically linked to -β-Gal-(1→4)-GlcNAc residue. That is why BSM, which contains *O*-acetylated sialic acids (mainly 9-*O*-acetyl and 8,9-di-*O*-acetyl-Neu5Ac) was completely non-inhibitory even at higher concentration.[24] Furthermore, colominic acid, which is a homopolymer of α-Neu5Ac-(2→8)-Neu5Ac sequence was non-inhibitory at a concentration of 2.5 mg/mL. Desialylated PTG was non-inhibitory, which indicates that the terminal Neu5Ac in the oligosaccharide chain is effective for binding.

With a view to understand the detailed carbohydrate specificity of MPL, ECLIA was carried out, wherein binding of MPL with fetuin and its binding inhibition in presence of different carbohydrates was monitored. The assay was standardized by monitoring the extent of binding between various amounts of fetuin (0.05–10 μg/100 mL) at varied times of incubation with the substrate. The binding between 5 μg fetuin and 10 ng/100 μL MPL-HRP resulted in the most effective binding condition, requiring the minimal amount of the lectin and glycoprotein. Concentration of sugars required for 50% inhibition was obtained from inhibition curves (Fig. 5) and is listed in Table 6. Except Neu5Ac, all the mono- and disaccharides tested in MPL-fetuin system were poor inhibitors, since their relative affinity in inhibiting the binding was very less compared to Neu5Ac which was 1. Neu5Ac (0.068 mM) required was 13 times less than Neu5Gc (0.875 mM) in 50% inhibition indicating that $–COCH_3$ group at C-5 is important for binding. The hydroxyl groups between C7–C8 and C8–C9 of the Neu5Ac residue were selectively cleaved by periodate oxidation. The periodate oxidized Neu5Ac exhibited less inhibitory potency (<27 times) than Neu5Ac. Furthermore, Neu5, 7(8), $9Ac_3$ was found to have reduced its inhibitory activity 16 times less compared to the Neu5Ac. Both the results strongly suggest that the cleavage or the modification of the glycerol chain of Neu5Ac drastically decreased the affinity of the entire sugar moiety towards MPL.

Neu5Ac methyl ester lost its inhibitory capacity over five times in comparison to its parent sugar, which definitely demonstrates that –COOH group at C1 is highly indispensable for binding to MPL. From these findings it may be said that Neu5Ac is prime inhibitor of MPL binding and the domain of Neu5Ac is confined around C2 to which the –OH group, –COOH group and the ring oxygen are linked.

In addition to Neu5Ac binding-specificity, another aspect of binding becomes most significant when sialic acid is α-(2→3) or α-(2→6) glycosidically linked to β-Gal-(1→4)-GlcNAc in the trisaccharide. Thus, α-Neu5Ac-(2→6)-β-Gal-(1→4)-Glc and α-Neu5Ac-(2→6)-β-Gal-(1→4)-GlcNAc were found to be over five and seven times more potent, respectively, than Neu5Ac itself. Comparing the inhibitory potency of 6′-*N*-acetylneuraminyllactose with 6′-*N*-acetylneuraminyllactosamine and 3′-*N*-acetylneuraminyllactose with 3′-*N*-acetylneuraminyllactosamine, it can be concluded that the presence of acetamido group at C2 of the reducing sugar at the reducing end facilitated the binding. It is evident from Table 6 that 3′-*N*-acetylneuraminyllactose and 3′-*N*-acetylneuraminyllactosamine were more potent inhibitors than 6′-*N*-acetylneuraminyllactose

Table 6. Relative potency of different carbohydrates towards *M. phaseolina* lectin

