Isolation and characterization of a Forssman antigen-binding lectin from velvet bean (*Mucuna derringiana*) seeds

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A Forssman antigen (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer)-binding lectin has been purified from velvet bean (*Mucuna derringiana*) seeds by a combination of affinity chromatography and reversed phase HPLC. This lectin agglutinates both native and trypsin-treated sheep erythrocytes as well as trypsinized rabbit erythrocytes, but neither native rabbit nor human erythrocytes, irrespective of blood group type. SDS-PAGE and gel filtration chromatography reveal the lectin to be a homodimer consisting of two 54 kDa subunits linked by non-covalent bonds. The results obtained by quantitative precipitation, haemagglutination inhibition and TLC overlay assays indicate that the *Mucuna* lectin specifically recognizes Forssman antigen and Forssman disaccharide (GalNAcα1-3GalNAc)-related structures.

Keywords: Mucuna (*Mucuna derringiana*) lectin; velvet bean lectin; haemagglutinin; Forssman antigen binding lectin

Abbreviations: The abbreviations and the trivial names used are: AH, 6-aminohexyl; BSA, bovine serum albumin; Cer, ceramide; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate-buffered saline, pH 7.2, containing 0.15 M NaCl; PMSF, phenyl methyl sulfonyl fluoride; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid; TBS, 20 mM tris-buffered saline, pH 7.2; TLC, thin-layer chromatography; A disaccharide, GalNAcα1-3Gal; A trisaccharide, GalNAcα1-3[Fucα1-2]Gal; Forssman disaccharide, GalNAcα1-3GalNAc; CDH (ceramide dihexoside or lactosyl ceramide) Galβ1-4Glcβ1-1Cer (LacCer); CTH (ceramide trihexoside or globotriosyl ceramide), Galα1-4Galβ1-4Glcβ1-1Cer (GbOse$_3$Cer or Gb$_3$); globoside (globotetraosyl ceramide), GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (GbOse$_4$Cer or Gb$_4$); Forssman antigen (globopentaosyl ceramide), GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (GbOse$_5$Cer).

Introduction

Lectins are specific carbohydrate-binding proteins (or glycoproteins) of nonimmune origin that agglutinate cells and/or precipitate glycoconjugates [1]. They are distributed ubiquitously in nature, being found in plants, fungi, viruses, bacteria, invertebrates and vertebrates [2].

In the plant kingdom, lectins are particularly abundant in the family leguminoseae [3–5], and it has been hypothesized that these lectins may play a critical role in the nitrogen-fixing symbiosis between leguminous plants and *Rhizobium* [4, 6–9]. Some leguminous seeds were claimed by earlier investigators to be 'non-haemagglutinating' [10–12]; nevertheless, the possibility of false negative results cannot be ruled out if, for example, the lectin co-exists with endogenous inhibitor(s) and/or endogenous ligand(s); or if the lectin receptors present in the cells used in the haemagglutination assays are insufficient in number or in a cryptic state.

As a part of our recent efforts to re-examine these 'non-haemagglutinating' legume seeds, a lectin with affinity towards Forssman glycolipid (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) and Forssman disaccharide (GalNAcα1-3GalNAc)- related structures has been isolated from velvet bean (*Mucuna derringiana*) seeds and its properties studied.

This paper deals with the purification of the *Mucuna* lectin and the characterization of its physicochemical and carbohydrate binding properties.

Materials and methods

Saccharides

Various monosaccharides and their derivatives were purchased from Sigma Chemical Co. (St Louis, MO) and

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Pfanstiehl Laboratories, Inc. (Waukegan, IL). Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside and methyl 2-acetamido-2-deoxy-β-D-galactopyranside were obtained from Calbiochem (La Jolla, CA). Phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (phenyl α GalNAcp), p-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (p-nitrophenyl α GalNAcp) and o-nitrophenyl-2-acetamido-2-deoxy-α-D-galactopyranoside (o-nitrophenyl α GalNAcp) were obtained from Glycoside Products Company (Evanston, IL). The disaccharides, GalNAcα1-3Gal and GalNAcα1-6Gal, were prepared by N. Plessas; 6-O-p-nitrophenyl methyl α-D-galactopyranoside was synthesized by Dr Y. Meah of this laboratory. The trivalent GalNAc glycoside (GalNAcAH)$_3$ was a gift from Dr Y. C. Lee of Johns Hopkins University.

