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Wistaria sinensis AGGLUTININ: PURIFICATION, CARBOHYDRATE SPECIFICITY, AND CHARACTERISATION OF THE COMBINING SITE

HAFIZ AHMED AND BISHNU P. CHATTERJEE*
Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Calcutta 700 032 (India)

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ABSTRACT

A 2-acetamido-2-deoxy-D-galactose-binding agglutinin from *Wistaria sinensis* seeds, purified by affinity chromatography on a 2-acetamido-2-deoxy-D-galactose–starch conjugate, was homogeneous as judged by poly(acrylamide) disc gel electrophoresis. It had a mol. wt. of 66,000 (gel filtration on Sephadex G-150); on electrophoresis on SDS–poly(acrylamide) gel in the presence of 2-mercaptoethanol, it dissociated into sub-units of mol. wt. 34,000, suggesting the agglutinin to be a dimer; and it was a glycoprotein containing 4.8% of carbohydrate. It agglutinated several vertebrate erythrocytes, including human regardless of the blood group. In hapten-inhibition assays, 2-acetamido-2-deoxy-D-galactose and its glycosides were found to be better inhibitors than D-galactose and its glycosides, but *N*-acetyl-lactosamine was the most potent inhibitor. The binding involved HO-3,4 of the haptens and HO-2 partially.

INTRODUCTION

Lectins, isolated chiefly from plants, bacteria, fungi, invertebrates, and vertebrates, are non-immunoglobulin-type carbohydrate-recognition molecules which can be involved in hemagglutination, lymphocyte transformation, inactivation of certain types of tumor cells, and precipitation of certain polysaccharides and glycoproteins[1–3]. Lectins are being used increasingly to probe the structure of carbohydrates on the surfaces of normal and malignant cells[4]. The presence of an agglutinin in an extract of the seeds of *Wistaria sinensis* was first reported by Mäkelä[5], and an agglutinin isolated from the seeds of *Wistaria floribunda* has been studied in detail[6–11] and both mitogenic and non-mitogenic lectins have been isolated from this species.

We now describe the purification of *W. sinensis* agglutinin and the characterisation of its combining site.

*Author for correspondence.

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EXPERIMENTAL

The seeds of *W. sinensis* were purchased from F. W. Schumacher (Sandwich, Mass). All operations were carried out at 4° unless otherwise stated.

Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside was prepared according to Sarkar and Kabat[12], and the following were kind gifts: D-gulose and stachyose (Dr. C. K. Rao, Department of Pathology, Case Western Reserve University, Cleveland), *N*-acetyl-lactosamine (Dr. Manju Sarkar, Indian Institute of Chemical Biology, Calcutta), methyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-α-D-galactopyranoside (Dr. N. Roy), α-D-Gal-(1→3)-D-Man (Dr. A. K. Sarkar), and [β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_8$-$CONH]_{30}$BSA, edible bird's nest glycoprotein, and pig erythrocyte mucoid (Professor G. Uhlenbruck, Medical University Clinic, Cologne). α-D-Gal-(1→6)-D-Man was isolated after hydrolysing fenugreek galactomannan with 0.3M trifluoroacetic acid for 2.5 h followed by h.p.l.c. (Waters Associates Model 440) on a μ-Bondapak carbohydrate column, using acetonitrile–water (70:30). Pronase P (*Streptomyces griseus*) was purchased from Serva (Heidelberg) and neuraminidase from *Vibrio cholerae* (500 U/mL) from Behring (Marburg). All other chemicals were commercial products.

Erythrocytes. — Samples of human blood were collected in citrate–dextrose solution by vein puncture from healthy donors. Blood was obtained from rabbit, rat, and mouse by cardiac puncture, from duck and pigeon after sacrificing the birds, and from cow, buffalo, pig, sheep, and chicken was collected from the slaughter house.

The erythrocytes were treated[13] with pronase P and neuraminidase.

