Lectinochemical Studies on the Binding Properties of a Toxic Lectin (Ricin) Isolated from the Seeds of *Ricinus communis*

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Background: Ricin (RCA_2 or RCA_{60}) is a highly toxic heterodimeric protein found in the seeds of the castor plant *Ricinus communis*. It is a potential biohazard. In the present study, the fine specificity of ricin was defined.

Methods: The combining site of ricin was characterized by quantitative precipitin (QPA) and precipitin inhibition assays (QPIA).

Results: Of 31 glycoproteins and *pneumococcus* type XIV capsular polysaccharide tested, only twelve of them precipitated over 50% of the toxin N added, reflecting poor precipitability of the lectin with the compounds tested. This can be explained by only a single chain (B chain of the molecules) participating in binding. The blood group active glycoproteins after mild acid hydrolysis or Smith degradation, as well as sialic-acid containing glycoproteins after removal of sialic acid, in general, had substantially increased activity. Of the monosaccharides tested for inhibition of precipitation of ricin, *p*-nitrophenyl βGal was the best; this compound was 1.3-fold better than its α-anomer. While methyl βGal was twice as active as its α anomer, Gal and blood group B active disaccharides (Galα1-3Gal) were 2.5 times more active than GalNAc. Among the oligosaccharides tested, Galβ1-3GalNAc (T) Galβ1-3/4GlcNAc (I/II), Galβ1-4Glc (L) and human blood group I Ma trisaccharide (Galβ1-4GlcNAcβ1-6Gal) were about equally active and the best inhibitors. They were about 2.0 and 2.4 more active than Galα1-4Gal (E) sequence and B determinant, respectively.

Conclusion: From the present results, it is concluded that: (a) this toxin has a broad range of affinity for the β-anomer of Gal; (b) its combining site is probably of a shallow groove type and as large as a trisaccharide; (c) Galβ- is the major combining site of the lectin; and (d) hydrophobic interaction gives a significant contribution for binding. This information should facilitate future usage of this lectin in glycobiological research and medical applications.

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Key words: carbohydrate specificities, glycoprotein binding, combining site, toxin, ricin.

Ricin (RCA_2 or RCA_{60}) is the highly toxic fraction of the carbohydrate binding proteins found in aqueous extracts of the castor bean *(Ricinus communis)*. It is a potential biohazard. It agglutinated

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weakly human red blood cells regardless of ABO blood group type, with or without neuraminidase treatment. Ricin is a dimeric protein *Mr* = 60-65 kDa, composed of two different polypeptide chains A and B linked by a single disulphide bridge.[1,2] It was found that the A chain is a catalytic unit capable of inactivating the 60S ribosomal subunit of eukaryotic cells resulting in inhibition of protein synthesis.[3] The B chain binds to cell surface glycoconjugate and thereby permits the entry of the toxic subunit into the cell. The combining site of this toxin has not yet been investigated, especially the binding relationship among many important mammalian oligosaccharide units, such as I/II (Galβ1-3/4GlcNAc), T determinant (Galβ1-3GalNAc), E (Galα1-4Gal, human blood group P_1 disaccharide), and B (Galα1-3Gal, human blood group B active disaccharide) as well as anomers of *p*-nitrophenyl Gal have not yet been investigated. Therefore, in the present study, we have examined the glycan affinity of ricin by quantitative precipitin (QPA) and precipitin inhibition assays (QPIA).[4,5] Comparison of the inhibition profile of ricin (RCA_2) with RCA_1 is also included.[6,7] From the present results, it is concluded that: (a) this toxin has a broad range of affinity for the β-anomer of Gal; (b) its combining site is probably of a shallow groove type and its size is as large as a trisaccharide; (c) Galβ- should be the major combining site of the lectin; and (d) hydrophobic interaction provide a significant contribution for binding.

METHODS

Lectin

Ricin, purified from the aqueous extracts of the castor bean (seeds of *Ricinus communis*) by affinity column chromatography using the method of Lin et al.[8] was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Glycoproteins and polysaccharides

Carbohydrate structural units in mammalian glycoprotein are shown in Table 1. The glycoproteins tested were prepared from human ovarian cyst fluid, saliva, and hog gastric mucosa.[9-15] The blood group A, B, H, Le^a, Le^b and Ii active substances were purified from human ovarian cyst fluid (HOC) by digestion with pepsin, precipitation with increasing concentrations of ethanol and extraction of the dried ethanol precipitates with 90% phenol. The insoluble fraction is named after the blood group substance

Table 1. Carbohydrate Structural Units in Mammalian Glycoproteins [46]

	Codes*	Structural units	Sources
1	Tn	GalNAc α1→Ser/Thr of protein core	Tn antigen, only in glycoproteins.
2	T_α	Gal β1→3GalNAc α1→Ser/Thr of protein core	The mucin-type sugar sequence on the human erythrocyte membrane.
3	I	Gal β1→3GlcNAc	Human blood group type I and II carbohydrate sequences. Branched or linear repeated II sequence is part of blood group I and i epitopes. I and II are precursors of ABH and Le^a, Le^b, Le^x, Le^y blood group active antigens. Most of the lectins reactive with II are also reactive with I. Tri-II and mII determinants are present at the nonreducing end of the carbohydrate chains derived from *N*- and *O*-glycans.
4	II	Gal β1→4GlcNAc	
	II_β	Gal β1→4GlcNAc β1→	
	Tri-II	Triantennary Gal β1→4GlcNAc	
	mII	Multivalent Gal β1→4GlcNAc	
5	A	GalNAc α1→3Gal	Human blood group A active di-saccharide.
	A_h	GalNAc α1→3[LFuc α1→2]Gal	Human blood group A active tri-saccharide.
6	B	Gal α1→3Gal	Human blood group B active di-saccharide.
	B_h	Gal α1→3[LFuc α1→2]Gal	Human blood group B active tri-saccharide.
7	E	Gal α1→4Gal	Blood group p^k and P_1 active disaccharide. Sheep hydatid cyst glycoproteins, salivary glycoproteins of the Chinese swiftlet, glycosphingolipids in human erythrocytes, and small intestine.
8	L	Gal β1→4Glc	Constituent of mammalian milk.
	L_β	Gal β1→4Glc β1→	Lactosyl ceramides in brain and part of carbohydrate structures in gangliosides.

