RESEARCH NOTE

The Carbohydrate-binding Specificity of a Highly Toxic Protein from *Abrus pulchellus* Seeds

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A highly toxic protein, which exhibits similar effects to ribosome inactivating proteins (RIPs type II), isolated from *Abrus pulchellus* seeds kills mice in a few hours after injection in the peritoneal cavity (MV Ramos et al. 1998a *Toxicon 36:* 477-484). It is well documented in the literature that some toxins mediate their incursion by recognizing glycoconjugates on the cell surface membrane before translation. Plant and microbial toxins have been studied in detail mainly to understand their intrinsic toxic pathway. However, the toxin recognition mechanism by membrane receptors and its translation through the membrane is poorly investigated, excepting abrin, the most investigated plant toxin. It has been determined that the initial recognition step must be mediated by carbohydrate-protein interaction. The ribosome inactivating protein from the seeds of *Viscum album* is a typical protein of this class and its carbohydrate-binding specificity was carefully investigated (H Debray et al. 1994 *Glycoconjugate J 11*: 550-557). Although the RIPs type II are commonly specific for galactose residues, more complex carbohydrates are distinctly recognized.

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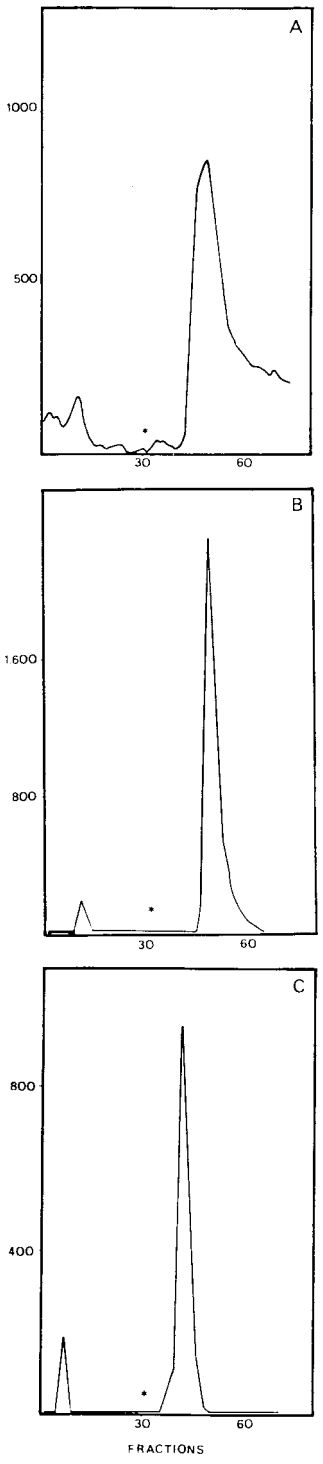
Thus, to well define the carbohydrate moiety that interacts with a toxin, a number of carbohydrates (monosaccharides, di- tri- and oligosaccharides) and complex glycans from glycoproteins should be tested. In this respect, we have investigated the carbohydrate-binding specificity of a toxin from *A. pulchellus* seeds by the combination of haemagglutination inhibition assay and affinity chromatography using immobilized glycoproteins.

The toxin was isolated following the procedure previously described (Ramos et al. 1998a *loc. cit.*) and the haemagglutinating activity induced by the toxin was studied using rabbit and human ORh$^+$ erythrocytes. Carbohydrates, glycoproteins and immobilized glycoproteins columns were obtained from Sigma Chemical Co. Haemagglutination inhibition assays were performed in U-bottomed plates as formally described (RA Moreira & JC Perrone 1977 *Plant Physiol 59*: 783-787). Affinity chromatography on avidin-Sepharose, fetuin-agarose and mucin-Sepharose columns with the purified toxin were carried out in the same conditions described in the Figure.