Miscellaneous materials

Velvet bean (*Mucuna derringiana*) seeds were purchased from Lawyer Nursery Inc. (Plains, MT). Synsorb-Forssman disccharide (GalNAcα1-3GalNAcβ-O-(CH$_2$)$_8$ CONH-Synsorb), Forssman disaccharide hapten (GalNAcα1-3GalNAcβ-O-(CH$_2$)$_8$COOCH$_3$), A-disaccharide (GalNAcα1-3Gal)-BSA, A-trisaccharide (GalNAcα1-3[L-Fucα1-2]Gal)-BSA and GalNAcα1-4Gal-BSA were obtained from Chembiomed Ltd. (Edmonton, Alberta, Canada) and Alberta Research Corp. (Edmonton, Alberta, Canada). N-acetyllactosamine (Galβ1-4GlcNAcβ-O-S-CONH)$_{16}$-BSA and GlcNAcβ1-6Gal-BSA were the products of Sockerbolaget, Swedish Sugar Co., Ltd (Arlov, Sweden). Pre-coated plastic-backed thin layer chromatography sheets, Polygram sil G, were the products of Macherey-Nagel (Germany). Polyisobutyl methacrylate (PIBM) was from Aldrich Chemical Company, Inc. (Milwaukee, WI). Gelatin and N-hydroxysuccinimidobiotin were from Sigma Chemical Co. Streptavidin-alkaline phosphatase, 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) were from Gibco BRL (Gaithersburg, MD). Trypsin was obtained from Worthington Biochemical Co. (New Jersey). Sephacryl S-200 HR was a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Standard neutral glycolipids, i.e. lactosyl ceramide, globotriosyl ceramide and globotetraosyl ceramide were kindly provided by Dr S. Basu of Notre Dame University. Authentic Forssman glycolipid (globopentaosyl ceramide) was a generous gift of Dr S-I. Hakomori of the Biomembrane Institute, Seattle. Fetuin, transferrin, thyroglobulin, bovine submaxillary mucin were obtained from Sigma Chemical Co. Ovine submaxillary mucin was a gift of Dr A. E. Eckhardt of Duke University; glycophorin was prepared by Dr S. J. Rinderle of this laboratory; α$_1$-acid glycoprotein was a gift of Dr G. W. Jourdian, University of Michigan. Asialoglycoproteins and asialomucins were prepared by heating the corresponding parental glycoproteins in 0.1 M hydrochloric acid at 80 °C for 1 h, followed by dialysis against distilled water and lyophilization. The
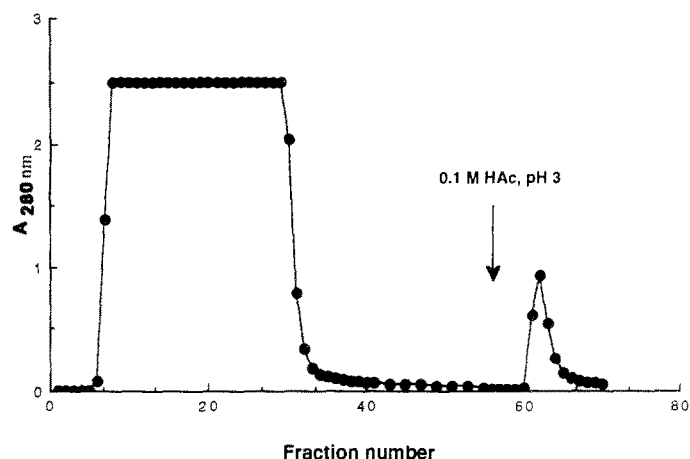

Figure 1. Isolation of the *Mucuna* lectin on a Forssman disaccharide-Synsorb column. *Mucuna* crude extract (400 mg) obtained from 60–80% ammonium sulphate fractionation was applied onto a Forssman disaccharide-Synsorb column (1.4 × 15 cm, bed volume 23 ml). The haemagglutinin was desorbed, at the arrow, with 0.1 M HAc, pH 3.0. Fractions of 80 drops per tube (approx. 2.5 ml per tube) were collected and monitored by absorbance at 280 nm.

removal of sialic acid from the glycoproteins was ascertained by the thiobarbituric acid assay [13–14]. Various blood type human erythrocytes and human sera were obtained from the Blood Bank, University of Michigan Hospital. Rabbit and sheep erythrocytes were obtained from mature and healthy animals; the trypsinized erythrocytes were prepared as described by Lis and Sharon [15].

