Studies on the binding of wheat germ agglutinin (*Triticum vulgaris*) to *O*-glycans

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Abstract The binding profile of *Triticum vulgaris* (WGA, wheat germ) agglutinin to 23 *O*-glycans (GalNAcα1→Ser/Thr containing glycoproteins, GPs) was quantitated by the precipitin assay and its specific interactions with *O*-glycans were confirmed by the precipitin inhibition assay. Of the 28 glycoforms tested, six complex *O*-glycans (hog gastric mucins, one human blood group A active and two precursor cyst GPs) reacted strongly with WGA and completely precipitated the lectin added. All of the other human blood group A active *O*-glycans and human blood group precursor GPs also reacted well with the lectin and precipitated over two-thirds of the agglutinin used. They reacted 4–50 times stronger than *N*-glycans (asialo-fetuin and asialo-human α_1 acid GP). The binding of WGA to *O*-glycans was inhibited by either *p*-NO_2-phenyl α,βGlcNAc or GalNAc. From these results, it is highly possible that cluster (multivalent) effects through the high density of weak inhibitory determinants on glycans, such as GalNAcα1→Ser/Thr (Tn), GalNAc at the non-reducing terminal, GlcNAcβ1→ at the non-reducing end and/or as an internal residue, play important roles in precipitation, while the GlcNAcβ1→4GlcNAc disaccharide may play a minor role in the precipitation of mammalian glycan-WGA complexes.

Key words: Wheat germ agglutinin; *O*-Glycan; Lectin binding

1. Introduction

Triticum vulgaris agglutinin (wheat germ, WGA) is one of the most popular applied lectins that has been frequently used as an investigative tool in glycobiology for over two decades [1]. Especially conjugates of WGA have been widely used to purify glycoproteins and glycopeptides, and to study neuronal cell adhesion molecules [2]. This agglutinin, which is not specific for human blood groups, shows a much higher affinity for GlcNAcβ1→4GlcNAc (*N*,*N*′-diacetylchitobiose) than for GlcNAc. The affinity of these chitin oligosaccharides for the lectin increases with size such that the disaccharide is over 100 times and the trisaccharide several thousand times more tightly bound to WGA than GlcNAc, while Galβ1→4GlcNAc (*N*-acetyllactosamine) is a poor inhibitor [1]. GlcNAcβ1→4GlcNAc is the essential structural feature found at the ends of *N*-linked chains in the carbohydrate-protein linkage region of *N*-glycans. WGA binds to peptidoglycans, chitin, glycosaminoglycans and glycolipids, and also interacts with some glycoproteins via sialic acid residues [3].

GalNAcα1→Ser/Thr-containing glycoproteins (*O*-glycans) are very common glycoconjugates in mammalians and play many important functions in life processes [4–9]. It was learned from studies on the binding properties of applied lectins that all *O*-glycans devoid of GlcNAcβ1→4GlcNAc (Fig. 1) precipitate strongly with WGA. Therefore, some other determinants should be involved in precipitation of the WGA-*O*-glycan complexes. To demonstrate this, we quantitated the affinity of WGA toward a panel of GalNAcα1→Ser/Thr-linked glycoproteins by the precipitin assay. Its reactivities toward *N*-glycans and colominic acid were also compared.