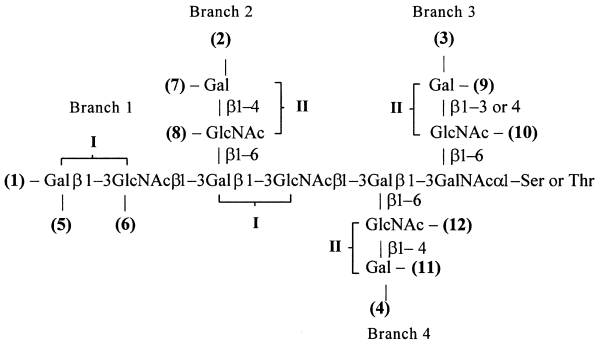
* α, β: anomer of sugars; m: multivalent.

(e.g. Cyst Tighe phenol insoluble, where "Tighe" denotes the HOC sample code). A similar principle was applied to other HOC collections (e.g. Beach, MSS, and N-1, etc). The supernatant was fractionally precipitated by addition of 50% ethanol in 90% phenol to the indicated concentrations.[9] The designation "10 (or 20)% (ppt)" denotes a fraction precipitated from phenol at an ethanol concentration of 10 (or 20)%; "2x" signifies that a second round of phenol extraction and ethanol precipitation was carried out (e.g. Cyst MSS 10% 2x and Cyst N-1 Le^a 20% 2x). The carbohydrate chains of HOC consist of multiple saccharide branches attached by *O*-glycosidic linkages at their internal reducing ends to serine or threonine residues of the polypeptide backbone.[11-13,16] In general, the "P-1" fractions (e.g. Cyst Beach P-1 and Cyst Tighe P-1) represent the nondialyzable portion of the blood group substances after mild hydrolysis at pH 1.5-2.0 for 2 h which removes most of the L-fucopyranosyl end groups, as well as some blood group A and B active oligosaccharide side-chains.[10,17,18] P-1 fractions from HOC gps which expose the internal structures equivalent to those on the blood group precursors are defined as "precursor equivalent gps" (Structure 1).

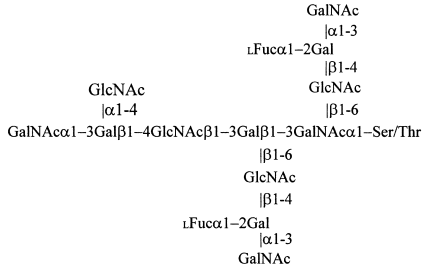
Human α_1-acid glycoprotein and porcine thyroglobulin were purchased from Sigma. Human α_1-acid glycoprotein contains tetra-, tri- and di-antennary complex type glycans in the ratio of 2:2:1.[19,20] Fetuin (Gibco Laboratories, Grand Island, NY, USA), which is the major glycoprotein in fetal calf serum and has six oligosaccharide side chains per molecule, three *O*-glycosidically-linked to Ser/Thr and three *N*-glycosidically-linked to Asn.[21] that contains tri- and di-antennary complex type glycans in the ratio of 1:2.

The rat sublingual glycoprotein (RSL) was prepared by the method of Moschera and Pigman.[22] The established carbohydrate side chains were found to be composed of 9, 10, 12, 13, and 15-sugar residues, respectively, and contain sialic acid, *N*-acetylglucosamine, galactose, and *N*-acetylgalactosaminitol.[23] RSL may also contain the Tn reactive determinants.[24]

Hog gastric mucin #4, a blood group A + H substance (Structure 2), was derived from crude hog stomach mucin as described previously.[25] Treatment of mucin #4 with HCl (pH 2, 100°C, 90 min) yields hog gastric mucin #9, while acid hydrolysis (pH 1.5, 100°C, 2 and 5 hrs) gives hog gastric mucins #14 and 21, respectively. Extensive hydrolysis leads to destruction of blood group activities.[4]



Structure 1. Proposed representative carbohydrate side chains of blood group active glycoproteins, prepared from human ovarian cyst fluid. This structure represents the internal portion of carbohydrate chains to which various human blood group determinants are attached. The four-branched structure (1 to 4) shown is the representative 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. This structure also represents precursor blood group active glycoproteins[13] and can be prepared by Smith degradation of A, B, H active glycoproteins, purified from human ovarian cyst fluids.[11-13,17] Numbers in parentheses indicate the site of attachment for the human blood group A, B, H, Le^a, and Le^b determinants. These determinants as well as the structural units at the nonreducing end are the sources of lectin reactive A/A_h, B, I/II, T, and Tn determinants.[4] This megalo-saccharide of twenty-four sugars has not been isolated. However, most of the carbohydrate chains isolated are parts of this structure.



Structure 2. Proposed structure of carbohydrate side chains of Hog A + H gastric glycoproteins.[25] Blood group A and H active key sugars for ricin. Most carbohydrate side chains are part of this structure.

Ovine, bovine, and porcine submandibular/salivary glycoproteins were purified according to the method of Tettamanti and Pigman[26] with modifications.[27,28] About 75% of the carbohydrate side chains of asialo OSM were GalNAcα1-Ser/Thr (Tn). Asialo PSM contains Galβ1-3GalNAcα1- (T_α) together with Tn and GalNAcα1-3Gal (A) sequences, as most of the outer fucosyl residues and sialic acids are cleaved by mild acid hydrolysis (Structure 3). Native ASG-Tn,[29] a salivary glycoprotein of nine-banded armadillo *(Dasypus novemcinctus mexicanus)* containing only Tn (GalNAcα1-Ser/Thr) as carbohydrate side chains, was isolated from 0.01 M PBS pH 6.8 gland extract after removal of ASG-A, which is one of the sialoglycoproteins in armadillo glands.[30]

Desialylation of sialoglycoproteins was performed by mild acid hydrolysis in 0.01 N HCl at 80°C for 90 min and dialyzed against distilled H_2O for 2 d to remove small fragments.[26,30]

The anti-freeze glycoprotein from the Antarctic fish *(Trematomus borchgrevinki)* which contains only T_α as carbohydrate chains.[31] was provided by Dr. R. E. Feeney (Department of Food Science and Technology, University of California, Davis, CA, USA) through the late Dr. E. A. Kabat (Columbia Medical Center, New York, USA).

The *Pneumococcus* type XIV polysaccharide, isolated from *Streptococcus pneumoniae* capsule,[32] was a generous gift from the late Dr. E.A. Kabat.