Isolation of mucuna lectin

All procedures were carried out at 4 °C. After peeling off the testa, the velvet bean (*Mucuna derringiana*) seeds were finely ground using a Cyclone Sample Mill (UD Corporation, Boulder, CO), defatted with methanol and dichloromethane, and air-dried at room temperature. The flour was extracted three times with 5 volumes (V/W) of PBS containing 1 mM PMSF (phenylmethylsulfonyl fluoride) and the extract was centrifuged (10000 × g, 30 min). The clear supernatant solutions were combined and subjected to ammonium sulphate fractionation. The precipitate formed between 60 and 80% ammonium sulphate saturation was collected and dialysed against PBS. After clarifying by centrifugation, the dialysate was applied onto a Synsorb-Forssman disaccharide (GalNAcα1-3GalNAcβ-O-(CH$_2$)$_8$-CONH-Synsorb) column (1.4 × 15 cm, bed volume 23 ml) pre-equilibrated with PBS and the eluate was monitored by absorbance at 280 nm. After washing the column with PBS, the affinity-adsorbed lectin was desorbed with 0.1 M acetic acid, pH 3.0 (Fig. 1), collected, dialysed immediately against distilled water and lyophilized.

The final purification was achieved by reversed phase HPLC on a C-4 column (1 × 25 cm) using a linear gradient

from 100% A (0.1% TFA) to 100% B (0.08% TFA, 75% acetonitrile) in 60 min.

Haemagglutination and inhibition assays

The haemagglutinating activity of the lectin and the inhibitory potency of various carbohydrates were determined by a two-fold serial dilution procedure as described previously [16] using various fresh and trypsin-treated erythrocytes.

Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Native gel electrophoresis was carried out in Tris/glycine buffer, pH 8.9, using a 10% polyacrylamide gel according to the method of Davis [17]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed using a 12% gel according to the method of Laemmli [18]. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. The proteins used as molecular mass markers were rabbit muscle phosphorylase b (97.4 kDa), BSA (67 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

Protein estimation

Protein concentration was determined by the method of Lowry et al. [19], using bovine serum albumin as standard.

Molecular mass estimation

The molecular mass of the purified *Mucuna* lectin was estimated by SDS-PAGE and by gel filtration chromatography was carried out on a Sephacryl S-200 HR column (0.9 × 104 cm; bed volume, 68 ml) operating at room temperature in PBS, pH 7.2, in the presence or in the absence of 0.2 M galactose. The column was calibrated with the following standard proteins: bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen A (25 kDa), and cytochrome C (12.5 kDa).

Amino acid composition analysis

The purified *Mucuna* lectin sample was hydrolysed for 24 h at 110 °C in 6 M HCl and the amino acid composition analysed using an Applied Biosystems model 420 A amino acid analyser.

Amino acid sequencing

N-terminal amino acid sequencing was conducted using an Applied Biosystems (Foster City, CA) model 470 A protein sequencer interfaced with an Applied Biosystems model 120 A on-line analyser. The cyanogen bromide cleavage was conducted using 2 μg CNBr per μg of protein in 70% formic acid at room temperature overnight in the dark [20];

lysylendopeptidase digestion was carried out as described by Stone et al. [21]. The fragments obtained from lysylendopeptidase digestion and cyanogen bromide cleavage were separated by HPLC and SDS-PAGE followed by electroblotting onto a polyvinylidene difluoride membrane (Applied Biosystems, Inc.), and sequenced using an Applied Biosystems model 473 protein sequenator according to the manufacturer's instruction.

Quantitative precipitation assays

Quantitative precipitation assays were performed by a microprecipitation technique described by So and Goldstein [22].

Preparation of biotin-mucuna lectin conjugate

Biotinylation of *Mucuna* lectin was performed as described by Bayer et al. [23], but in the presence of 0.1 M N-acetylgalactosamine to protect the carbohydrate combining site. After coupling, the lectin activity was reassayed by haemagglutination assay.