Inhibitors	IC_{50} (mM)	Relative potency
Glucose	7.95	0.0086
Galactose	8.23	0.00826
Mannose	9.2	0.00739
L-Fucose	12.11	0.00562
L-Rhamnose	9.2	0.00739
Galactosamine	5.9	0.0115
Glucosamine	3.93	0.017
Chitotriose	2.7	0.0252
Lactose	3	0.0227
N-Acetyllactosamine	2.3	0.0296
Melibiose	3.48	0.0195
Chitobiose	3.8	0.0179
GlcNAc	4.4	0.0155
GalNAc	4.5	0.0151
Glucuronic acid	5.24	0.0129
Galacturonic acid	5.4	0.0126
Muramic acid	3.1	0.0219
N-Acetyl neuraminic acid	0.068	1
6′-*N*-Acetylneuramin-*N*-acetyllactosamine	0.0092	7.39
6′-*N*-Acetylneuraminyllactose	0.013	5.23
3′-*N*-Acetylneuraminyl-*N*-acetyllactosamine	0.0068	10
3′-*N*-Acetylneuraminyllactose	0.0082	8.29
3′-*N*-Acetylneuraminyl-3-fucosyllactose	0.045	1.5
N-Acetylneuramin-lacto-*N*-tetraose b	0.055	1.13
N-Aceylneuraminyl-lacto-*N*-neo-tetraose c	0.053	1.28
N-Acetylneuraminyl-fucosyllacto-*N*-neo-tetraose	0.052	1.3
N-Acetyl neuraminic acid methyl ester	0.384	0.177
N-Glycolyl neuraminic acid	0.875	0.078
Neu5Ac, 7(8), 9 Ac_3	1.12	0.061
Periodate treated *N*-acetyl neuraminic acid	1.89	0.036

and 6′-*N*-acetylneuraminyllactosamine, respectively, which indicated that α-Neu5Ac-(2→3) linkage is more preferable in binding than α-Neu5Ac-(2→6). Moreover, the attachment of more sugars in the neuraminic acid containing trisaccharides viz, 3′-*N*-acetylneuraminyl-3-fucosyllactose, *N*-acetylneuramin-lacto-*N*-tetraose b, *N*-acetylneuraminyl-lacto-*N*-neo-tetraose c, and *N*-acetylneuraminyl-fucosyllacto-*N*-neo-tetraose decreased the inhibitory potency as compared to trisaccharides. This may be due to steric hindrance offered by the additional sugar unit.

Enzyme-conjugated lectin inhibition assay on MPL gave an insight into its specificity and size of the combining site. Neu5Ac was the prime carbohydrate ligand of receptor-protein MPL, while lactose and lactosamine in conjugation strengthened the binding, which themselves were almost non-inhibitory. Similarly, a lectin isolated from the fruiting bodies of *Agrocybe cylindraceae* (ACL) showed weak affinity towards Neu5Ac and lactose but strongly interacted with glycoconjugates containing α-Neu5Ac-(2→3)-β-Gal-(1→3)-GlcNAc/GalNAc sequence[13] and the extracellular lectin from the dermatophyte *Tricophyton rubrum* (TRA) did not bind free neuraminic acid, but recognized terminal Neu5Ac having both α-(2→6) and α-(2→3) linkages with either β-Gal-(1→3)-GalNAc or β-Gal-(1→4)-GlcNAc units present in glycoproteins.[18] Lectins from the polypore mushroom *Polyporus squamosus* (PSA) and from tubers of *Tricosanthes joponica* (TJA) although bound to β-galactoside, showed enhanced binding by α-(2→6) sialylation.[11,25] Again the elderberry (*Sambucus nigra* L.) bark lectin (SNA) exhibited extremely high inhibitor potency towards the oligosaccharides containing α-Neu5Ac-(2→6)Gal sequence (>1600–10,000 times) than Gal.[26] Thus, it is presumed that these lectins have extended carbohydrate-combining site that allows them to exhibit highest affinity towards non-reducing terminal α-Neu5Ac-(2→6)-β-Gal-(1→4)-Glc/GlcNAc chain of N-glycans.