(i) GalNAcα1-Ser/Thr (**Tn**)

(ii) Galβ1-3GalNAcα1-Ser/Thr (**T**)

(iii) GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr (**A**)

(iv) LFucα1,2Galβ1-3GalNAcα1-Ser/Thr (**H**)

(v) GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr ($\mathbf{A_h}$)
| α1, 2
LFuc

Structure 3. Porcine salivary mucin gp (PSM). The carbohydrate side chains of this glycoprotein are *O*-glycosidically linked through GalNAc at the reducing end of the carbohydrate side chain to Ser or Thr of the protein core.[28] Twelve kinds of carbohydrate side chains have been isolated. They are composed of one to five sugar residues with Gal β1-3GalNAc α-1-*O*-Thr or Ser as the carbohydrate core region. Substitution by NeuNAc, NeuNGc, GalNAc, or LFuc exhibits blood group A and H activities, respectively. The mild acid treated product of porcine salivary glycoprotein contains mainly mixtures of the above sequences. H, LFucα1-2Gal.

Sugars used for inhibition studies

Mono-, di-, oligo-saccharides and their derivatives were purchased from or prepared by Dextra (Berkshire, UK) and Sigma.

Immunochemical Assays

Quantitative precipitin and precipitin-inhibition assays were performed by a microprecipitin technique[33] using 6.0 µg of lectin nitrogen (*N*) for each determination: total *N* in the washed precipitates was estimated by the ninhydrin method.[34]

RESULTS

Quantitative Precipitin Assays (QPA)

Quantitative precipitin reactions of the purified ricin with various blood group active substances and glycoproteins are seen in Figure 1 and Table 2. Reactions were carried out in 250 µl and some of the differences reflect solubility effects (Fig. 1). The most reactive glycoproteins, cyst Mcdon P-1 and Beach P-1 precipitated over 80% of the lectin N with less than 4 µg being required for 50% precipitation (Fig. 1b and 1d). 1st Smith degraded product of cyst MSS 10% 2x (Fig. 1a), hog gastric mucin before and after mild acid treatment (Fig. 1c), *Pneumococcus* type XIV capsular polysaccharide (Fig. 1f), asialo fetuin (Fig. 1f), asialo RSL (Fig. 1g) and desialized PSM and BSM (Fig. 1h) gave intermediate reaction and precipitated about 2/3 of the lectin nitrogen. Of the soluble complex carbohydrates tested, only twelve of them precipitated over 50% of the toxin N added. This result indicates that RCA_2 has a poor precipitability. This can be explained by only a single chain (B chain of the molecules) responsible for binding. The blood group active glycoproteins after mild acid hydrolysis or Smith degradation as well as sialic acid containing glycoprotein after removal of sialic acid, in general, had substantially increased activity. This indicates that ricin-glycoprotein interaction was strongly masked by sialic acid or Gal substituted residues at non-reducing end.

Quantitative precipitin inhibition assays (QPIA)

The abilities of various sugars to inhibit the precipitation of ricin with desialized porcine sub-

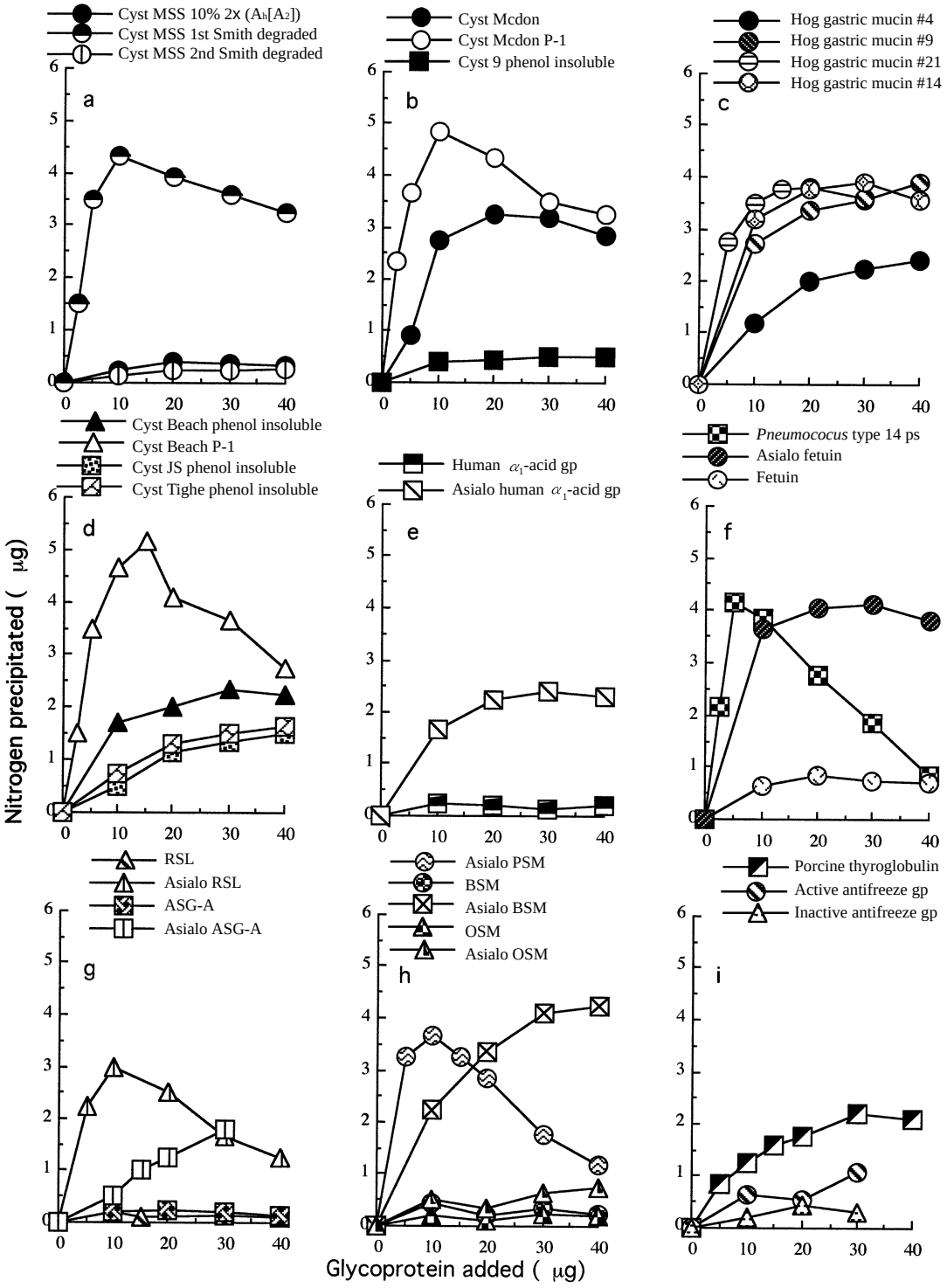


Fig. 1 Quantitative precipitin curves of ricin with glycoproteins and *Pneumococcus* type XIV capsular polysaccharide. Conditions: 6.0 μg ricin nitrogen; total volume was 250 μl.