Thin-layer chromatography (TLC) and TLC immunostaining

Thin layer chromatography and immunostaining were conducted on pre-coated, plastic-backed silica gel, Polygram Sil G, sheets, as described by Smith [24] and Brockhaus et al. [25] with slight modifications as follows: prior to chromatography, the TLC sheet was pre-developed with chloroform:methanol 2:1 (v/v) and allowed to dry. Neutral glycolipids were separated using chloroform:methanol:water 65:25:4 (by volume) as the developing solvent. After chromatography, the dried chromatogram was immersed for 1 min in a solution of 0.1% (w/v) polyisobutylmethacrylate in hexane. The sheet was then blocked by dipping in PBS, pH 7.2, containing 1% gelatin and 0.1% NaN$_3$ at room temperature overnight. After draining off the excess blocking solution, the TLC sheet was overlayered with biotinylated lectin diluted to 50 μg ml^{-1} with Tris-buffered saline (TBS), pH 7.2, containing 1% BSA and incubated at room temperature in a petri dish under a humidified atmosphere for 24 h. After removing the biotin-lectin conjugate solution, the TLC sheet was washed five times with PBS containing 0.05% Tween 20, 2 min for each wash, and overlayered with streptavidin-alkaline phosphatase (1:1000 diluted with TBS containing 1% BSA) for 4 h at room temperature. After six washings in TBS, the lectin binding glycolipids were visualized with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT). A replicate chromatogram was sprayed with orcinol reagent for visualizing the total neutral glycolipids.

Results

Haemagglutinating activity

The crude extract of *Mucuna derrigiana* seeds agglutinated fresh and trypsin-treated sheep erythrocytes as well as
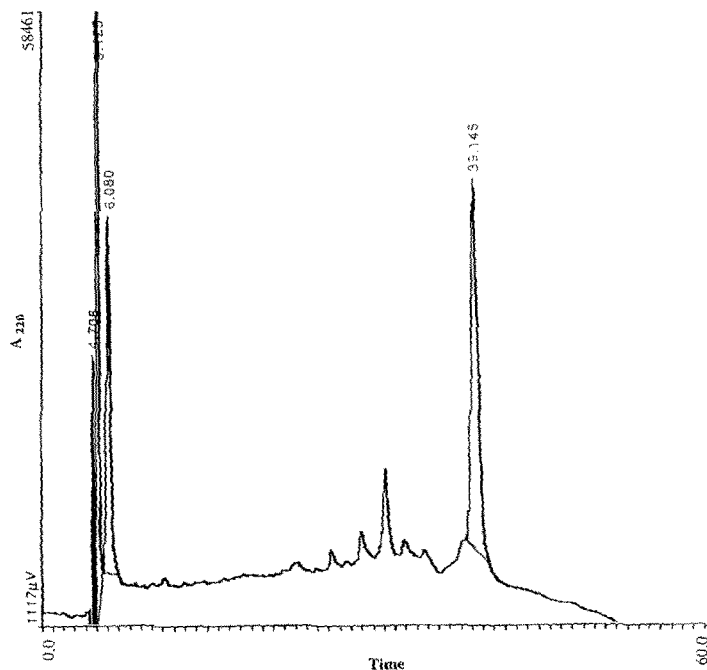
Figure 2. Purification of the *Mucuna* lectin by reversed phase HPLC on a C-4 column. The crude *Mucuna* lectin obtained from affinity chromatography on a Forssman-disaccharide-Synsorb column was applied onto a reversed phase HPLC C-4 column (1 × 25 cm). The separation was achieved using a linear gradient from 100% A (0.1% TFA) to 100% B (0.08% TFA, 75% acetonitrile) in 60 min. The major protein peak was the purified *Mucuna* lectin.

Figure 3. Native polyacrylamide gel electrophoretic pattern of the *Mucuna* lectin. From left to right: Lane 1: Crude *Mucuna* lectin isolated from affinity chromatography on a Forssman-disaccharide-Synsorb column contains one major protein band and several slow moving minor bands. Lane 2: HPLC-purified *Mucuna* lectin.

Figure 4. SDS-PAGE of the HPLC-purified *Mucuna* lectin. From left to right: Lane 1: HPLC-purified *Mucuna* lectin (treated with β-mercaptoethanol). Lane 2: Molecular mass standards. From top to bottom: phosphorylase b (97.4 kDa); bovine serum albumin (67 kDa); ovalbumin (45 kDa); carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa); lysozyme (14.4 kDa).

trypsin-treated rabbits erythrocytes, but failed to agglutinate fresh rabbit, and both fresh trypsin-treated human red blood cells, irrespective of the ABO blood group type. The haemagglutinating activity did not require bivalent cations.

The minimum concentration of the purified *Mucuna* lectin required for agglutinating trypsin-treated erythrocytes was 0.3 µg ml^{-1}.