Although MPL did not show specific linkage of sialic acid to penultimate sugar, yet it showed binding preference to α-Neu5Ac-(2→3)-β-Gal-(1→4)-GlcNAc. In this respect, MPL resembles ACL[13] and *Psathyrella velutina*,[27] which exhibited enhanced affinity towards trisaccharides containing the α-Neu5Ac-(2→3)-β-Gal sequence. Conversion of the sialic acid residue to its seven carbon analogue decreased the inhibitory potency of MPL suggesting that the –OH groups in the glycerol chain of Neu5Ac is an important loci for binding. Similar modification of sialic acid residue was reported to decrease its binding ability to *Achatina fulica* lectin (ATN_H),[24] influenza virus lectin[28] and *S. Nigra* lectin

(SNA).[26] In contrast, the binding of wheat germ lectin (WGA, *Triticum vulgaris*) was hindered by the exocylic part of Neu5Ac, thus the binding is increased with the C_7 analogue of Neu5Ac.[29] Furthermore, in MPL, inhibition by the trisaccharide was stronger compared to the tetrasaccharide. This is analogous to ATN_H and SNA in which the presence of a second sialic acid molecule may sterically interfere with the ability of these lectins for binding.

The above findings explicitly tell us that the binding affinity of MPL was not exclusively towards on Neu5Ac. Further addition of lactose or lactosamine residue to Neu5Ac molecule improved the binding potency, indicating the presence of an extended binding pocket, which can accommodate a trisaccharide.

The carbohydrate binding specificity of MPL described here is unique as it exhibits linkage specificity. The present study not only enriches our knowledge regarding the sugar binding properties of a fungal lectin but obviously renders it as a very useful tool in separation and analysis of glycoconjugates, which are important molecules for the study of glycobiology and are frequently mediated by carbohydrate–protein interaction.

3. Experimental

3.1. Materials

Reagents for gel electrophoresis, ELISA and chemical modification, carbohydrate inhibitors, horseradish peroxidase type VI, pronase P from *Streptomyces griseus* type XIV, neuraminidase from *Clostridium perfringens* type X were purchased from Sigma, USA. CNBr activated Sepharose 4B, DEAE Sephadex A-50 were purchased from Pharmacia Biotech, Uppsala, Sweden. Most monosaccharides, oligosaccharides, their derivatives and glycoproteins were procured from Sigma, USA. Neu5,7(8),9Ac_3 was the generous gift of Prof. R. Schauer, Biochemisches Institut, Kiel, Germany. Edible bird's nest glycoprotein was the kind gift of Prof. J. F. G. Vliegenthart, Utrecht University, Utrecht, The Netherlands.

3.2. Organism and culture condition

The phytopathogenic fungus *M. phaseolina* (MTCC 166) was obtained from the Institute of Microbial Technology, Chandigarh, India.[30] The fungus was grown in Richard's medium under stationary condition as described earlier.[30] After 7 days, the culture medium was separated from mycelia by filtration and then lyophilized. Hemagglutination activity and the protein content of the culture filtrate were determined as before.[30] All experiments were carried out at 4 °C unless stated otherwise.

The activity of the lectin at different stages of purification was assayed by hemagglutination using human red blood cells (A, B, O and AB groups) in a 96-well plastic plate by serially diluting the lectin (25 μL) in tris-buffered saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 7.2) and 2% (v/v) erythrocytes suspension. The same was also performed with pronase and neuraminidase treated human as well as animal erythrocytes.[31]