Table 2. Comparison of Precipitating Activities of Ricin with Various Glycoproteins and Polysaccharides

Curve in Fig. 1	Glycoprotein / Polysaccharide*	Maximum precipitated (μgN)†	GP or PS giving 50% ppt (μg)
a	Cyst MSS 10% 2x (A_1 or A_h)	0.5 (8%)	-
a	Cyst MSS 1st Smith degraded (I, II, T, Tn)	4.4 (73%)	4
a	Cyst MSS 2nd Smith degraded (I, II, T, Tn)	0.3 (5%)	-
b	Cyst Mcdon (A_h)	3.3 (55%)	13
b	Mcdon P-1 (I, II, T, Tn)	4.8 (80%)	3
b	Cyst 9 (A_h)	0.5 (8%)	-
c	Hog gastric mucin #4 (A_h, H)	2.4 (40%)	-
c	Hog gastric mucin #9 (A_h, H)	3.9 (65%)	13
c	Hog gastric mucin #14 (A, I/II)	3.9 (65%)	9
c	Hog gastric mucin #21 (I/II)	3.8 (63%)	6
d	Cyst Beach phenol insoluble (B)	2.3 (38%)	-
d	Beach P-1 (I, II, T, Tn)	5.1 (85%)	4
d	Cyst JS phenol insoluble (H)	1.5 (25%)	-
d	Cyst Tighe phenol insoluble (H, Le^b)	1.6 (27%)	-
e	Human α_1-acid gp (sialyl mII)	0.3 (5%)	-
e	Asialo human α_1-acid gp (mII)	2.4 (40%)	-
f	*Pneumococcus* type 14 ps (II)	4.2 (70%)	2
f	Fetuin (sialyl II, T)	0.8 (13%)	-
f	Asialo fetuin (II, T)	4.1 (68%)	6
g	RSL (sialyl II)	0.2 (3%)	-
g	Asialo RSL (II)	3.0 (50%)	10
g	ASG-A (sialyl Tn)	0.3 (5%)	-
g	Asialo ASG-A (Tn only)	1.8 (30%)	-
h	Asialo PSM (T, Tn, A_h, H)	3.9 (65%)	4
h	OSM-major (sialyl Tn)	0.3 (5%)	-
h	Asialo OSM (Tn)	0.7 (12%)	-
h	BSM (sialyl Tn, GlcNAcβ1-3Tn)	0.4 (7%)	-
h	Asialo BSM (Tn, GlcNAcβ1-3Tn)	4.2 (70%)	15
i	Active antifreeze gp (T_α)	1.1 (18%)	-
i	Inactive antifreeze gp (T_α)	0.5 (8%)	-
i	Porcine thyroglobulin	2.2 (37%)	-

* The symbol in parentheses indicates the human blood group activity and/or lectin determinants.[46] Expressed in bold are: Tn (GalNAcα1-Ser/Thr); T (Galβ1-3GalNAc); I/II (Galβ1-3/4GlcNAc); A (GalNAcα1-3Gal); A_h (GalNAcα1-3 [LFucα1-2]Gal); B (Galα1-3Gal); H (LFucα1-2Gal); RSL: Rat sublingual glycoprotein; ASG-A: Armadillo submandibular glycoprotein; PSM: Porcine submandibular mucin: OSM-major: Ovine salivary glycoprotein; BSM: Bovine submandibular glycoprotein.
† The value in parantheses indicates the % of μgN precipitated at maximum when the amount of lectin added is expressed as 100% (= 6.0 μgN).

mandibular glycoprotein are shown in Figure 2, and the quantities (μmoles) required for 50% inhibition are listed in Table 3. Among the oligosaccharides tested, blood group I Ma-active trisaccharide (Galβ1-4GlcNAc β1-6Gal, line 2 in Fig. 2, Table 3), type II (Gal β1-4GlcNAc, line 3), L (Gal β1-4Glc, line 4), Gal β1-4Man (line 5), type I (Gal β1-3GlcNAc, line 6) and T structure (Gal β1-3GalNAc, line 7), respectively were equally active and about 2.4 times more potent then Gal (line 17). Of the monosaccharides tested, *p*-nitrophenyl βGal, *p*-nitrophenyl αGal and methyl βGal were the most powerful inhibitors with 0.066, 0.084 and 0.084 μmol being required for 50% inhibition (lines 1, 10 and 9). *p*-nitrophenyl βGal was (line 1) was 1.3 and 2.4 times better than *p*-nitrophenyl and methyl αGal (lines 10 and 15), while methyl βGal (line 9) was 1.9 times potent than its α anomer (line 15). This group of inhibitors was about 4.8 and 2.5 times better than GalNAc (line 21), indicating that the *N*-acetamido group at C-2 in the