Isolation of Mucuna lectin

Although the carbohydrate binding specificity of the crude extract of *Mucuna* seeds was found to be toward galactose and *N*-acetyl-D-galactosamine, the lectin activity was not retained by galactose-based affinity matrices including: Sepharose, acid-treated Sepharose, divinylsulfone-activated, galactose or lactose derivatized Sepharose and an asialo-fetuin-Sepharose column. The lectin activity was finally found to be adsorbed by a synthetic affinity matrix having nonreducing terminal GalNAcα1-3GalNAc disaccharide units linked to crystalline silica beads, i.e. Forssman disaccharide (GalNAcα1-3GalNAc)-Synsorb. The result of affinity chromatography of the crude *Mucuna* extract on a Forssman disaccharide-Synsorb column is shown in Fig. 1. The haemagglutinating activity was eluted with 0.1 M acetic acid, and both reversed phase HPLC and polyacrylamide gel electrophoresis revealed that this fraction contained one
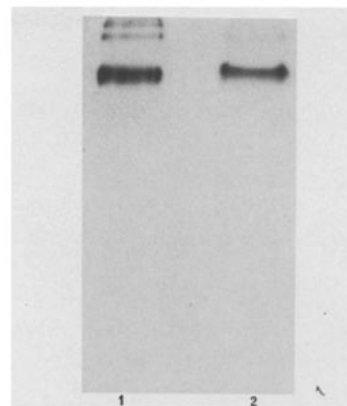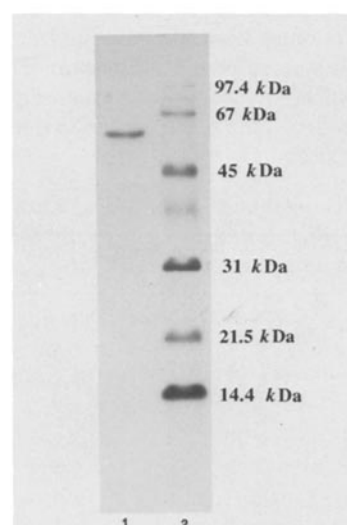
major protein and several minor components (Figs 2 and 3); the major protein peak from HPLC was collected and used as the purified *Mucuna* lectin for physicochemical characterization.

Molecular mass and molecular structure

On SDS-PAGE, both in the presence and in the absence of β-mercaptoethanol, the HPLC-purified *Mucuna* lectin gave a single band with an apparent molecular mass of 54 kDa (Fig. 4); under reducing conditions this protein band migrated slightly slower than it did under nonreducing
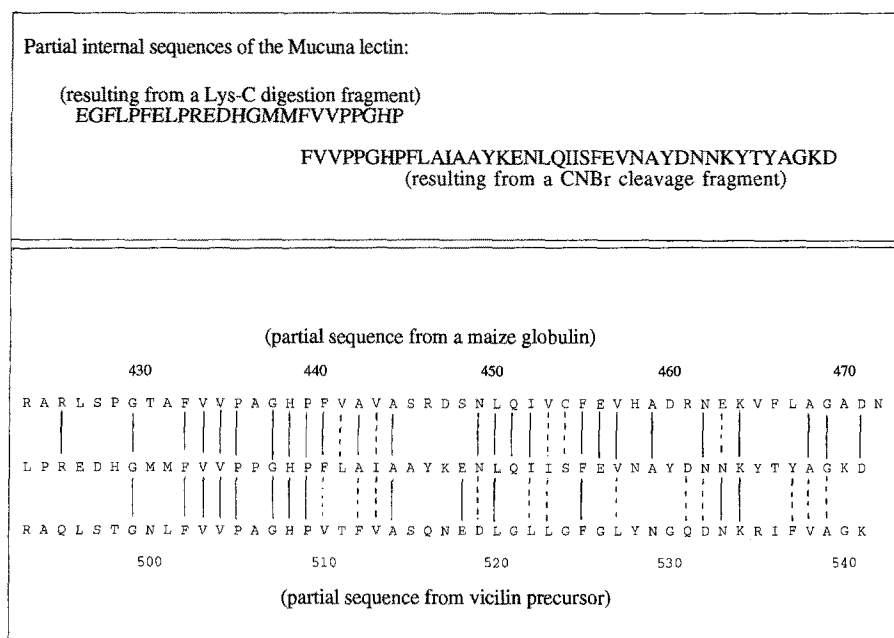
Partial internal sequences of the Mucuna lectin:

(resulting from a Lys-C digestion fragment)
EGFLPFELPREDHGMMFVVPPGHP

FVVPPGHPFLAIAAYKENLQIISFEVNAYDNNKYTYAGKD
(resulting from a CNBr cleavage fragment)

(partial sequence from a maize globulin)

(partial sequence from vicilin precursor)

Figure 5. Partial internal sequence resulting from CNBr cleavage (Top) and endopeptidase Lys-C digestion and its comparison with those of two plant storage proteins (Bottom). The solid lines show identical amino acids and the dashed lines indicate homologous amino acids. The first row shows the partial sequence from a maize globulin; the middle row, the partial sequence from the *Mucuna* lectin; and the third row, the one from the vicilin precursor.

Table 1. Amino acid composition of *Mucuna* lectin.

Amino acid	Mol %
Asx	11
Glx	10.5
Lys	8.7
Gly	8.3
Leu	8.2
Ser	7.5
Pro	6.5
Ala	6.1
Val	5.3
Phe	5.3
Arg	5.2
Thr	4.8
Ile	4.7
Tyr	3.1
His	2.7
Met	1.6
Cys	0.4
Trp	ND

ND, not determined.

conditions (data not shown), indicating the probable presence of intrasubunit, and the absence of intersubunit, disulfide bond(s). Gel filtration chromatography on a Sephacryl S-200 HR column conducted in PBS, pH 7.2, both in the presence or in the absence of 0.2 M galactose, gave a single symmetric peak at an elution volume corresponding to an apparent molecular mass of 90 kDa. Taken together, these data suggest that at neutral pH, the *Mucuna* lectin exists as a homodimer joined by non-covalent bond(s).

The amino acid composition of the HPLC purified *Mucuna* lectin is shown in Table 1. The lectin contains high concentrations of Asx, Glx, hydrophobic amino acids, and relatively high amounts of lysine and glycine. Attempts to sequence the *Mucuna* lectin's N-terminal amino acids failed due to a blocked amino terminus which was inert to the Edman degradation; however, an internal 56 amino acids sequence was determined by two overlapping peptides resulting from CNBr degradation and endopeptidase Lys-C digestion (Fig. 5).

Carbohydrate binding specificity

As will be elaborated in the Discussion, the *Mucuna* lectin failed to form a precipitate with any of a very large number of natural and synthetic glycoconjugates; this obviated the possibility of conducting sugar hapten inhibition of precipitation. Therefore the carbohydrate binding specificity of the *Mucuna* lectin was investigated by haemagglutination inhibition assay. The results are tabulated in Table 2. Of the monosaccharides tested, only D-galactose and N-acetyl-D-galactosamine were good inhibitors, whereas epimers of D-galactose, i.e. D-talose (the C-2 epimer) D-gulose (the C-3 epimer), and D-glucose (C-4 epimer) were all noninhibitory up to 200 mM. N-Acetyl-D-galactosamine was eight times more potent than D-galactose as an inhibitor. The methyl

Table 2. The minimum concentrations of saccharides required for complete inhibition of four haemagglutinating units of *Mucuna* lectin.

Saccharide	Concentration required (mM)	Relative potency
Galactose	1.04	1
Methyl α-D-Galactopyranoside	0.52	2
Methyl β-D-Galactopyranoside	2.08	0.5
GalNAc	0.13	8
Methyl α-D-GalNAc*p*	0.065	16
Methyl β-D-GalNAc*p*	0.52	2
Phenyl α-D-GalNAc*p*	0.033	32
o-Nitrophenyl α-D-GalNAc*p*	0.065	16
p-Nitrophenyl α-D-GalNAc*p*	0.033	32
GalNAcα1-3Gal	0.065	16
GalNAcα1-6Gal	0.13	8
Melibiose	0.26	4
Lactose	33.3	0.03
LacNAc	No inhibition at 50 mM	
L-Arabinose	33.3	0.03
D-Fucose	16.7	0.062
Forssman disaccharide-hapten (GalNAcα1-3GalNAcβ-*O*-(CH$_2$)$_8$COOCH$_3$)	0.035	30

[a] The values were the mean of three independent experiments.
There was no inhibition by L-fucose, D-arabinose, D-glucose, *N*-acetyl-D-glucosamine, D-gulose, D-mannose, *N*-acetyl-D-mannosamine, methyl D-mannoside, D-talose, L- and D-xylose, L- and D-rhamnose, ribose and 2-deoxy-ribose, up to 200 mM.