2. Materials and methods

2.1. Glycoproteins and polysaccharide

The glycoproteins tested were prepared from human ovarian cyst fluid and hog gastric mucosa [8,10–12]. The blood group active glycoproteins from human ovarian cyst fluids were purified by digestion with pepsin and precipitation with ethanol; the dried ethanol precipitates were extracted with 90% phenol, the insoluble fraction being named according to its blood group glycoprotein (e.g. cyst Beach-phenol-OH insoluble). The supernatant was fractionally precipitated by addition of 50% ethanol in 90% phenol to the indicated concentrations. The designation 10 or 20% (ppt) denotes a fraction precipitated from phenol at an ethanol concentration of 10 or 20%; 2× signifies that a second phenol extraction and ethanol precipitation was carried out (e.g. cyst MSS 10% 2×). A proposed representative carbohydrate side chain is shown in Fig. 1. The P-1 fraction represents the non-dialyzable portion of the blood group substances after mild hydrolysis at pH 1.5–2.0 and 100°C for 2 h which removed most of the terminal L-fucopyranosyl groups, as well as some blood group A and B active oligosaccharide side chains [11–14]. The first Smith degradation products of the blood group A active substance (MSS 10% 2×), in which almost all of the sugar residues at the non-reducing end were removed, were prepared according to Wu et al. [8,10–12].

Rat sublingual glycoprotein was prepared by the method of Moschera and Pigman [7,15,16]. Ovine and porcine salivary glycoproteins were purified according to the modified method of Tettamanti and Pigman [17]. Hog stomach mucin #4, hog A+H (structure I in Fig. 1) [18], was partially purified from porcine stomach mucin (type II, M-2378, Sigma) by centrifugation at $10\,000\times g$ for 3 h and exhaustive dialysis against distilled water (molecular weight cut-off, 8.0×10^3). The non-dialyzable fraction was centrifuged to remove insoluble material, and lyophilized. Hog gastric mucin #9 was the product of hog gastric mucin #4 after treatment at 80°C, pH 2.0 for 90 min (same condition as desialylation) and hog mucin #14 was the non-dialyzable fraction (molecular weight cut-off 8.0×10^3) of hog mucin #4, after hydrolysis at 100°C, pH 1.5 for 2 h (≈cyst glycoprotein P-1). Fetuin [19] and human α_1 acid glycoprotein [20,21] were purchased from Gibco (Grand Island, NY, USA) and Sigma Chemical Company (St. Louis, MO, USA), respectively. Asialo fetuin and asialo salivary glycoproteins were prepared by mild acid hydrolysis with 0.01 N HCl at 80°C for 90 min, and were dialyzed against water

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Abbreviations: GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; Gal, D-galactopyranose; LFuc, L-fucopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; NeuAc/Neu5Ac, *N*-acetylneuraminic acid; GP, glycoprotein; BSM, major fraction of bovine submandibular glycoprotein; OSM, major fraction of ovine submandibular glycoprotein; PSM, porcine salivary glycoprotein; RSL, rat sublingual glycoprotein major; WGA, wheat germ agglutinin

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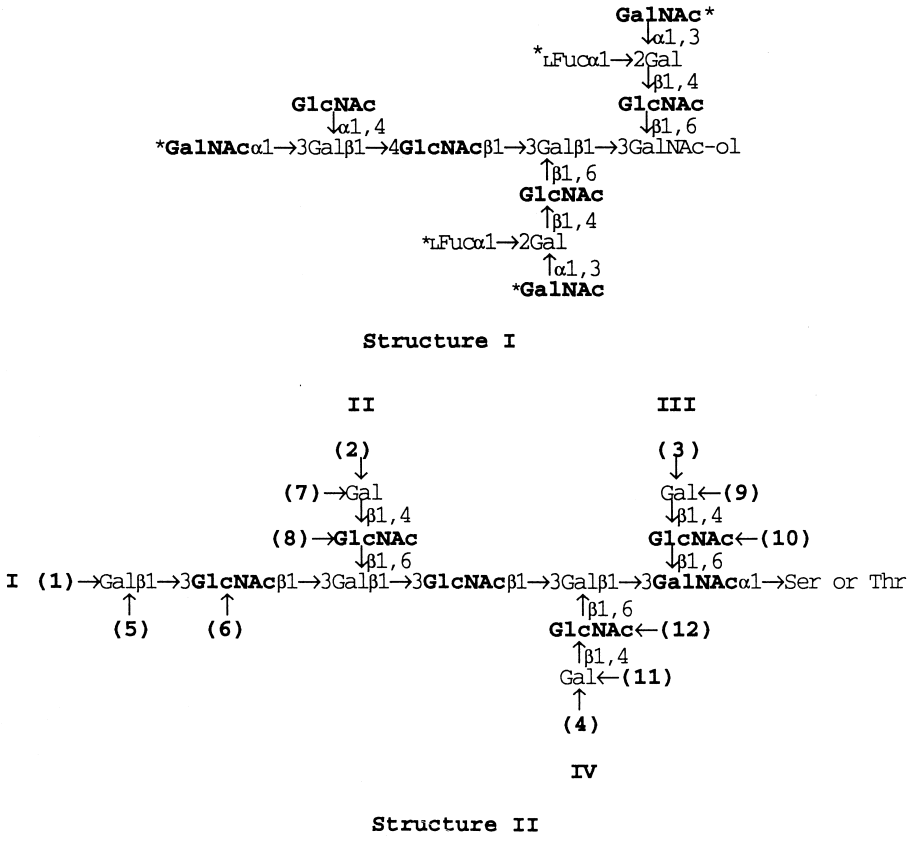