Purification of W. sinensis *agglutinin.* — Powdered seeds (100 g) of *W. sinensis* were stirred with 0.15M NaCl (1 L) overnight and then centrifuged (Sorvall RC-5B refrigerated centrifuge) at 12,000 r.p.m. for 40 min. The clear supernatant solution was fractionated by saturation to 0–40, 40–70, and 70–100% with solid $(NH_4)_2SO_4$. A solution of each precipitate in the minimum quantity of distilled water was dialysed against water until free of NH_4^+ ions, then centrifuged, and lyophilised. The most-active (40–70%) agglutinin fraction (30 mg in 2.5 mL of saline) was applied to a column (1.2 × 3 cm) of starch conjugated with 2-acetamido-2-deoxy-D-galactose[14] equilibrated with 0.15M NaCl. Protein was eluted with the same solvent, and the active protein with 0.05M glycine–HCl buffer (pH 4.0) containing 0.5M NaCl. The fractions were assayed for protein (absorption at 280 nm) and for hemagglutinating activity with pronase-treated erythrocytes after neutralisation with saturated aqueous $NaHCO_3$. The active fractions were combined, dialysed against water, lyophilised, and stored at -20°.

Hemagglutination and hemagglutination-inhibition assays. — Hemagglutination assays were performed[13] in Takatsy microtitre plates in saline with 2-fold serial dilution of the lectin. Titre was expressed as the reciprocal of the highest dilution showing visible agglutination; specific activity was expressed as titre per mg of protein per mL. Hemagglutination-inhibition tests were carried out as follows.

TABLE I

PURIFICATION OF *Wistaria sinensis* AGGLUTININ

Fraction	*Protein (mg/mL)*	*Titre*[a]	*Specific activity*[b]	*Purification (fold)*
Crude extract	7.60	8	1	1
Ammonium sulfate fractionation				
0–40%	2.54	64	25	25
40–70%	1.44	64	44	44
70–100%	1.36	4	3	3
Affinity chromatography-purified lectin	0.87	256	294	294

[a]Determined against human untreated erythrocytes. [b]Expressed as titre per mg of protein per mL.

To 2-fold serial dilution of the test sugar solution (25 μL) was added an equal volume of two hemagglutinating doses of lectin. After incubation for 1 h at room temperature, 25 μL of a 2% suspension of erythrocytes was added, and the mixture was stored for 1 h at room temperature. The maximun dilution of sugar solution showing inhibition was determined.

Disc electrophoresis. — A 7.5% poly(acrylamide) gel was used at pH 4.3 in β-alanine-acetic acid buffer[15] and at pH 8.9 in Tris-glycine buffer[16]. Staining was performed with Coomassie Brilliant Blue or Amido Black in aqueous 7% acetic acid, and destaining in aqueous 7% acetic acid in an electric field.

Determination of molecular weight. — This was done by sodium dodecylsulfate(SDS)-poly(acrylamide) gel electrophoresis[17] on 10% poly(acrylamide) gel. Dissociation and reduction of the protein were performed by heating for 5 min at 100° in aqueous 0.1% SDS with or without the addition of 0.1% of 2-mercaptoethanol. Protein markers employed were bovine serum albumin (mol. wt. 68,000), ovalbumin (43,000), chymotrypsinogen A (25,500), myoglobin (17,200), and cytochrome C (12,400). The molecular weight of the lectin was determined[18] also by comparison of its elution volume with those of molecular weight markers from a column (2 × 95 cm) of Sephadex G-150.

Protein and carbohydrate analyses. — Protein was determined by the method of Lowry *et al.*[19], using bovine serum albumin as standard, and total neutral carbohydrate content by the phenol-H_2SO_4 method[20] with D-galactose as the standard.

RESULTS

Purification of W. sinensis *agglutinin (WSA).* — The lectin present in the 40–70% saturated $(NH_4)_2SO_4$ fraction was found to be most active (see Table I). This fraction, on elution from a column of starch conjugated to 2-acetamido-2-deoxy-D-galactose with saline (aqueous 0.9% NaCl), yielded a large protein peak

devoid of hemagglutinating activity. WSA was released by elution with a buffer (0.05M glycine-HCl, 0.5M NaCl, pH 4.0) as a single peak (see Fig. 1). The affinity column retained 1 mg of WSA per mL of gel, and the yield was 3 mg with a 294-fold purification. The minimum amount of WSA required to agglutinate human erythrocytes was 3.4 μg per mL.

On poly(acrylamide) disc gel electrophoresis at pH 4.3 or 8.9, WSA moved as a single band (Fig. 2).