The haemagglutinating activity induced by the *A. pulchellus* seed toxin was not inhibited by a large range of mannose/glucose monosaccharides and their derivatives. Carbohydrates as D-idose, D(+)xylose, D(+)ribose and most of the glycoproteins tested were not recognized by the toxin (Table I). Although the RIPs type II are commonly described as galactose-specific proteins, some galactose derivatives are more potent inhibitors, like methylated forms or lactose (b-D-Gal-[1® 4]-a-D-Glc) (Table II). Interestingly, the *p*-nitrophenyl groups attached to C1 of galactose ring did not increase the inhibitory potency. In general, for plant carbohydrate-specific proteins, such as true lectins, it has been shown that the hydrophobic interactions established between amino acid residues in the vicinity of the carbohydrate-binding site and the *p*-nitrophenyl group, enhances the affinity of the complex (MV Ramos et al. 1996 *Rev Bras Fisiol Veg 8*: 193-199). Remarkably, the inhibition patterns obtained using rabbit erythrocytes differ from those with human ORh$^+$ cells. This may be due to the differential distribution of potential receptors on the cell surface membrane in each erythrocyte, or their accessibility to the toxin binding-site. Therefore, the use of two cell lines is a strategy to better determine the inhibitory effect of sugar and glycoproteins. Regarding the inhibitory effect of glycoproteins, only asialofetuin (possessing a triantennary *N*-linked glycan), glycophorin (*O*-linked and *N*-linked glycans), human lactotransferrin (biantennary *N*-linked glycan, usually fucosylated) and porcine thyroglobulin (possess-



Affinity chromatography on fetuin-agarose (A), porcine stomach mucin-Sepharose (B) and avidin-Sepharose (C) columns. Samples: 10 $mg.ml^{-1}$, flow rate 30 $ml.h^{-1}$. The columns were first washed with equilibrium solution (tris-HCl 0.1 M pH 7.4) containing 0.15 M NaCl and $CaCl_2/MnCl_2$ 5 mM. After elution of unbound material, the retained fraction was obtained using 0.1 M glycine-HCl, pH 2.6 containing NaCl 0.15 M (*). Absorbance of fractions (1 ml) was recorded at 280 nm.

ing highly heterogeneous *N*-linked glycans) were recognized by *A. pulchellus* toxin. Glycophorin was only active when tested against human ORh^+ erythrocytes. With the excepting of glycophorin which presents both *O*- and *N*-linked glycan structures, these glycoproteins are characterized by the presence of complex glycan *N*-acetyllactosamine type of high heterogeneity, branched to asparagine residues. However, glycophorin a glycoprotein largely present in human erythrocytes membranes possesses a well defined glycan structure *O*-linked to serine residues, composed of Ser-GalNAc-[b1® 3Gal-a2® 3NANA]a2® 6NANA which may be a receptor for the toxin in human erythrocytes. The interaction with human lactotransferrin, asialofetuin and porcine thyroglobulin glycan structures of high heterogeneity may explain why the plant toxin RIPs can invade almost all types of Eukariotic cells of unrelated organisms, systems or organs.

Affinity chromatography on fetuin-agarose and porcine stomach mucin-Sepharose did not reproduce the result from inhibition tests (Fig.). The toxin interacted with the immobilized glycoproteins although inhibition tests failed to reveal the interactions. According to the glycan structure of the desyalylated fetuin (asialofetuin), the presence of *N*-acetylneuraminic acid in the terminal nonreducing extremity of fetuin could mask the recognition when the inhibition process between the free glycoprotein and a membrane receptor is carried on. However, the toxin strongly interacted with immobilized fetuin. Avidin and porcine stomach mucin columns were also good matrix to fix the toxin. These results suggest that differences in the spatial conformation of a glycan chain in glycoprotein solution may significantly differ from that of immobilized glycoproteins. The high flexibility of these structures in glycoproteins may interfere on the recognition process. In fact, conflicting results concerning lectin-receptor interaction was previously discussed (ME Etzler 1994 *Glycoconjugate J 11*: 395-399). Although the direct interaction of the toxin with either matrix (agarose or Sepharose) could occur, in our extensive work with different carbohydrate-binding proteins, it has never been observed that a protein interacts with the matrix in the presence of an attached ligand. A typical example of this, is the galactose-specific lectin from *Artocarpus incisa* seeds which binds strongly to agarose-galactose column but not to agarose-fetuin or Sepharose-mucin (MV Ramos et al. 1998b *Physiol Mol Biol Plants 4:* 157-163). Furthermore, when a macromolecule (glycoprotein) is attached, the accessibility of a protein (the toxin in this case) to the main carbohydrate-chain (oli-