Table 3. Structures of neutral glycolipids used to characterize the carbohydrate binding specificity of the *Mucuna* lectin.

Glycolipid	Structure
Lactosyl ceramide	Galβ1-4Glcβ1-1Cer
Globotriosyl ceramide (GbOse$_3$)	Galα1-4Galβ1-4Glcβ1-1Cer
Globotetraosyl ceramide (GbOse$_4$) (Globoside)	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer
Globopentaosyl ceramide (GbOse$_5$) (Forssman glycolipid)	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer

α-glycosides were better, whereas the corresponding β-glycosides were poorer inhibitors than the parental sugars. Of the disaccharides examined, both lactose and *N*-acetyllactosamine were inferior inhibitors compared to galactose, whereas melibiose was four-fold better an inhibitor than galactose, signifying that the lectin has a strong preference for the α-anomeric configuration. Among the disaccharides containing nonreducing terminal *N*-acetyl-D-galactosaminyl residue, the Forssman disaccharide hapten (GalNAcα1-3GalNAcβ-*O*-(CH$_2$)$_8$COOCH$_3$) was by far the best inhibitor, being 30-fold more potent than galactose, and four times better than *N*-acetyl-D-galactosamine. GalNAcα1-3Gal was two times more active than GalNAc as an inhibitor, whereas the disaccharide GalNAcα1-6Gal showed an inhibitory potency similar to that of the monosaccharide GalNAc.

The carbohydrate binding specificity of the *Mucuna* lectin was also studied with four neutral glycolipids (Table 3) containing either a nonreducing terminal D-Gal or D-GalNAc group using a TLC overlay technique. The results are shown in Fig. 6. *Mucuna* lectin specifically bound Forssman glycolipid (globopentaosyl-ceramide, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer), whereas binding to lactosylceramide, globotriosylceramide (globoside) was not observed under the conditions of this assay.

Discussion

The crude extract of *Mucuna derringiana* seeds agglutinates trypsin-treated, but not native, rabbit erythrocytes, implying that the lectin 'receptors' on the native rabbit red blood cell surface are in a cryptic state, possibly glycolipids in nature. This was later confirmed by TLC immunostaining experiments.

Although N-terminal amino acid sequencing of the *Mucuna* lectin failed due to the blocked amino terminus, some partial internal sequences were obtained from CNBr
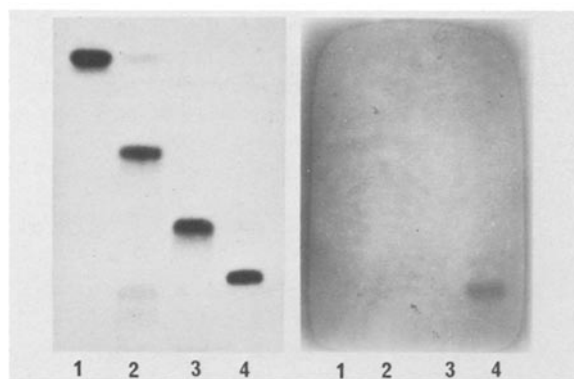
Figure 6. Thin-layer chromatogram of neutral glycolopids (Table 3). From left to right: Lane 1: LacCer. Lane 2: Gb₃ (globotriosylceramide). Lane 3: Gb₄ (globoside). Lane 4: Forssman glycolipid (globopentaosylceramide). Left panel: Visualized by spraying with orcinol reagent. Right panel: A replicate chromatogram stained with biotinylated-*Mucuna* lectin. The purified *Mucuna* lectin bound to Forssman glycolipid, but not to lactosylceramide, globotriosylceramide or globotetraosylceramide.

cleavage and lysylendopeptidase digestion (Fig. 5). It was interesting to note that this internal peptide sequence had considerable homology with the sequences of two plant storage proteins: vicilin precursor (34.1% identity in 41 amino acids overlap) and maize globulin (52.1% identity in 48 amino acids overlap (Fig. 5), because it has been well documented that in mature seeds, plant lectins are localized in the protein bodies of cotyledons, the major storage organelle [4]. Homology with other legume lectins was not observed.