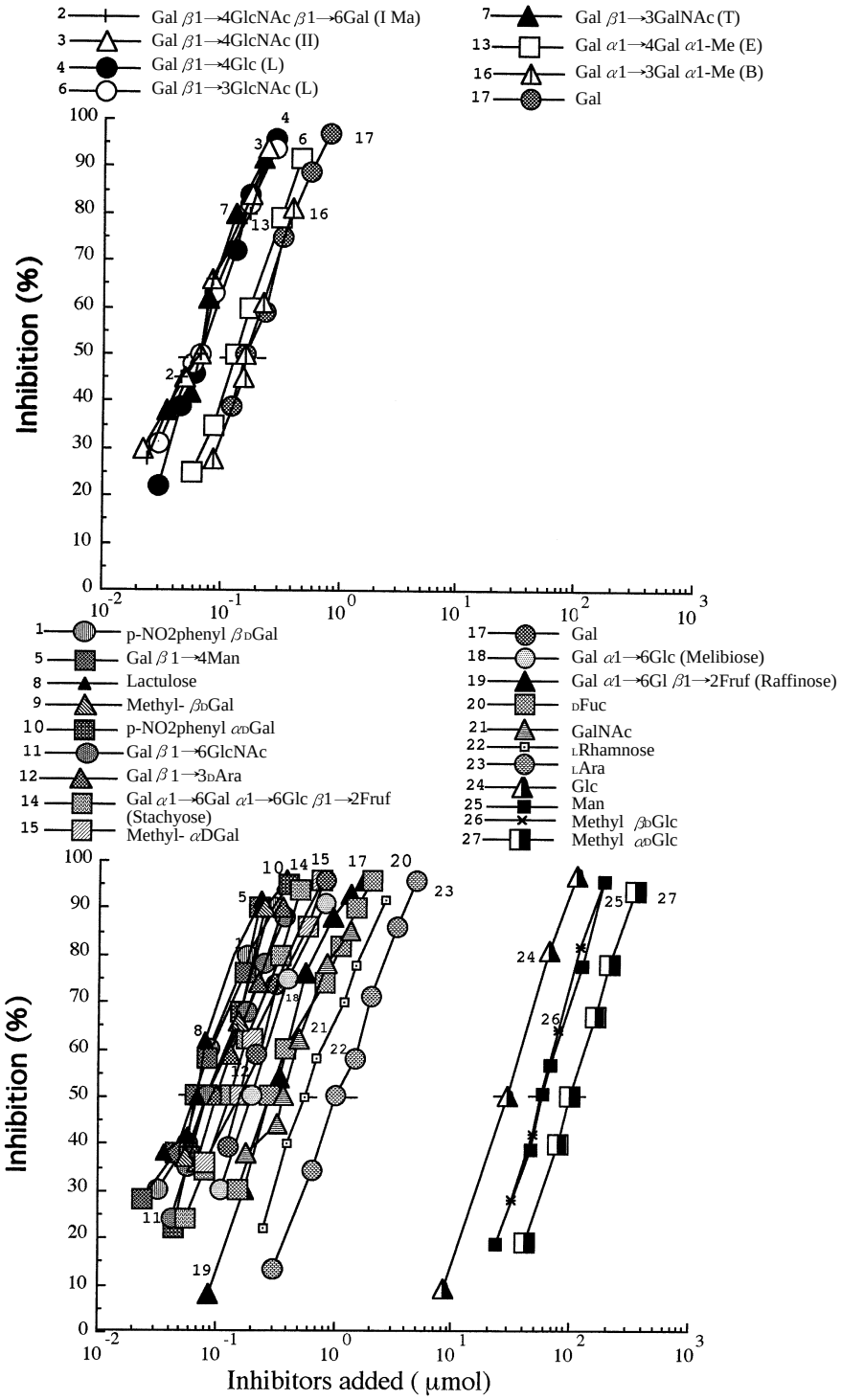


Fig. 2 Inhibition by various monosaccharides and oligosaccharides of the precipitation of ricin with desialized porcine salivary glycoprotein. Total volume was 250 μl.

Table 3. Amount of Various Saccharides Giving 50% Inhibition of Precipitation of Ricin by Desialized Porcine Salivary Glycoprotein*

Line No. in Fig. 2	Inhibitor	Quantity giving 50% inhibition (μmol)	Ka (10^3 M^{-1})	Relative Potency† Ricin (RCA_2)	RCA_1[6,7]‡
1	*p*-NO_2phenyl βGal	0.066	8.5	2.4	14.6
2	Galβ1-4GlcNAcβ1-6Gal (Human blood group I Ma trisaccharide)	0.066	8.5	2.4	9.1
3	Galβ1-4GlcNAc (II)	0.066	8.5	2.4	6.4
4	Galβ1-4Glc (L)	0.066	8.5	2.4	4.1
5	Galβ1-4Man	0.066	8.5	2.4	3.7
6	Galβ1-3GlcNAc (I)	0.066	8.5	2.4	3.2
7	Galβ1-3GalNAc (T)	0.066	8.5	2.4	3.2
8	Lactulose	0.066	8.5	2.4	ND
9	Methyl βGal	0.084	6.7	1.9	2.3
10	*p*-NO_2phenyl αGal	0.084	6.7	1.9	0.6
11	Galβ1-6GlcNAc	0.084	6.7	1.9	3.3
12	Galβ1-3DAra	0.10	5.6	1.6	3.3
13	Galα1-4Gal (E)	0.13	4.3	1.2	1.7
14	Galα1-6Galα1-6Glcβ1-2Fruf (Stachyose)	0.13	4.3	1.2	0.6
15	Methyl αGal	0.16	3.5	1.0	0.8
16	Galα1-3Galα1-Me (B)	0.16	3.5	1.0	1.5
17	Gal	0.16	3.5	1.0	1.0
18	Galα1-6Glc (Melibiose)	0.20	2.8	0.8	1.5
19	Galα1-6Glcβ1-2Fruf (Raffinose)	0.28	2.0	0.6	1.1
20	DFuc	0.28	2.0	0.6	ND
21	GalNAc	0.37	1.5	0.4	0.02
22	LRhamnose	0.54	1.0	0.3	0.8
23	LAra	1.0	0.41	0.2	0.2
24	Glc	30.0	0.019	0.005	0.006
25	Man	56.0	0.010	0.003	ND
26	Methyl βGlc	56.0	0.010	0.003	ND
27	Methyl αGlc	100.0	0.006	0.001	ND

* 6.0 μg N of ricin + 5 μg of desialized porcine salivary glycoprotein. Total volume was 250 μl.
† Relative potency of Gal taken as 1.0.
‡ Data taken from references 6 and 7.
Lectin abbreviation: RCA_1 = *Ricinus communis* agglutinin 1; ND = not determined.

pyranose ring blocks reactivity. DFuc (line 20) was 1/2 as active as Gal (line 17), implying that the OH group on C-6 participates in binding; LArabinose which has the same configuration as DGal but lacks the CH_2OH of C_6 was about 1/5 as active as Gal, an indication that the CH_2OH of C_6 is necessary for binding. Glc (line 24) and Man (line 25) showed 1/200 and 1/333 of Gal activity, demonstrating that the configuration of carbon-4 and carbon-2 in Gal are essential for binding. Melibiose (Galα1-6Glc) and raffinose (Galα1-6Glcβ1-2DFruf) were equally active but 1.5 and 2.0 times less active than stachyose (Galα1-6Galα1-6Glcβ1-2Dfruf) (lines 18 and 19 vs. line 14), demonstrating that the carbohydrate-binding site of the lectin for the α-anomer of Gal can accommodate up to trisaccharide structure.

