Fraction A of armadillo submandibular glycoprotein and its desialylated product as sialyl-Tn and Tn receptors for lectins

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Abstract Fraction A of the armadillo submandibular glycoprotein (ASG-A) is one of the simplest glycoproteins among mammalian salivary mucins. The carbohydrate side chains of this mucous glycoprotein have one-third of the NeuAcα2→6GalNAc (sialyl-Tn) sequence and two thirds of Tn (GalNAcα→Ser/Thr) residues. Those of the desialylated product (ASG-Tn) are almost exclusively unsubstituted GalNAc residues (Tn determinant). When the binding properties of these glycoproteins were tested by a precipitin assay with Gal, GalNAc and GlcNAc specific lectins, it was found that ASG-Tn reacted strongly with all of the Tn-active lectins and completely precipitated *Vicia villosa* (VVL both B_4 and mixture of A and B), *Maclura pomifera* (MPA), and *Artocarpus integrifolia* (jacalin) lectins. However, it precipitated poorly or negligibly with *Ricinus communis* (RCA_1); *Dolichos biflorus* (DBA); *Viscum album*, ML-I; *Arachis hypogaea* (PNA), and *Triticum vulgaris* (WGA). The reactivity of ASG-A (sialyl-Tn) was as active as that of ASG-Tn with MPA and less or slightly less active than that of ASG-Tn with VVL-A+B, VVL-B_4, HPA, WFA, and jacalin, as one-third of its Tn was sialylated. These findings indicate that ASG-A and its desialylated product (ASG-Tn) are highly useful reagents for the differentiation of Tn, T (Galβ1→3GalNAc), A (GalNAcα1→3Gal) or Gal specific lectins and monoclonal antibodies against such epitopes.

Key words: Lectin reactivity;
Armadillo submandibular glycoprotein

1. Introduction

Tn (GalNAcα1→Ser/Thr) and sialyl-Tn (NeuAcα2–6GalNAcα1→Ser/Thr) sequences are the simplest carbohydrate chains that have been proposed as markers of cancerous tissues [1–5]. They are also found in many mammalian salivary mucus glycoproteins [6–10], especially armadillo, hamster, and ovine salivary glycoproteins.

At the surface of the red cell membrane, the Tn transformation indicates an acquired disorder characterized by the exposure of normally cryptic GalNAc residues α→linked to the hydroxyl group of Ser or Thr of membrane sialoglycoproteins [11]. The Tn syndrome is the result of a selective deficiency of the 3-β-D-galactosyltransferase involved in the biosynthesis of the T structure: Galβ-1→3GalNAcα1→Ser(Thr) [12]. The Tn antigen can also be detected at the cell surface of erythrocytes, granulocytes, platelets, and B and T lymphocytes of patients presenting the Tn syndrome [13].

Over the past two decades, we have been using many water-soluble glycoproteins and oligosaccharides to study the binding properties of many lectins [14–21], especially glycoproteins that contain F (GalNAcα1→3GalNAc), A (GalNAcα1→3Gal), A_h (GalNAcα1→3[Fucα1→2]Gal), Tn (GalNAcα1→Ser/Thr), B (Galα1→3Gal), E (Galα1→4Gal), I/II (Galβ-1→3/4GlcNAc), L (Galβ-1→4Glc) and T (Galβ-1→3GalNAc) determinants [16,17]. To broaden this panel, new glycoprotein reagents, especially Tn and sialyl-Tn containing glycoproteins have been intensively searched for. Fraction A of the armadillo submandibular glycoprotein (ASG-A) was chosen for this study as this glycoprotein is one of the simplest among mammalian salivary mucins [8,9]. It is composed of approximately 53.4% protein, 26% hexosamine, and 13% sialic acid [9]. Thr and Ser in the protein core account for more than half of the total amino acid residues, while Gly, Glu, Val, and Ala are the major components of the remaining amino acids. Its carbohydrate side chains are composed of about only two-thirds of Tn determinant and one third of the sialyl-Tn sequence. Desialylation yields a single Tn- glycoprotein (ASG-Tn). Thus, the results of the lectin-ASG-A and ASG-Tn interactions can be expressed as molar ratios reflecting the Tn residues present in both glycoproteins, thereby affording a clear molar interaction profile while such patterns are not easily obtainable from other glycoproteins. The above glycoprotein can also be used as an ideal reagent for detecting the affinity for epitopes of Tn-active lectins or monoclonal antibodies. The purpose of this study is to examine the binding property of these simple sialyl-Tn and/or Tn containing glycoproteins by quantitative precipitin assay.