3.3. Purification of extracellular lectin

The purification of extracellular lectin involves three consecutive steps. Solid $(NH_4)_2SO_4$ was added to the culture filtrate (2 mg/mL, 20 mL) by stirring to give 80% saturation and stirred overnight. The precipitate collected by centrifugation (10,000*g*, 10 min) was dialyzed against deionised water and lyophilized. The lyophilized material (0.7 mg/mL, 1.5 mL) was dissolved in 50 mM TBS (pH 7.2) containing 50 mM $CaCl_2$ (TBS-Ca) and applied to a column of fetuin-Sepharose 4B (6 × 1 cm) previously equilibrated with the same buffer. The unbound proteins were eluted with TBS-Ca until the A_{280} of the effluents was less than 0.02. The bound protein from the column was desorbed with citrate buffer (50 mM, pH 5). The eluted fractions showing hemagglutination activity were pooled, concentrated by lyophilization and immediately dialyzed against TBS. The dialyzed sample was applied (0.075 mg/mL, 1 mL) on a DEAE Sephadex A-50 column (15 × 0.5 cm) previously equilibrated with 50 mM Tris–HCl/50 mM $CaCl_2$ (TB-Ca) (pH 7.2). The column was washed thoroughly with TB-Ca. The adsorbed material(s) was eluted and then with the same buffer containing 1 M NaCl and dialyzed against TBS. Both adsorbed and unadsorbed proteins after dialysis against deionised water and lyophilization were tested for hemagglutination activity against pronase-treated human erythrocytes. The active protein was present in the unadsorbed material.

3.4. Homogeneity and molecular weight

Homogeneity of the lectin was assessed by polyacrylamide gel electrophoresis (12.5%) under non-denaturing conditions[32] and it was further confirmed by gel filtration in HPLC using protein PAC 300 SW column (7.5 × 300 mm) connected to a precolumn Ultra PAC TSK SWP (7.5 × 75 mm, LKB, Sweden). The column was eluted with 10 mM PBS (pH 6.8) and monitored spectrophotometrically.

The extracellular lectin was subjected to SDS-PAGE (12.5%) according to Laemmli.[33] Dissociation and reduction of the protein were performed by heating it for 5 min at 100 °C with 0.1% SDS in the presence and absence of 2-mercaptoethanol (0.1%). The protein was stained with 0.05% Coomassie Brilliant Blue R

250. The molecular weight of the lectin was further judged by HPLC system as above.

3.5. Physico-chemical properties

The pH dependence of the lectin was determined by dialysing the protein against the buffers of desired pH [acetate buffer (pH 4), citrate buffer (pH 5–6), Tris–HCl buffer (pH 7.2–9) and glycine NaOH (pH 10)], followed by hemagglutination assay in presence of the aforesaid buffers.

The effect of temperature on the lectin activity was assessed by the erythroagglutination assay after incubating the lectin (0.065 mg/mL) in TBS (pH 7.2) during 30 min at 10–100 °C.

The effect of divalent cations on the lectin activity was judged as follows. The purified protein was dialysed extensively against 1 M acetic acid followed by 100 mM EDTA. The dialyzed protein was further dialyzed extensively against deionized water and finally against TBS (pH 7.2) without Ca^{2+}. Hemagglutination of the dialyzed lectin was performed with or without addition of Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (10–100 mM, all as chlorides).

3.6. Carbohydrate and amino acid analyses

Total neutral sugars in the lectin was determined by the phenol–sulfuric acid using D-glucose as the standard.[34] The identification and quantitation of neutral sugars present in the lectin were determined by GLC as alditol acetates[35] of the monosaccharides released in the hydrolyzates.

Amino acid analysis was performed by hydrolyzing the lectin (100 μg) using 6 N HCl at 105 °C for 24 h. The hydrolyzates were then analyzed in HPLC (Waters USA) using PICO TAG AMINO ACID SYSTEM. Tryptophan was determined separately in the unhydrolyzed lectin using spectrophotometric method.[36]

3.7. Metal ion analysis

The lectin was refluxed with a mixture of concentrated HNO_3 and 70% $HClO_4$ (1:1) for 30 min. The solution was cooled and filtered through Whatman No. 40 filter paper. Metal contents in the purified lectin were measured by atomic absorption spectrophotometer (Perkin–Elmer model 2380) using air-acetylene flame and hollow cathode lamp.