DISCUSSION

Ricin, like other plant toxins such as abrin from the seeds of Jequiriti beans (*Abrus precatorious*), mistletoe lectin from mistletoe plant (*Viscum album*) and modeccin from the roots of *Adenia digitata* are galactose specific lectins,[35] has played interesting and important roles in the history of clinical medicine and biomedical research.[36] During the past two

decades, this cytotoxic lectin has been cloned and sequenced.[37-39] and its protein crystal structure resolved,[40,41] but its fine specificity profile and the precise combining sites have not been established. Furthermore, the binding relationship of lectin determinants among II, T and Tn in ricin was obscure.[42-44] Therefore, in the present study, we characterized the binding properties of ricin by quantitative precipitin (QPA) and precipitin-inhibition assays (QPIA) which can provide insight into the specificities and size parameters for its combining site. During the past two to three decades, this system has been used as an important tool to characterize the affinity of lectins.[4]

In this study, the blood group precursor equivalent gps (Structure 1), such as cyst Mcdon P-1 and Beach P-1 were the best and precipitated over 80% of the lectin N. Of the soluble complex carbohydrates tested, only twelve of them precipitated over 50% of the toxin N added. This result indicates that ricin has a poor precipitability. This can be explained by only a single chain (B chain of the molecules) responsible for binding. The blood group active glycoproteins after mild acid hydrolysis or Smith degradation as well as sialic acid containing glycoprotein after removal of sialic acid, in general, had substantially increased binding activity. This result is in good agreement with Baenziger and Fiete[43] who reported that the presence of sialic acid substituents on Gal of I/II or T/Tn markedly reduced the binding reactivity. Thus, sialic acid plays an important masking effect on cell interactions. The poor precipitation of ricin with the active and inactive antifreeze glycoproteins, which are composed of repeat units of Gal β1-3GalNAc can be ascribed to the glycotopes masked by steric effects or GalNAc for reaction. This case may be similar to the result obtained by another T-specific lectin, amaranthin of the seeds of *Amaranthus caudatus*.[45] The poor reactivity of ricin with desialized ovine submandibular gps, which contains over 75% of Tn determinants and asialo armadillo salivary gps whose carbohydrate side chains are exclusively made up of Tn determinants (Table 2) can be explained by the high-density of Tn residues along the peptide chains which prevents the lectin domains to access the Tn glycotopes.

Among the monosaccharides tested for inhibition of precipitation of ricin, *p*-nitrophenyl βGal was the best; this compound was 1.3-fold better than its α anomer. While methyl βGal was twice as active as its α anomer, Gal and blood group B active disaccharides (Galα1-3Gal) were 2.5 times more active than GalNAc. Among the oligosaccharides tested, Gal β1-3GalNAc (T) Gal β1-3/4GlcNAc (I/II), Gal β1-4Glc (L) and human blood group I Ma trisaccharide (Gal β1-4GlcNAc β1-6Gal) were about equally active and the best inhibitors. They were about 2.0 and 2.4 more active than Gal α1-4Gal (E) sequence and B determinant, respectively.

It is of interest to compare ricin (RCA_2) with RCA_1 (*Ricinus communis* agglutinin 1; Tables 3 and 4).[6,7] Ricin is a potent cytotoxin but a weak hemagglutinin, whereas RCA_1 is a weak cytotoxin and a powerful hemagglutinin. As illustrated in Table 3, the interaction of ricin with simple sugars is similar, but not identical to that of the agglutinin (RCA_1). Melibiose and raffinose were 80% and 60% less active than Gal in causing inhibition of the precipitin reactions of glycoproteins by ricin; both results are different from those found with the agglutinin. Though N-acetyllactosamine (Gal β1-4GlcNAc) and human blood group I Ma trisaccharide (Gal β1-4GlcNAc β1-6Gal) are 6.4 and 9.1 times more effective as inhibitors of the agglutinin than Gal, *p*-NO_2 phenylβGal was the best inhibitor of the agglutinin and 6.3 times more potent than the methyl analogue. On the otherhand, ricin showed equal potency with type II, L (Galβ1-4Glc), T structure (Galβ1-3GalNAc), blood group I Ma-active trisaccharide and Galβ1-4Man. Besides, in both toxin and agglutinin, the specificity for *p*-nitrophenyl and methyl glycosides, the β-anomer of Gal is preferred over the α-anomer, and that hydrophobic forces are important for interaction. However, the ratio of GalNAc/Gal in ricin was 20 times less than that of agglutinin (Table 4). Furthermore, the relative inhibitory power of some important oligosaccharides with ricin and RCA_1 differs considerably. For instance, the values of the relative potencies of Gal β1-4GlcNAc (II), Galβ1-4GlcNAcβ1-6Gal (I Ma), p-NO_2 phenyl βGal, and Gal β1-3GalNAc (T) are strikingly different. These variations support the concept that every lectin with its unique amino acid sequence has its own binding characteristics.[46]

Overall, the conclusions of this study are the following: [a] from the present and previous results, it can be deduced that the combining site of lectin domains in ricin should be a shallow groove-type, recognizing Galβ1-4Glc as the major binding site;

Table 4. Variations in Binding to Monosaccharides and Their Glycosides of Ricin and RCA_1*

Lectin	Specificity for *p*-nitrophenyl glycosides	Specificity for methyl glycosides	Hydrophobicity (*p*-nitrophenyl vs methyl glycosides)	GalNAc/Gal (RP)†
Ricin	$\beta > \alpha$	$\beta > \alpha$	yes	2.5
RCA_1 (6, 7)	$\beta > \alpha$	$\beta > \alpha$	yes	50.0

* Values are calculated from moles required for 50% inhibition.

† Expressed as reciprocal of relative potency.