Fig. 1. Proposed representative carbohydrate side chains of *O*-linked glycoproteins (*O*-glycans), prepared from pig stomach cell linings (Hog A+H), and human ovarian cyst fluid (Cyst gp). In hog (A+H) (**structure I**), the terminal sequences (*) are considered to be blood group A+H and crypto H antigenic determinants. **Structure II** represents precursor blood group active glycoproteins, which can also be prepared by Smith degradation of A, B, H active glycoproteins, purified from human ovarian cyst fluids [8,11]. The four-branched structure (**I–IV**) is the internal portion of the carbohydrate moiety of blood group substances to which the residues responsible for A, B, H, Le^a, and Le^b activities are attached. Numbers in parentheses indicate the site of attachment for the human blood group A, B, H, Le^a, and Le^b etc. determinants. They are attached as follows:

Curve	Blood group active glycoprotein purified from human ovarian cyst fluid	Human blood group determinant present	Sugar added	Site of determinant addition to structure II
b	MSS 1st Smith	Ii		
c	Beach P-1		None	
b	Mcdon P-1			
c	Cyst JS phenol insoluble	H	LFucα1→2	(5), (7), (9), (11)
c	Tighe O cyst phenol insoluble	Le^b	LFucα1→4	(6)
		Le^x	LFucα1→3	(8), (10) and/or (12)
b	Cyst MSS, native		**GalNAcα1→3***	(1), (2), (3)
b	Cyst MSM	A or A_1	and as in	and/or (4)
b	Cyst Mcdon		H, and Le^b	(5), (7), (9) and (12)
c	Cyst Beach phenol insoluble		Galα1→3 and	(1), (2), (3)
		B	same as H, Le^b, Le^x or Le^y	and/or (4), (5), (7), (9), (6), (8), (10) and or (12)

The sugar shown in bold indicates the possible determinants contributing to precipitation and binding (Table 2). Most of the carbohydrate side chains of Hog (A+H) or cyst gp are part of its corresponding structures shown above.

to remove small fragments [17,22]. Colominic acid was purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Sugar inhibitors

GalNAc, *p*-NO_2-phenylβGlcNAc, *p*-NO_2-phenylαGlcNAc and Glc were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.3. Lectins

Wheat germ agglutinin (*Triticum vulgaris*) was purchased form Sigma Chemical Company (St. Louis, MO).

2.4. Lectinochemical (binding) assays [11,23–25]

Quantitative precipitin and precipitin inhibition assays were performed by a microprecipitin technique [26] using 5.0 μg of lectin nitrogen for each determination; total N in the washed precipitates was estimated by the ninhydrin method [27].