The results suggest that ASG-A and its desialylated product (ASG-Tn) are highly useful reagents for the differentiation of Tn , T(Galβ-1→3GalNAc), A (GalNAcα1→3Gal) and Gal-specific lectins and monoclonal antibodies against such epitopes.

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Abbreviations: Gal, D-galactopyranose; Glc, D-glucopyranose; LFuc or Fuc, L-fucopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; NeuAc, *N*-acetylneuraminic acid. Abbreviations of lectins and lectin determinants are given in Table 1. ASG-A, armadillo submandibular glycoprotein, fraction A [9] one of the sialic acid containing glycoproteins in armadillo submandibular glands (see text); ASG-Tn, the desialylated ASG-A; HSM, hamster submaxillary glycoproteins; the carbohydrate side chains of HSM were mainly of sialyl-Tn sequence; HSM-Tn, the desialylated HSM containing only Tn (GalNAcα1→Ser/Thr) residues as carbohydrate side chains [21].

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2. Materials and methods

2.1. Lectins

Ricinus communis agglutinin (RCA1) and ricin were purchased from Boehringer Mannheim Biochemical GmbH, Germany. Peanut lectin (PNA), wheat germ agglutinin (WGA) and *Vicia villosa* B_4 (VVA-B_4) were from Sigma Chemical Co., St. Louis, MO, USA. The *Maclura pomifera* (MPA), *Helix pomatia* (HPA), *Dolichos biflorus* (DBA) and *Wistaria floribunda* (WFA) lectins were purified by adsorption to insoluble polyleucyl hog gastric (A+H) mucin [22,23] and eluted by melibiose [15], GalNAc [15,24,25] and lactose [26], respectively. The mistletoe toxic lectin-I (ML-I), provided by Dr. Uwe Pfüller, Universität Witten/Herdecke, Institute of Phytochemistry, Berlin (Germany), was isolated from mistletoe grown on the locust tree (*Robinia pseudoacacia*). It was purified by affinity chromatography using acid-treated agarose as a carrier. Elution was performed with 0.15 M NaCl [27]. *Abrus precatorius* agglutinin (APA) as well as abrin-a were given by Drs. L.P. Chow and J.Y. Lin, Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan. These lectins were purified from the seeds of *A. precatorius* (jequirity bean) by Sepharose 4B and DEAE-cellulose column chromatographies, as described [28].

2.2. Preparation and characterization of sialyl-Tn and Tn glycoproteins

Fraction A of armadillo submandibular glycoprotein was prepared according to the modified method of Tettamanti and Pigman [9,29]. Its chemical composition was analyzed according to the methods described previously [9,29].

For desialylation, a sample of the native glycoprotein, dissolved in 0.01 M HCl at 4 mg/ml was hydrolyzed at 80°C for 90 min and dialyzed for 48 h against frequently changed water. The nondialyzable material was freeze-dried.

2.3. Immunochemical assays

Quantitative precipitin with purified lectins was performed by a microprecipitation technique [30]. Five to 6.0 μg N of lectin was mixed with varying amounts of glycoprotein; the mixture was incubated at 37°C for 1 h and kept at 4°C for 1 week. The N in the washed precipitates was determined by the ninhydrin procedure [31].

3. Results and discussion

The armadillo submandibular glycoprotein (ASG-A) used for this study contained 26.6% (131.5 μmol/100 μg) GalNAc, 13.3% (43 μmol/100 μg) NeuAc and 53.4% total amino acids in which six amino acids (Thr + Ser, 55%; Glu, 9.9%; Gly, 14.4%; Ala, 6.0% and Val, 13.2%) constituted 98.8 mol% of the protein core [9]. Alkaline β-elimination and borohydride reduction [6–9] showed that the carbohydrate side chains of ASG-A were two-thirds Tn determinant and one-third sialyl-Tn sequence. As expected, the desialylated product contained only Tn (GalNAcα1→Ser/Thr) residues as carbohydrate side chains, which contained 31.1% (153.8 μmol/100 μg) GalNAc, and it was defined as ASG-Tn.