[b] its binding size may be as large as a trisaccharide of the β-anomer of Gal; [c] illustration of the high specificity of ricin for clustered II/I and Tn glycotopes (Fig. 1; Table 2); [e] configuration at carbon-2, -3, -4 and -6 are essential for binding; [f] hydrophobic forces are important for Gal β- interaction (Fig. 2); [g] Gal was 2.5 times more active than GalNAc, indicating that CH_3CO in $NH.CH_3CO$ decreases reactivity; [h] sialic acid blocks the access of reactive glycotopes to ricin binding; and [i] on a micromolar basis, the carbohydrate specificity of ricin for mammalian disaccharide structural units can be mapped as: II/I ≥ L ≥ T > E > B. This study not only demonstrates the structural basis for the interaction of ricin with natural glycans and oligosaccharides, but also explains the biological activities of ricin with respect to toxin-receptor recognition processes.

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REFERENCES

1. Olsnes S, Pihl A. Different biological properties of two constituent peptide chain of ricin, a toxic protein inhibiting protein synthesis. Biochemistry 1973;12:3121-6.
2. Despeyroux D, Walker N, Pearce M, Fisher M, McDonnell M, Bailey SC, Griffiths GD, Watts P. Characterization of ricin heterogeneity by electrospray mass spectrometry, capillary electrophoresis and resonant mirror. Anal Biochem 2000;279:23-36.
3. Sharon N, Lis H. Lectins, London: Kluwer Academic Publishers, 2003.
4. Wu AM, Lin SR, Chin LK. Defining the carbohydrate specificities of *Abrus precatorius* agglutinin as T (Galβ1 →3GalNAc) > I/II (Gal β1-3/4GlcNAc). J Biol Chem 1992;267:19130-9.
5. Wu AM, Song SC, Chang SC, Wu JH, Chang KSS, Kabat EA. Further characterization of the binding properties of a GalNAc specific lectin from *Codium fragile* subspecies tomentosoids. Glycobiology 1997;7:1061-6.
6. Wu AM, Sugii S, Gruezo FG, Kabat EA. Immunochemical studies on the Galβ1→4(3)GlcNAc specificity of *Ricinus communis* agglutinin. Carbohydr Res 1988;178:243-57.
7. Wu JH, Herp A, Wu AM. Defining carbohydrate specificity of *Ricinus communis* agglutinin as Gal β1→ 4GlcNAc (II) > Gal β1→3GlcNAc (I) > Galα1→3Gal (B) > Gal β1→3GalNAc (T). Mol Immunol 1993;30:333-9.
8. Lin JY, Lee TC, Hu ST, Tung TC. Isolation of four isotoxic proteins and one agglutinin from jequiriti bean (Abrus precatorius). Toxicon 1981;19:41-51.
9. Kabat EA. Blood Group Substances: Their Chemistry and Immunochemistry. New York: Academic Press, 1956:135-9.
10. Allen PZ, Kabat EA. Immunochemical studies on blood groups XXII. Immunochemical studies on the nondialyzable residue from partially hydrolyzed blood group A, B, and O (H) substances (P_1 fraction). J Immunol 1959;82:340-57.
11. Lloyd KO, Kabat EA. Immunochemical studies on blood groups. XLI. Proposed structures for the carbohydrate portion of blood group A, B, H, Lewis^a and Lewis^b substances. Proc Nat Acad Sci USA 1968;61:1470-7.
12. Maisonrouge-McAuliffe F, Kabat EA. Immunochemical studies on blood groups. Heterogeneity of oligosaccharides liberated by degradation with alkaline borohydride of two human ovarian cyst fractions differing in B, I, and i activities and in reactivity toward concanavalin A. Arch Biochem Biophys 1976;175:81-9.
13. Wu AM. Structural concepts of the blood group A, B, H, Le^a, Le^b, I and i active glycoproteins purified from human ovarian cyst fluid. Adv Exp Med Biol 1988;228:351-94.
14. Wu AM, Kabat EA, Pereira MEA, Gruezo FG, Liao J. Immunochemical studies on blood groups: the internal structure and immunological properties of water-soluble human blood group A substance studied by Smith degradation, liberation and fractionation of oligosaccharides and reaction with lectins. Arch Biochem Biophys 1982;215:390-404.