3. Results and discussion

Both the quantitative precipitin assay and the quantitative

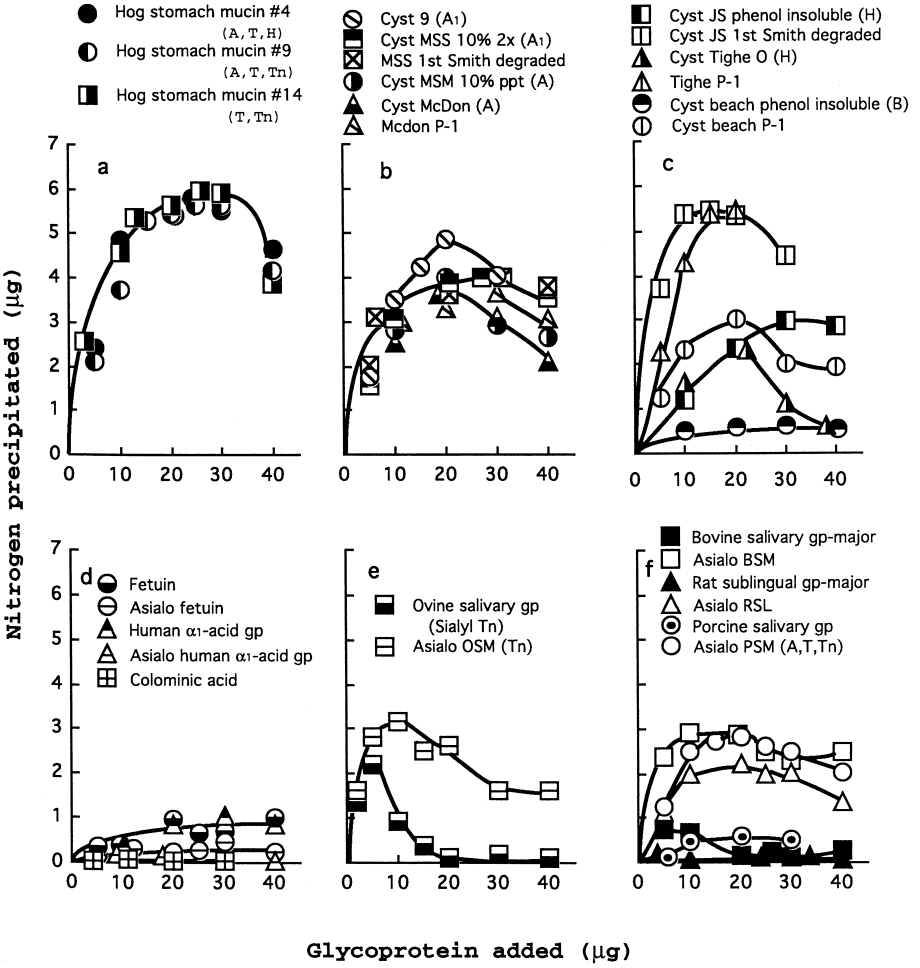

Fig. 2. Quantitative precipitin curves of wheat germ agglutinin with *O*-glycans, *N*-glycans and polysaccharide. Conditions: 5.0 μg WGA nitrogen; Total volume 300 μl.

precipitin inhibition assay are established methods that can provide insight into the specificities and size parameters of lectin-sugar interaction sites. These assays have been successfully used to characterize the combining sites of lectins and the binding properties of glycans for over two decades [28–31].