To examine the binding properties of ASG-A and ASG-Tn, their reactivities with lectins were characterized by quantitative precipitin assay [30,31]. The lectins tested, their carbohydrate specificity and molecular weight are listed in Table 1. The amount of lectin nitrogen (N) used ranged from 5.0 to 6.0 mg per sample (Table 2) and the washed lectin–glycoprotein precipitates are estimated as microgram nitrogen and also expressed as molar % of precipitation. As shown in Fig. 1 and Table 2, ASG-Tn completely precipitated four Tn specific lectins – *Maclura pomifera* (MPA) (Fig.1a), *Artocarpus integrifolia* (jacalin, AIL) (Fig. 1b), *Vicia villosa* B_4 (VVA-B_4) (Fig. 1c), and *Vicia villosa* A&B (Fig. 1d); less than 1.5 μg of ASG-Tn was required to achieve 50% precipitation. This is one of the best reagents with the ability to completely precipitate MPA and jacalin. In combination with our previously reported data [15,16], we conclude that the carbohydrate specificity of MPA and jacalin should be of a dual nature, and is most specific for the Tn and T sequences or crypto-Tn masked by β-linked Gal. As expected, ASG-Tn was the best reagent to precipitate VVA-B_4,(Fig. 1c). ASG-Tn also reacted strongly with two blood group A specific lectins – *Helix pomatia* (edible snail) (Fig. 1e) and *Wistaria floribunda* (WFA) (Fig. 1f) and precipitated over 94% of the lectin N added. Thus, its specificity can be extended to include the Tn determinant as active determinant and its carbohydrate specificities should be defined as F >A ($\geq A_h$) Tn >T for HPA, and A ($>A_h$), F, Tn>I/II for WFA. This Tn glycoprotein reacted moderately with a T specific lectin – *Abrus precatorius* agglutinin (APA) (Fig. 1g), while its precipitation with DBA (Fig. 1h), RCA1, ML-I, PNA and WGA was weak or negligible (Table 2). Native ASG-A (with sialyl-Tn and Tn determinants) showed complete precipitation with MPA; it also reacted strongly with jacalin, *Vicia villosa*-B_4, *Vicia villosa* A&B, and *Helix pomatia* agglutinins, intermediately with *Wistaria floribunda* agglutinin, and weakly or not at all with other

Table 1
Gal, GalNAc and GlcNAc specific lectins, and their determinants tested for binding properties of armadillo and hamster salivary glycoproteins

No.	Lectin (agglutinin)	MW × 10^{-3}	Determinants[a] (active carbohydrate sequence)
1	*Dolichos biflorus* [DBA]	110–120	F > A_h^b > A > Tn
2	*Helix pomatia* [HPA]	79	F > A(> A_h^c) ≥ Tn, T
3	*Wistria floribunda* [WFA]	68	A (A_h^b,F > Tn,I (II)
4	*Vicia villosa* A&B [VVA-A&B]	134–143	A > A_h and F[d]
5	*Arachis hypogaea* [peanut, PNA]	110	T ⩾ I (II)
6	*Abrus precatorius* (APA)	134	T > I/II > E > B ⩾ Tn
7	*Maclura pomifera* [MPA]	40–43	T > Tn
8	*Artocarpus integrifolia* [jacalin, AIL]	39.5	T > Tn ⋙ I (II)
9	*Vicia villosa* [VVA-B_4]	134	Two Tn ⩾ one Tn ⩾ one or two T
10	Abrin-a	60	Galα1→
11	Ricin	63	T, I/II, L > E & B[e]
12	*Ricinus communis* [RCA_1]	120	II > I > B > T ⩾ Tn
13	Mistletoe toxic lectin-I [ML-I]	115–120	E, L, T, I/II
14	*Triticum vulgaris* [WGA]	43	C_3 > C_2

[a]Carbohydrate specificity of lectins as expressed by lectin determinants – F, GalNAcα1→3GalNAc; A, GalNAcα1→3Gal; [b]A_h, GalNAcα1→3(Fucα1→2)Gal; Tn, GalNAcα1→Ser/Thr; B, Galα1→3Gal; E or P_1 Galα1→4Gal; I/II, Galβ1→3/4GlcNAcβ1→; L, Galβ1→4Glc; T, Galβ1→3GalNAc; C_2, GlcNAcβ1→4GlcNAc. Substitution of Fucα1→2 to subterminal Gal is an important factor for binding. [c]Substitution of Fucα1→2 to subterminal Gal blocks binding. [d]The affinity to Tn has not been determined. [e]Our unpublished results.