The mol. wt. of WSA was ~66,000 (Fig. 3) as determined by gel filtration on Sephadex G-150. The same mol. wt. was obtained by electrophoresis with SDS

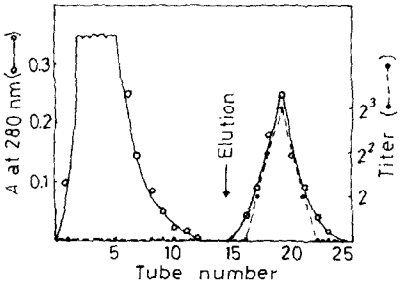


Fig. 1. Affinity chromatography of *W. sinensis* agglutinin on a column of starch conjugated to 2-acetamido-2-deoxy-D-galactose. The 40–70% ammonium sulfate fraction (30 mg in 2.5 mL of saline) was applied to a column (1.2 × 3 cm) at 4°, and, after elution of proteins with 0.15M NaCl (52 mL, 4-mL fractions), the agglutinin was eluted (3-mL fractions) with 0.05M glycine-HCl buffer containing 0.5M NaCl (pH 4.0) (indicated by the arrow).

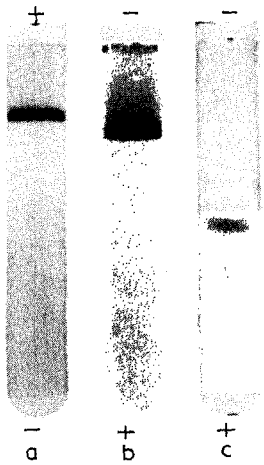


Fig. 2. Poly(acrylamide) disc gel electrophoresis of *W. sinensis* agglutinin in 7.5% gel with 50–100 μg of purified lectin (*a*) at pH 4.3 for 4 h at 3 mA, (*b*) at pH 8.9 for 5 h at 5mA in a 10% gel, and (*c*) in the presence of 0.1% of SDS and 0.1% of 2-mercaptoethanol for 6 h at 8 mA. The migration of protein was from the top. The gels were stained for protein with Amido Black or Coomassie Brilliant Blue R-250.

whereas, after reduction with 2-mercaptoethanol, only one band corresponding to a mol. wt. of 34,000 (Fig. 4) was obtained, indicating WSA to be a dimer.

Hemagglutination assay. — The data for WSA with human and animal erythrocytes are shown in Table II; erythrocytes of blood groups A, B, and O were agglutinated equally well, thus showing WSA to be non-specific. The agglutinating activity of WSA was enhanced by treatment of the cells with pronase and neuraminidase. WSA strongly agglutinated pig, rabbit, and rat erythrocytes, whereas those of sheep, dog, chicken, buffalo, mouse, and duck were weakly agglutinated. Bovine erythrocytes were agglutinated only after the cells were treated with pronase and neuraminidase.

Hemagglutination-inhibition assay. — The results with WSA and haptenic sugars are summarised in Table III. D-GalNAc and its methyl α-glycosides (each 0.78mM) were 32 times more effective than D-Gal (25mM) and 16 times more potent than its methyl α- or β-glycoside (12.5mM). A similar relationship was found for the nitrophenyl glycosides of D-GalNAc and D-Gal. α-D-Galactose 1-phosphate, L-arabinose, and 2-deoxy-D-*lyxo*-hexose (each 25mM) were as effective as D-Gal, which was twice as potent as methyl 1-thio-β-D-galactopyranoside and D-talose (each

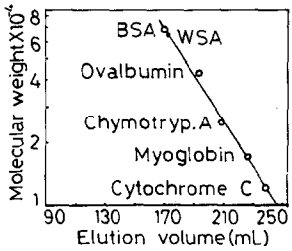


Fig. 3. Determination of the molecular weight of *W. sinensis* agglutinin by gel filtration. The protein solution (0.5 mL, 10 mg/mL in the eluent) was applied to a column (2 × 95 cm) of Sephadex G-150 and eluted with 0.15M NaCl at 10 mL/h (5-mL fractions). Elution volumes were determined from the position of the maxima of the elution profiles.

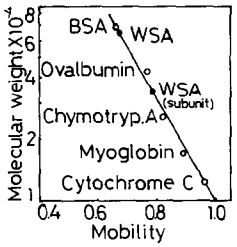


Fig. 4. Determination of molecular weight of the sub-unit of *W. sinensis* agglutinin by poly(acrylamide) gel (10%) electrophoresis in the presence of 0.1% of SDS and 0.1% of 2-mercaptoethanol. Standard protein samples together with *W. sinensis* lectin (50 μg of each) were applied, and, after staining with Coomassie Brilliant Blue R-250, their mobility was measured from the top of the gel to the leading edge of the protein band.