Many native glycoproteins, mucins and their desialylated derivatives were tested for their ability to precipitate the *Mucuna* lectin, but none succeeded; among those examined were: both native and disialylated fetuin, thyroglobulin, glycophorun, α₁-acid glycoprotein, transferrin, ovine submaxillary mucin, bovine submaxillary mucin, porcine blood group type A substance, defucosylated A substance and various blood type human sera. The following neoglyco-proteins and glycosyl derivatives also failed to precipitate the lectin: A-disaccharide (GalNAcα1-3Gal)-BSA, A-trisaccharide (GalNAcα1-3[Fucα1-2]Gal)-BSA, LacNAc(Galβ1-4GlcNAc)-BSA, GalNAcα1-4Gal-BSA, Galα1-4Galβ1-4GlcNAc-BSA and a trivalent GalNAc glycoside (GalNAcAH)₃.

The carbohydrate binding specificity of the *Mucuna* lectin was investigated by haemagglutination inhibition. Of the monosaccharides examined, only D-galactose and N-acetyl-D-galactosamine were good inhibitors, whereas epimers of D-galactose were all noninhibitory up to 200 mM. This result indicates that each of the hydroxyl groups of galactose may be involved, though to a different degree, in lectin binding; the C-3 equatorial and the C-4 axial hydroxyl groups of the D-pyranose ring are the most important loci for lectin binding. The fact that N-acetyl-D-

galactosamine is 8 times more effective than D-galactose as an inhibitor and D-fucose is a much weaker inhibitor than D-galactose (Table 2) suggests that an equatorial acetamido group at the C-2 position and a free hydroxyl group at the C-6 position of the pyranose ring significantly enhance its binding to the *Mucuna* lectin.

Both methyl α-D-galactoside and the α-anomer of methyl N-acetyl-D-galactosaminide were four to eight times more potent as an inhibitor than the corresponding β-glycosides, indicating a distinct preference of the lectin for an α-anomeric configuration of the glycoside bond over the β-configuration. This conclusion was further confirmed by the observation that both lactose and N-acetyllactosamine were very poor inhibitors whereas melibiose (Galα1-6Glc) was a reasonably good inhibitor.

It was of great interest to note that the introduction of a hydrophobic aglycon such as a phenyl or nitrophenyl group significantly enhanced the affinity of the sugar for lectin binding; in fact, both phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside and p-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside showed the same inhibitory potency as the Forssman disaccharide hapten. This finding suggests the presence of a nonpolar region of the lectin adjacent to the carbohydrate binding site similar to the one reported may years ago for concanavalin A [26].

Inasmuch as GalNac occurs more frequently in glyco-sphingolipids (especially in globo and ganglio series), four glycolipids from the globo series were chosen to explore the binding specificity of the *Mucuna* lectin. The results are presented in Fig. 6. Among the four glycosphingolipids tested, only globopentaosylcermide, i.e. Forssman glycolipid, bound to *Mucuna* lectin.

The fact that the *Mucuna* lectin was isolated on a Forssman-disaccharide-Synsorb column, that the Forssman disaccharide was the best inhibitor of the haemagglutination induced by the *Mucuna* lectin, and that the biotinylated-*Mucuna* lectin specfically bound to Forssman glycolipid on TLC prompts us to suggest that the *Mucuna* lectin is specific for the Forssman disaccharide (GalNAcα1-3GalNAc) and Forssman antigen (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer)-related structures. This might also account for the failure of the lectin to precipitate all the glycoproteins and neoglycoproteins aforementioned, inasmuch as to the best of the authors' knowledge, none of these glycoproteins contains Forssman disaccharide-related structures.

Several haemagglutinins including *Dolichos biflorus* agglutinin, *Helix pomatia* agglutinin and *Wistaria floribunda* agglutinin have been reported to be reactive with the Forssman antigen [27]. However, all these lectins also agglutinate human type A erythrocytes and cross-react with blood group A substances [27], whereas the *Mucuna* lectin does not agglutinate either natural or trypsin-treated human red blood cells, irrespective of the blood group type, and fails to precipitate both native and defucosylated blood

group A substance. This property distinguishes the *Mucuna* lectin from other Forssman-reactive lectins.

Humans are generally considered to be Forssman antigen negative species; however, Forssman and Forssman-like glycolipids have been detected and isolated from various human cancer tissues and carcinoma cell lines [28–32]. The unique ability of *Mucuna* lectin to recognize the Forssman and Forssman-like glycolipids makes it a potentially important diagnostic and therapeutic tool.

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