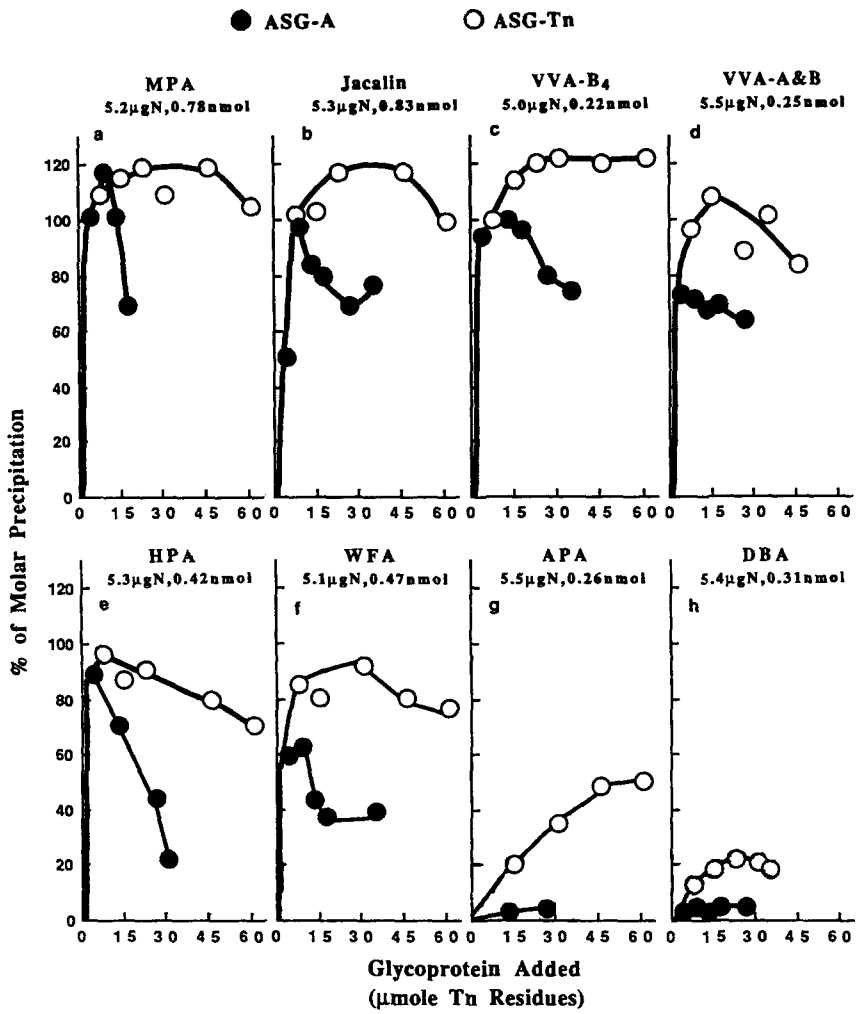

Fig. 1. Quantitative molar precipitin curves of ASG-A (sialyl-Tn and Tn) and asialo- ASG (ASG-Tn) with Gal, GalNAc and GlcNAc specific lectins. The amount of lectin nitrogen ranged from 5.0 to 6.0 μg (0.22 nmol to 0.78 nmol) (Table 1) and that of glycoprotein added, expressed as Tn residues, up to 60 μmol.

$$\text{Mol\% precipitation} = \frac{\text{mol of lectin precipitated} \times 100}{\text{total moles of lectin added}}$$

$$= \frac{\mu\text{g N lectin–glycoprotein complex precipitate} \times 6.25/\text{MW of lectin} \times 100}{\mu\text{g N of lectin added} \times 6.25/\text{MW of lectin}}$$

$$= \frac{\mu\text{g N of lectin–glycoprotein complex precipitate} \times 100}{\mu\text{g N of lectin added}}$$

Here, the contribution of glycoprotein N was not corrected, as the N contribution from the glycoproteins was about or less than 20% when the maximum precipitate was 20 μg. Glycoprotein added (Tn residues in ASG-A added) = μg ASG-A added × (132 μmol total carbohydrate side chains/100 μg − 43 μmol sialyl-Tn residues/100 μg). Tn residues in ASG-Tn added = μg ASG-Tn added × 154 μmol/100 μmol. Total volume: 300 μl; ASG-A and ASG-Tn reacted weakly or negligibly with *Arachis hypogaea* (peanut) agglutinin; Mistletoe toxic lectin-I, ML-I; *Ricinus communis* agglutinin; *Triticum vulgaris* (wheat germ) agglutinin, abrin-a and ricin.