TABLE II

HEMAGGLUTINATION PATTERN OF DIFFERENT TYPES OF ERYTHROCYTES BY *Wistaria sinensis* AGGLUTININ

Erythrocytes	*Titre*		
	Untreated	*Pronase-treated*	*Neuraminidase-treated*
Human (O,A,B)	8	512	256
Cow	0	32,768	8
Pig	128	1024	256
Rabbit	256	65,536	2048
Rat	256	65,536	4096
Sheep	8	32	256
Dog	8	32	256
Chicken	32	131,072	2048
Buffalo	16	512	16
Mouse	8	128	16
Duck	16	4096	32

50mM). The inhibitory potencies of D-galacturonic acid, D-fucose, melibiose, raffinose, and stachyose were the same (each 12.5mM) and twice that of D-Gal (25mM). *N*-Acetyl-lactosamine [β-D-Gal-(1→4)-D-GlcNAc] was the most potent inhibitor (0.195mM).

Table IV contains data for the inhibition of agglutination of WSA by polysaccharide and glycoproteins. The *Pneumococcus* Type XIV (S-XIV) polysaccharide was the most potent inhibitor.

Carbohydrate analysis. — WSA gave a precipitin band with Con A, indicating it to be a glycoprotein. Its neutral sugar content was found to be 4.8%.

DISCUSSION

The purification procedure of *Wistaria sinensis* agglutinin (WSA) involved extraction of the seeds with saline, fractionation of the extract with ammonium sulfate, and affinity chromatography on starch conjugated to 2-acetamido-2-deoxy-D-galactose. The electrophoretically homogeneous WSA was similar in its physical and chemical properties to many other purified lectins[3]. The molecular weights of WSA (66,000) and its sub-units (34,000) are nearly the same as those of *Wistaria floribunda* agglutinin[9] (WFA) (68,000 and 32,000, respectively) and *Pseudomonas aeruginosa* H 8 (Habs type 8) lectin[21] (61,000 and 31,000, respectively). The latter lectin comprises two apparently identical sub-units joined non-covalently.

WSA agglutinated human erythrocytes regardless of the blood group and thus resembled WFA and another *N*-acetyl-lactosamine-specific lectin from *Erythrina cristagalli*[22]. Like many other lectins, WSA agglutinated animal red cells both before and after treatment with pronase or neuraminidase, except bovine erythro-

TABLE III

HEMAGGLUTINATION-INHIBITION ASSAY OF *Wistaria sinensis* AGGLUTININ BY CARBOHYDRATES

Carbohydrate	*Minimum inhibitory concentration (mM)*[a]
D-Galactose	25.0
Methyl α-D-galactopyranoside	12.5
Methyl β-D-galactopyranoside	12.5
o-Nitrophenyl α-D-galactopyranoside	6.25
o-Nitrophenyl β-D-galactopyranoside	3.12
p-Nitrophenyl α-D-galactopyranoside	6.25
p-Nitrophenyl β-D-galactopyranoside	3.12
Methyl 1-thio-β-D-galactopyranoside	50.0
α-D-Galactopyranose 1-phosphate	25.0
2-Acetamido-2-deoxy-D-galactose	0.78
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	0.78
o-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	0.39
p-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	0.39
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside	0.39
D-Galacturonic acid	12.5
D-Fucose	12.5
2-Deoxy-D-*lyxo*-hexose	25.0
D-Talose	50.0
L-Arabinose	25.0
α-D-Gal-(1→6)-D-Glc (melibiose)	12.5
α-D-Gal-(1→6)-D-Man	12.5
α-D-Gal-(1→3)-D-Man	25.0
β-D-Gal-(1→4)-D-Glc (lactose)	3.12
β-D-Gal-(1→4)-D-GlcNAc	0.195
α-D-Gal-(1→6)-β-D-Glc-(1→2)-D-Fru*f* (raffinose)	12.5
α-D-Gal-(1→6)-α-D-Gal-(1→6)-β-D-Glc-(1→2)-D-Fru*f* (stachyose)	12.5

[a]Required for complete inhibition of two hemagglutinating doses of lectin. Galactitol, D-tagatose, D-glucose, D-mannose, 2-acetamido-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, L-fucose, and D-arabinose were non-inhibitory up to 400mM. D-Gulose, methyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-α-D-galactopyranoside, β-D-Gal-(1→3)-D-GalNAc, methyl 2-*O*-methyl-α-D-galactopyranoside, and sialic acid were non-inhibitory up to 100mM.

cytes which required treatment with the enzymes probably to remove the mucoid layer of cell membrane and unmask the cell receptors[23].