The quantitative precipitation curves of WGA with various glycoforms, especially mammalian *O*-glycans with blood group activity and sialylated *O*-glycans, are shown in Fig. 2. Of the 28 glycoforms tested, six *O*-glycans (hog gastric glycoprotein in Fig. 2a; cyst 9 (A_1) in Fig. 2b; cyst JS 1st Smith degraded in Fig. 2c; cyst Tighe P-1 in Fig. 2c) which do not contain GlcNAcβ1→4 GlcNAcβ1→ related oligosaccharides, reacted strongly with WGA. They completely precipitated the lectin added with less than 5.5 μg of glycoprotein required for 50% precipitation of 5.0 μg WGA nitrogen (Table 1), indicating that some other precipitating factors should be involved in the WGA-*O*-glycan interaction. Structures of these glycoproteins, as shown in Fig. 1, contain only several weak WGA inhibitory determinants such as GlcNAcβ1→6Gal, GlcNAcβ1→6GalNAc, GlcNAcβ1→3, GalNAcα at the non-reducing terminal and GalNAcα1→Ser/Thr at the reducing end [32–34]. Therefore, the strong precipitation of this group of glycans most likely depends on the high density of these weak determinants and the resulting cluster (multivalent) effects [34–36].

In contrast to the poor reactivity of human blood group B active glycoprotein (Beach phenol insoluble in Fig. 2c), the strong reactivity of cyst blood group A_1 active glycoproteins (Fig. 2b) indicates that structural (cluster) features of GalNAcα1→ at the reducing and non-reducing ends of the sugar chains should be potent precipitating agents. The precipitability of the Smith degraded or P-1 products derived from other cyst glycoproteins (Fig. 2b,c) can be attributed to the ability of the WGA to interact with **Tn** residues, as well as GlcNAcβ1→ residues.

Cyst beach glycoprotein with blood group B activity (Fig. 2c) reacted poorly with WGA and precipitated only 12% of the lectin, but its mild acid hydrolyzed product had an increased reactivity by 48% (Fig. 2c). This difference in binding can be assumed to be attributable to the strong shielding effect of blood group B (Galα1→3) key sugar and/or LFucα1→ determinants. The weakly reactive bovine and porcine submandibular and rat sublingual glycoproteins showed increased reactivities by 40% after removal of sialic acid (Fig. 2f), demonstrating the possibility of a masking effect of sialic acid. The asialo products of the *N*-glycans – fetuin and human α_1 acid glycoprotein as tested by QPA – showed a loss of reactivity of

Table 1
Comparison of precipitation activities of wheat germ agglutinin (*T. vulgaris*) with various glycoproteins and polysaccharides

Curve in Fig. 2	Glycoprotein or polysaccharide	Maximum precipitated[c] (μg N)	GP[a] or PS giving 50% ppt (μg)
a	Hog gastric mucin #4	5.8 (116%)	4
	Hog gastric mucin #9	5.6 (112%)	4
	Hog gastric mucin #14	5.9 (118%)	3
b	Cyst 9 (A_1)[b]	5.0 (100%)	5.5
	Cyst MSS 10% 2× (A)[b]	4.0 (80%)	7
	Cyst MSS 1st Smith degraded	4.0 (80%)	7
	Cyst MSM 10% ppt (A_1)[b]	4.0 (80%)	8
	Cyst Mcdon (A)[b]	3.6 (72%)	9
	Mcdon P-1 (mild acid hydrolyzed)	3.7 (74%)	6
c	Cyst JS phenol insoluble (H)[b]	3.0 (60%)	21
	Cyst JS 1st Smith degraded	5.5 (110%)	2
	Cyst Tighe O	2.3 (46%)	–
	Tighe P-1	5.5 (110%)	5
	Cyst Beach phenol insoluble (B)[b]	0.6 (12%)	–
	Cyst Beach P-1 (mild acid hydrolyzed)	3.0 (60%)	11
d	Fetuin (GP)	1.0 (20%)	–
	Asialo fetuin	0.5 (10%)	–
	Human α_1 acid GP	1.1 (22%)	–
	Asialo human α_1 acid GP	0.2 (4%)	–
	Colominic acid	0.1 (2%)	–
e	Ovine submandibular GP major (OSM)	2.2 (44%)	–
	Asialo OSM	3.2 (64%)	4
f	Bovine submandibular GP major (BSM)	0.7 (14%)	–
	Asialo BSM	2.9 (58%)	6
	Rat sublingual GP major	0.2 (4%)	–
	Asialo RSL	2.2 (44%)	–
	Porcine submandibular GP (PSM)	0.6 (10%)	–
	Asialo PSM	2.8 (56%)	11