lectins employed (Figs. 1 and 2). The reactivity of ASG-A (sialyl-Tn and Tn) was almost identical to that of ASG-Tn with MPA and weaker or slightly weaker than that of ASG-Tn with other lectins (Table 2 and Fig. 2). The high reactivity of MPA, VVA-B_4 with ASG-A and with asialo-ASG-A indicates that these lectins can cross-react with sialyl-Tn structures, with Tn residues, or both of which present in ASG-A. Carbohydrate analysis of ASG-A indicated that the molar ratio of the NeuAc: GalNAc content was only equal to one-third [9] which indicates that more than two thirds of GalNAc residues are nonsialylated. As shown in Fig. 1, when the ASG-A and ASG-Tn precipitation profiles are expressed as mol% precipitation vs. mol Tn residues added, interaction with bare Tn residues seems to be the major contributor, as ASG-A provides sufficient Tn residues to yield 50% precipitation. Compared to the precipitation profile of ASG-A before and after desialylation, they were very similar before reaching the hight of the peak (up to 10 mmol Tn residues, Fig. 1), but were quite different beyond the peak

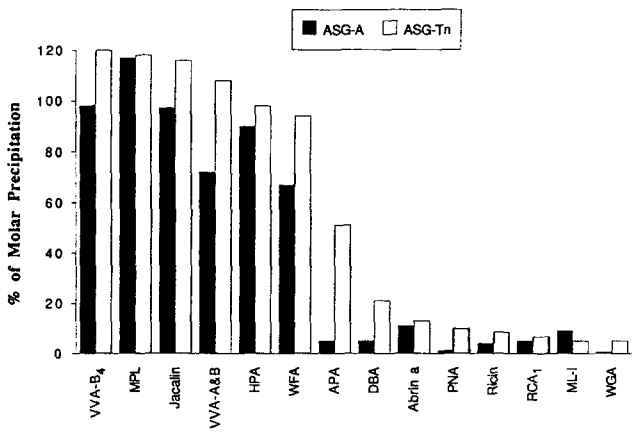

Fig. 2. Comparison of the binding of various lectins with ASG-A (sialyl-Tn and Tn) and asialo-ASG (ASG-Tn). % of μg N precipitated at maximum with the amount of lectin nitrogen added expressed as 100%.

hight (i.e. between 10 to 60 mmol Tn residues, Fig. 1a–f), most likely, because ASG-A requires more lectin molecules to precipitate as lectin–glycoprotein complex, i.e. if a constant number of lectin molecules has to interact with (compete for) an increased number of ASG-A molecules, the resulting dispersal yields a glycoprotein–lectin interaction to weak to trigger a precipitate. The unexpected behaviour of ASG-A with jacalin and WFA at the higher glycoprotein concentration is largely and most likely due to that a higher concentration of glycoprotein results in a broader dispersion of the glycoprotein in the medium, thereby providing greater accessibility which in turn requires a higher amount of glycoprotein to form a lectin complex and a precipitate, (rather than having a small amount of glycoprotein being reacted with a larger proportion of lectin molecules). Thus, the higher molar precipitation values reflect the fact that the amount of nitrogen contributed by the glycoprotein in the complex was not corrected for. From these results, it is being concluded that ASG-A and its asialo product (ASG-Tn) are the best models for studying sialyl-Tn and Tn binding lectins or antibody interactions. They are also the most valuable reagents for studying the differential binding properties of T (Galβ1 → 3GalNAc), Tn (GalNAcα1 → Ser/Thr), and A (GalNAcα1 → 3Gal), and A_h (GalNAcα1 → 3[LFucα1 → 2]-Gal) specific lectins, as well as of anti-T, -Tn, -A and -A_h monoclonal antibodies.