Among the monosaccharide inhibitors of WSA-induced hemagglutination were D-GalNAc and its α-glycosides, which were more potent than D-Gal and its α-glycosides, indicating the important role of NHAc-2 for binding.

HO-1 of D-Gal is not important in the derivatives of monosaccharides for binding, since the 1-phosphate, the methyl α- and β-glycosides, and methyl 1-thio-glycosides were equally effective in inhibiting the agglutination. However, the *o*- or *p*-nitrophenyl glycosides showed increased binding with WSA. The inhibitory potency of *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-α- or β-D-galactopyranoside was twice that of the methyl glycoside of α-D-GalNAc or D-GalNAc. Likewise, *o*-

TABLE IV

HEMAGGLUTINATION-INHIBITION ASSAY OF *Wistaria sinensis* AGGLUTININ BY GLYCOSUBSTANCES

Glycosubstances	*Minimum inhibitory concentration (mg/mL)*[a]
Pneumococcus Type XIV polysaccharide	0.038
Human chorionic gonadotropin	0.62
Blood-group A substance	0.31
Fetuin	5.0
Asialo fetuin	5.0
Bovine submaxillary mucin	5.0
Asialo human erythrocyte glycoprotein	1.25
Ant-egg glycoprotein	0.62
C_1 activator	5.0

[a]Required for complete inhibition of two hemagglutinating doses of lectin. Bird's nest glycoprotein, pig erythrocyte mucoid and its asialo derivative, BSA-T antigen, IgA, IgE, and IgM were non-inhibitory up to 10 mg/mL.

and *p*-nitrophenyl α- or β-D-galactopyranoside were 2–4 times better inhibitors than methyl α- or β-D-galactopyranoside. Similar findings were also noted in other D-Gal/D-GalNAc binding lectins from *Maclura pomifera*[24], *Artocarpus integrifolia*[25], and *W. floribunda*[11], although, with the last, this enhancement was observed only for the *p*-nitrophenyl β-glycosides of D-Gal or D-GalNAc. By comparing inhibition results of *o*- and *p*-nitrophenyl α- and β-D-galactopyranoside, lactose, and melibiose, it is evident that WSA preferentially binds β-carbohydrates although the inhibitory potencies of methyl α- and β-D-galactopyranoside are comparable. Similar behaviour was also observed with other D-Gal/D-GalNAc specific lectins from *Sophora japonica*[26], *W. floribunda*[11], *Psophocarpus tetragonolobus*[27], and *E. cristagalli*[22].

That HO-2 is not of critical importance is shown by the similar inhibitory potencies of D-talose (axial HO-2), 2-deoxy-D-*lyxo*-hexose (HO-2 absent), and D-galactose (equatorial HO-2). However, the presence of a 2-keto group (D-tagatose) or 2-amino group (D-GalNH$_2$) caused loss of binding, whereas a 2-acetamido group (D-GalNAc) promoted binding, presumably due to additional Van der Waal's interactions or hydrogen bonding. The non-inhibition of methyl 2-*O*-methyl-α-D-galactopyranoside is probably due to steric hindrance.

Inversion (D-gulose) or substitution (methyl 2-acetamido-2-deoxy-3-*O*-methyl-α-D-galactopyranoside) of HO-3 caused loss of inhibitory potency. Likewise, β-D-Gal-(1→3)-D-GalNAc, BSA-T antigen, [β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_8$-CONH]$_{30}$ BSA, edible bird's nest glycoprotein[28], pig erythrocyte mucoid and its asialo derivative, and serum glycoproteins[29], each of which contains a β-D-Gal-(1→3)-D-GalNAc residue, did not inhibit the lectin up to 10 mg/mL (Table IV).

That HO-4 is also important in the lectin carbohydrate interaction is shown by the facts that inversion (D-glucose, D-mannose, 2-acetamido-2-deoxy-D-glucose) or

substitution (methyl 2-acetamido-2-deoxy-4,6-*O*-isopropylidene-α-D-galactopyranoside, methyl 3,4-*O*-isopropylidene-α-D-galactopyranoside) caused loss of inhibitory potency.