3.8. Carbohydrate specificity

3.8.1. Hemagglutination-inhibition assay. The hemagglutination-inhibition assay was performed in 96-well U-bottomed microtiter plate in TBS-Ca (pH 7.2), as described previously.[37] The glycoprotein inhibitors and their asialo derivatives were dissolved in 150 mM saline at a concentration of 1%. Asialo derivatives of glycoproteins were prepared by heating the samples (100 mg each) with 0.05 M H_2SO_4 at 80 °C for 1 h, followed by dialysis. The removal of sialic acid was ascertained by the method of Aminoff.[38]

3.8.2. Enzyme-conjugated lectin inhibition assay. Different sugar inhibitors (50 μL) of varying concentrations were incubated separately with 50 μL of *M. phaseolina* lectin–HRP conjugate (10 ng/100 μL), for 3 h at 37 °C. The mixtures were then added to the wells of immunoassay plate pre-incubated with fetuin (5 μg/well). The plates were further incubated for 1 h at 37 °C, washed with TBS containing 0.05% Tween-20 for three times followed by the addition of 0.04% OPD in 0.05 M citrate-phosphate buffer, containing 0.01% H_2O_2 as the substrate. The absorbance was recorded after 20 min at 492 nm in a Titertek Multiskan MK Plus II automatic ELISA reader.

3.9. Chemical modification of *M. phaseolina* lectin

3.9.1. Periodate oxidation. The lectin (2 mg/mL) in 50 mM sodium acetate buffer (pH 5.1) was mixed with an equal volume of 50 mM sodium metaperiodate in the same buffer and kept at 4 °C in the dark. Aliquots were withdrawn at an interval of 2, 4, 8, 12, 24 and 48 h, respectively, 0.1 mL ethylene glycol was added to each aliquot and the product was dialyzed extensively against TBS (pH 7.2). Vial containing only lectin and that containing only ethylene glycol were set up as controls. The hemagglutination activity of the periodate treated and untreated lectin was determined.

3.9.2. Modification of particular amino acids. The modifications of lysine, tyrosine, arginine, tryptophan and carboxy groups were done essentially by the same method as described before.[39] All the treatments were performed under mild conditions with specific reagents that could modify only particular amino acids and the reaction was monitored by appropriate control experiments. The modified proteins were freed from excess reagents by extensive dialysis against TBS.

3.10. Fluorescence quenching

Fluorescence measurement of Neu5Ac-*M. phaseolina* lectin complex was performed on Perkin–Elmer MPF-44B fluorescence spectrophotometer at 25 °C. The lectin (3 mL, 0.35 mg/mL) was incubated separately with increasing concentrations of Neu5Ac acid (0.25–2.0×10^{-4} M) for 2 h at 37 °C. After incubation, the samples were excited at 285 nm and the emission spectra were recorded between 300 and 400 nm. The association constant, K_a for Neu5Ac–lectin interaction was

determined using the peak heights of the spectra of the lectin obtained with different concentrations of Neu5Ac in Scatchard equation

$$r/c = K_a n - K_a r$$

r is concentration of the bound ligand (ligand bound/protein); c is free ligand concentration; n is no. of binding sites/protein.

A straight line was obtained by plotting r/c against r, and from the intersection of X-axis and the line, the value of n can be obtained.

3.11. Equilibrium dialysis

Equilibrium dialysis was carried out between *M. phaseolina* lectin (0.5 mg/mL) and Neu5Ac acid (60–200 μM) for 48 h at 20 °C. The concentration of the unbound Neu5Ac was estimated.[38]

The number of sugar-binding site(s) was obtained from the intercept of Scatchard plot on X-axis and the association constant K_a was determined from the Scatchard equation

$$1/r = 1/nK_a \times 1/c + 1/n$$

where n is number of sugar-binding sites; c is concentration of free sugar; r is ratio of mole of sugar bound/mol of lectin.

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