The carbohydrate side chains of armadillo, hamster and ovine salivary glycoproteins are mainly of the Tn (GalNAc α1 → Ser/Thr) and/or sialyl-Tn (NeuAcα2–6GalNAcα1 → Ser/Thr) sequence [7]. After desialylation, almost all of the carbohydrate chains are of the Tn determinant (GalNAcα1 → Ser/Thr). Recently, the binding property of hamster submaxillary glycoprotein (HSM) and of HSM-Tn (desialylated) with lectins was established [21]. When their lectin affinity was compared with the results obtained with ASG-A and ASG-Tn, it was found that both ASG-Tn and HSM-Tn, as shown in Table 2, demonstrate similar reactivity toward all lectins tested, although HSM-Tn is rich in proline (16.2 mol%) and lysine (8.3 mol%). However, the lectin affinity of HSM is quite different from that of ASG-A. Since most of Tn in HSM is sialylated, it reacted mainly with the lectins that have the ability to recognize internal (crypto)-Tn, such as jacalin. ASG-A reacted well with all Tn-active lectins (HPL, WFL, VVA-A4, MPL, jacalin and VVA-B4), because only one-third of Tn in ASG-A is sialylated. Ovine and hamster salivary glycoproteins are also rich in sialyl-Tn or crypto-Tn [7]. Thus, it will be interesting to characterize the binding property of OSM or OSM-Tn (desialylated OSM) with lectins or Tn and sialyl-Tn mono-clonal antibodies, and to compare their differential binding specificities.

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Table 2
Comparative precipitation activities of armadillo and hamster salivary glycoproteins before and after mild acid hydrolysis with Gal, GalNAc, and GlcNAc lectins

No.	Lectins[a] (agglutinins)	Amount of lectin used for precipitation reaction (μg N)	Maximum lectin N precipitated[b] μg N (%)[c]				Amount of glycoprotein required for 50% precipitation μg (μmol Tn residues)[d]			
			ASG-A	HSM	ASG-Tn	HSM-Tn	ASG-A	HSM	ASG-Tn	HSM-Tn
1	DBL	5.4	Tr	Tr	1.2 (21%)	0.9 (17%)	–	–	–	–
2	HPL	5.3	4.8 (90%)	1.2 (23%)	5.2 (98%)	4.7 (87%)	1.0 (0.9)	–	0.5 (0.8)	4
3	WFL	5.1	3.4 (67%)	Tr	4.8 (94%)	4.2 (82%)	2.5 (2.2)	–	2.5 (3.9)	2
4	VVA-A&B	5.5	3.9 (72%)	0	6.0 (108%)	6.2 (113%)	1.5 (1.3)	–	1.0 (1.5)	3
5	PNA	6.0	Tr	Tr	Tr	0	–	–	–	–
6	APA	5.5	Tr	0	2.8 (51%)	–	–	–	25.0 (38.5)	–
7	MPL	5.2	6.0 (117%)	3.1 (60%)	6.2 (119%)	5.5 (106%)	2.5 (2.2)	8	1.0 (1.5)	2
8	Jacalin	5.3	5.2 (97%)	5.0 (94.8%)	6.3 (118%)	5.3 (100%)	3.5 (3.2)	4	1.5 (2.3)	2
9	VVA-B_4	5.0	4.9 (98%)	1.6 (32%)	6.0 (120%)	6.0 (120%)	1.0 (0.9)	–	1.5 (2.3)	2
10	Abrin-a	5.7	0.6 (11%)	Tr	0.7 (13%)	0.4 (7%)	–	–	–	–
11	Ricin	6.0	Tr	Tr	0.5 (8.5%)	1.9 (32%)	–	–	–	–
12	RCA_1	5.9	Tr	Tr	Tr	Tr	–	–	–	–
13	ML-I	5.1	Tr	Tr	Tr	0.4 (8.0%)	–	–	–	–
14	WGA	5.0	Tr	Tr	Tr	0.4 (8.0%)	–	–	–	–

[a] Full name of lectin and carbohydrate specificities are listed in Table 1. [b] The maximum lectin precipitated as μg N of glycoproteins was tested up to 40 μg. [c] The value in parentheses indicates the % of N precipitated at maximum when the amount of lectin nitrogen added is expressed as 100%. Tr, trace or less than 5%; '–', not available; Gp, glycoprotein. [d] μmol Tn residues in ASG-A = μg of ASG-A required for 50% precipitation × μmol of Tn residues in ASG-A/100 μg (= 89 μmol/100 μg); μmol Tn residues in ASG-Tn = μg of ASG-Tn required for 50% precipitation × μmol of Tn residues in ASG-Tn/100 μg (= 154 μmol/100 μg).

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