15. Wu AM, Kabat EA, Nilsson B, Zopf DA, Gruezo FG, Liao J. Immunochemical studies on blood groups. Purification and characterization of radioactive ^{3}H-reduced di- to hexasaccharides produced by alkaline beta-elimination-borohydride ^{3}H-reduced of Smith degraded blood group A active glycoproteins. J Biol Chem 1984;259:7178-86.
16. Rovis L, Anderson B, Kabat EA, Gruezo FG, Liao J. Structures of oligosaccharides produced by base-borohydride degradation of human ovarian cyst blood group H, Le^{b}, and Le^{a} active glycoproteins. Biochemistry 1973;12:5340-54.
17. Kabat EA, Baer H, Bezer AE, Knaub V. Immunochemical studies on blood groups VII. Chemical changes associated with destruction of blood group activity and enhancement of the type XIV cross reactivity by partial hydrolysis of hog and human blood group A, B, and O substances. J Exp Med 1948;88:43-57.
18. Leskowitz S, Kabat EA. Immunochemical studies on blood groups XV. The effect of mild acid hydrolysis on the glucosamine and galactosamine in blood group substances. J Amer Chem Soc 1954;76:5060-5.
19. Fournet B, Montreuil J, Strecker G, Dorland L, Haverkamp J, Vliegenthart JFG, Binette JP, Schmid K. Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma α_1-acid glycoprotein by 360-MHz ^{1}H-NMR spectroscopy and permethylation analysis. Biochemistry 1978;17:5206-14.
20. Fournier T, Medjoubi NN, Porquet D. Alpha-1-acid glycoprotein. Biochim Biophys Acta 2000;1482:157-71.
21. Nilsson B, Norden NE, Svensson S. Structural studies on the carbohydrate portion of fetuin. J Biol Chem 1979;254:4545-53.
22. Moschera J, Pigman W. The isolation and characterization of rat sublingual mucus-glycoprotein. Carbohydr Res 1975;40:53-67.
23. Slomiany A, Slomiany BL. Structures of the acidic oligosaccharides isolated from rat sublingual glycoprotein. J Biol Chem 1978;253:7301-6.
24. Wu AM, Herp A, Song SC, Wu JH, Chang KSS. Interaction of native and asialo rat sublingual glycoproteins with lectins. Life Sci 1995;57:1841-52.
25. van Halbeek H, Dorland L, Vliegenthart JFG, Kochetkov NK, Arbatsky NP, Derevitskaya VA. Characterization of the primary structure and the microheterogeneity of the carbohydrate chains of porcine blood-group H substance by 500-MHz ^{1}H-NMR spectroscopy. Eur J Biochem 1982;127:21-9.
26. Tettamanti G, Pigman W. Purification and characterization of bovine and ovine submaxillary mucins. Arch Biochem Biophys 1968;124:41-50.
27. Herp A, Wu AM, Moschera J. Current concepts of the structure and nature of mammalian salivary mucous glycoproteins. Mol Cell Biochem 1979;23:27-44.
28. Herp A, Borelli C, Wu AM. Biochemistry and lectin binding properties of mammalian salivary mucous glycoproteins. Adv Exp Med Biol 1988;228:395-435.
29. Wu AM, Wu JH, Shen F. Interaction of a novel Tn (GalNAc alpha 1→Ser/Thr) glycoprotein with Gal, GalNAc and GlcNAc specific lectins. Biochem Biophys Res Commun 1994;198:251-6.
30. Wu AM, Pigman W. Preparation and characterization of Armadillo Submandibular Glycoproteins. Biochem J 1977;161:37-47.
31. De Vries AL, Komatsu SK, Feeney RE. Chemical and physical properties of freezing point-depressing glycoproteins from antarctic fishes. J Biol Chem 1970;245:2901-8.
32. Lindberg A, Lönngren J, Powell DA. Structural studies on the specific type-14 *pneumococcal* polysaccharide. Carbohydr Res 1977;58:177-86.
33. Kabat EA, Mayer MM. Experimental Immunochemistry (2nd Ed). Thomas, Springerfield, IL , 1961.
34. Schiffman G., Kabat EA, Thompson W. Immunochemical studies on blood groups. XXX. Cleavage of A, B and H blood group substance by alkali. Biochemistry 1964;3:113-20.
35. Goldstein IJ, Poretz RD. Isolation, physiochemical characterization, and carbohydrate -binding specificity of lectins. In: Liener IE, Sharon N, Goldstein IJ, eds. The Lectins, Properties, Functions and Applications in Biology and Medicine. New York: Academic press, 1986:33-247.
36. Olsnes S. The history of ricin, abrin and related toxins. Toxicon 2004;44:361-70.
37. Halling KC, Halling AC, Murray EE, Ladin BF, Houston LL, Weaver RF. Genomic cloning and characterization of a ricin gene from Ricinus communis. Nucl Acid Res 1985;13:8019-33.
38. Lamb FI, Roberts LM, Lord JM. Nucleotide sequence of cloned cDNA coding for preproricin. Eur J Biochem 1985;148:265-70.
39. Montfort W, Villafranca JE, Monzingo AF, Ernst S, Katzin B, Rutenber E, Xuong NH, Hamlin R, Robertus JD. The three-dimensional structure of ricin at 2.8A. J Biol Chem 1987;262:5398-403.
40. Rutenber E, Katzin BJ, Collins EJ, Mlsna D, Ernst S, Ready MP, Robertus JD. The crystallographic refinement of ricin at 2.5A resolution. Proteins 1991;10:240-50.
41. Lord JM, Roberts LM, Robertus JD. Ricin: structure, mode of action, and some current applications. FASEB J 1994;8:201-8.
42. Nicolson GL, Blaustein J, Etzler ME. Characterization of two plant lectins from *Ricinus communis* and their quantitative interaction with a murine lymphoma. Biochemistry 1974;13:196-204.
43. Baenziger JU, Fiete D. Structural determinants of *Ricinus communis* agglutinin and toxin specificity for oligosaccharides. J Biol Chem 1979;254:9795-9.
44. Debray H, Decout D, Strecker G, Spik G, Montreuil J. Specificity of twelve lectins towards oligosaccharides and

glycoeptides related to *N*-glycosylproteins. Eur J Biochem 1981;117:41-55.
45. Rinderle SJ, Goldstein IJ, Matta, KL, Ratcliffe MR. Isolation and characterization of Amaranthin, a lectin present in the seeds of *Amaranthus caudatus*, that recognizes the T- (or Cryptic T)-antigen. J Biol Chem 1989;264:16123-131.
46. Wu AM. Carbohydrate Structural Units in glycoproteins and polysaccharides as important ligands for Gal and GalNAc reactive lectins. J Biomed Sci 2003;10:676-88.

用凝集素化學法探討研究篦麻毒素 (Ricin) 的結合性質

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背 景： 篦麻毒素是從篦麻種子中萃取之有毒性蛋白。

方 法： 利用定量沉澱反應 (QPA) 及定量沉澱抑制反應 (QPIA) 檢測篦麻毒素之結合位置。

結 果： 它和 31 種醣蛋白、肺炎雙球菌 XIV 類型莢膜的多醣體進行定量沉澱反應時，當其活性與篦麻凝集素 (RCA1) 比較時，結果僅有 12 種醣蛋白會與篦麻毒素引起 50% 含氮 (N) 量的沉澱-表示這種毒素與這些醣蛋白有不佳的沉澱反應。可能是因爲篦麻毒素僅有一個單鏈能參與醣蛋白結合。當血型活性蛋白在中等酸性下水解或史密斯降級作用及醣蛋白在移除唾液酸後與篦麻毒素反應的活性會適度增加。另外用單糖及寡糖與篦麻毒素進行定量沉澱抑制反應時，顯示β異位體要比α異位體物有更大的抑制效果，而 Gal 和血型 B 活性雙糖比 GalNAc 有 2.5 倍大抑制效果。另外 Galβ1-3GalNAc(T)、Galβ1-3/4GlcNAc (I/II)、Galβ1-4Glc (L) 和人體血型 I Ma 三元糖的抑制效果相等，而且比 Galα1-4Gal (E) 與血型 B 決定因子有 2 倍及 2.4 倍抑制效果。

結 論： 根據以上結果顯示 (1) 篦麻毒素對大多數(異位體的醣有較佳親和力 (2) 此凝集素的化合位置是一個淺凹槽型大小至少有三元糖的尺寸 (3)Galβ- 是凝集素主要之結合位置 (4) 疏水性交互作用對結合作用有重大貢獻。

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關鍵字： 篦麻毒素，碳水化合物的特異性，醣蛋白結合性。

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