[a]GP, glycoproteins; ppt, precipitate.
[b]The symbol in parentheses indicates the human blood group activity.
[c]The value in parentheses indicates the % of μg N precipitated at maximum when the amount of lectin added is expressed as 100% (= 5.0 μg N), in which the nitrogen contributed by glycoprotein was small.

more than 50% (Fig. 2d), indicating that sialic acid in both fetuin and human α_1 acid glycoprotein plays a significant role in the binding but the binding is weak. Colominic acid, which is a poly-2,8-*N*-acetylneuraminic acid, was inactive (Fig. 2d). Thus, the effect of sialic acids in the interaction of sialoglycoproteins with WGA is variable, i.e. structural features of the sialic acid configuration determine its role in binding (Fig. 2d) or masking (Fig. 2e,f).

The poor precipitability of WGA by the above two *N*-glycans after mild acid hydrolysis (Fig. 2 and Table 1) can be explained by an insufficient number of GlcNAcβ1→4GlcNAc sequences in their carbohydrate moiety to precipitate the lectin.

In order to establish that the *O*-glycan-lectin interaction occurs through sugar determinants rather than being non-specific, four sugar inhibitors (GalNAc, *p*-NO_2-phenyl βGlcNAc, *p*-NO_2-phenyl αGlcNAc and Glc) were used to inhibit the lectin-*O*-linked glycoprotein association. When the inhibition assay was carried out with 5.0 μg N of WGA and 10 μg asialo BSM or asialo OSM (Table 2), nearly 100% of the precipitation was inhibited by 28.5, 2.7 and 2.3 μmol of GalNAc, *p*-NO2-phenyl βGlcNAc and *p*-NO2-phenyl αGlcNAc, respectively, but insignificantly with 126 μmol of Glc. The interaction of the *O*-glycan, purified from human ovarian cyst fluid (MSM 10% ppt) with WGA was significantly inhibited by *p*-NO_2-phenyl α,βGlcNAc and GalNAc, but not by Glc.

In summary, we conclude that cluster effects afforded through a high density of many weak determinants, such as GalNAcα1→ at non-reducing ends; GalNAcα1→Ser/Thr (**Tn**) residues and their resulting structural features; GlcNAcβ linked at the non-reducing end and crypto GlcNAcβ→, play a crucial role in the precipitation of WGA, and that

Table 2
Inhibition of various glycoprotein-WGA interactions by *p*-nitrophenyl-α,β-D-GlcNAc, GalNAc and Glc

Glycoprotein tested	Amount of glycoprotein (μg)	Inhibition (%)			
		28.5 μmol D-GalNAc added	2.7 μmol *p*-nitrophenyl-β-D-GlcNAc added	2.3 μmol *p*-nitrophenyl-α-D-GlcNAc added	126 μmol D-Glc added
Asialo-BSM major	10	100	99.4	100	8.6
Asialo-OSM	10	100	100	98.8	17.3
Human ovarian cyst glycoprotein (MSM 10% PPT)	15	37.1	84.2	64.0	0

5.0 μg N of lectin in the centrifuge tube was mixed with or without (control) 28.5 μmol GalNAc, 2.7 μmol *p*-nitrophenyl-β-D-GlcNAc, 2.25 μmol *p*-nitrophenyl-α-D-GlcNAc or 126 μmol Glc. After incubation at 37°C for 30 min, 10 or 15 μg of glycoprotein was added, and incubated at the same temperature for 1 h and at 4°C for 6 days.

GlcNAcβ1→4GlcNAc is not an essential precipitating motif in mammalian *O*-glycan-WGA interaction. It is conceivable that other precipitating factors are also involved.

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