Compounds (D-fucose, D-galacturonic acid, and L-arabinose) modified at position 6 of D-Gal were inhibitors of agglutination, suggesting that HO-6 is not critical for binding. Similar findings have been observed with other D-Gal/D-GalNAc specific lectins, *viz., B. simplicifolia*[30], *S. japonica*[31], *M. pomifera*[24], *W. floribunda*[11], and soybean[32].

Like *A. integrifolia* (jackfruit)[25] and *B. simplicifolia I*[30] lectins, but unlike peanut agglutinin[33], the combining site of WSA does not recognise galactitol.

Since there was no difference in the inhibitory potencies of methyl α-D-galactopyranoside and di- and oligo-saccharides containing terminal α-D-Gal (Table III), it appears that the sub-terminal sugar(s) did not play any role in binding.

The inhibition of WSA-induced hemagglutination by asialo human erythrocyte glycoprotein[34] was due to the presence of terminal α-D-GalNAc. The presence of sialic acid in the glycoproteins did not hamper the binding of the lectin, since bovine submaxillary mucin[35], in which 22.4% of the terminal D-GalNAc is substituted by sialic acid, inhibited the agglutination. Also, both fetuin and asialo fetuin[36] were inbihitors of equal potency. Human chorionic gonadotropin (HCG)[37], which contains Neu5Ac-α-D-Gal-β-D-GlcNAc at the non-reducing end, was also an inhibitor. WSA differs from WFA[38] in that the latter did not bind with fetuin. Another D-Gal-binding lectin from the tunicate *Didemnum candidum*, unlike WSA, bound these glycoproteins only when the sub-terminal D-Gal residues were exposed by desialylation[39].

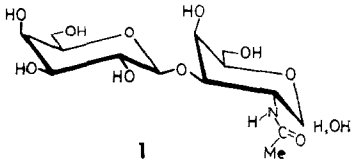
Blood group A substance interacted with the lectin because of the terminal α-D-GalNAc unit[40].

β-D-Gal-(1→4)-D-GlcNAc was the most potent inhibitor found and confirmed by the inhibition of *Pneumococcus* Type XIV polysaccharide (S-XIV), which contains the following repeating unit[41].

[→6)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-β-D-Glc-(1→]$_n$
4
↑
1
β-D-Gal

The inhibitory potency of asialo fetuin[36] is probably due to the presence of nine terminal β-D-Gal-(1→4)-D-GlcNAc units since the other disaccharide unit, β-D-Gal-(1→3)-D-GalNAc, present was inactive (see Table III). In the T-disaccharide [**1**, β-D-Gal-(1→3)-D-GalNAc], the D-Gal residue is at an angle to the plane of the D-GalNAc residue and is not recognised by the lectin combining site. In contrast, in *N*-acetyllactosamine [**2**, β-D-Gal-(1→4)-D-GlcNAc], the sugar residues are in the same plane and accessible to the combining site. The behaviour of WSA towards *N*-acetyl-lacto-

samine resembles those of the lectins from *E. cristagalli*[22], calf heart[42], *Geodia cydonium*[43], and the agglutinin from *Achatina fulica*[44], each of which was inhibited by *N*-acetyl-lactosamine. *N*-Acetyl-lactosamine was a poor inhibitor of WFA and had only 33% of the activity of D-GalNAc[11].



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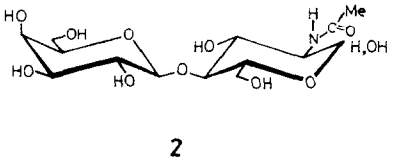

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Thus, it is concluded that the combining site in WSA interacts only with HO-3, HO-4, and (partially) HO-2. The D-GalNAc-specific lectins from soybean, lima bean, and *Dolichos biflorus*[32] also interacted with HO-2 and HO-4; the importance of HO-3 was not ascertained. For the D-GalNAc-specific lectin of *Helix pomatia*[32], additional interaction involves positions 1 and 4 although partially, since the methyl glycosides of α-D-GalNAc (C-1) and α-D-GlcNAc (C-1,4) inhibited better than the corresponding β-anomers. However, WFA interacted with HO-2,3,4, thus showing the apparent similarity of its combining site to that of WSA.

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