TABLE I

List of non-inhibitory substances of the *Abrus pulchellus* seed toxin haemagglutinating activity

Substances	
D-Idose[a]	a-D(+) Glucopyranoside[a]
a-D-Glc-[1® 4]-a-D-Glc-[1® 4]-D-Glc[a]	b-D(+) Glucopyranoside[a]
D-glucuronic acid[a]	Methyl-a-Glucopyranoside[a]
D(-)Fructose[a]	D(+)-Glucopyranoside-6-Phosphate[a]
b-D-Gal-[1® 4]-b-D-Glc[a]	r-Nitrophenyl-b-D-Glucopyranoside[a]
2-acetamido-2-deoxi-4-*O*-b-D-galactopyranosyl-D-glucopyranose[a]	2-amino-2-deoxy-D-glucopyranoside[a]
D(+)Xylose[a]	2-acetamido-2-deoxy-D-glucopyranoside[a]
L(+)Arabinose[a]	*N*-Acetyl-a-D-Glucosamine-1-Phosphate[a]
D(-)Arabinose[a]	Poly-[1® 4]-b-D-glucosamine[b]
D(-)Ribose[a]	Poly-[1® 4]-b-*N*-acetyl-D-glucosamine[b]
D-Ribose-5-Phosphate[a]	D(+)Mannopyranoside[a]
b-D-Glc-[1® 4]-D-Glc[a]	D(+)Mannose-6-Phosphate[a]
a-D-glucopyranosyl-b-D-fructofuranoside[a]	2-amino-2-deoxy-D-mannopyranoside[a]
4-*O*-a-D-Glucopyranosyl-D-glucopyranoside[a]	2-acetamido-2-deoxy-a-D-mannopyranoside[a]
O-a-D-Glucopyranosyl-[1® 3]-*O*-b-D-Fructofuranosyl-[2® 1]-a-D-Glucopyranoside[a]	2-acetamido-2-deoxy-b-D-mannopyranoside[a]
4-*O*-b-D-Galactopyranosyl-D-fructofuranoside[a]	2-acetamido-2-deoxy-3-*O*-[D-1'-carboxyethyl]-D-glucopyranose[a]
a-D-Glucopyranosyl-a-D-glucopyranoside[a]	2-amino-3-*O*-[1-carboxyethyl]-2-deoxy-D-glucopyranoside[a]
bovine serum albumin[b]	*N*-acetylneuraminic acid[a]
fetuin[b]	human apotransferrin[b]
bovine apotransferrin[b]	ovalbumin[b]
Orosomucoid[b]	ovomucoid[b]
bovine submaxilary mucin[b]	stomach porcine mucin[b]
bovine g-globulin[b]	ribonuclease A[b]
human lactotransferrin[b]	human albumin[b]
carraginin[b]	mannan[b]

Not inhibitory even at the following concentrations – *a*: 33.3 mM, and *b*: 330 mg.ml^{-1} using 2% human ORh^{+} and rabbit erythrocytes suspensions.

gosaccharide) composing the matrix is highly reduced.

Although the haemagglutinating activity is not a powerful technique to define minor differences in affinity between molecular partners, the recognition of complex *N*-linked antennary glycan chain and *O*-linked shorter carbohydrate moiety (as in glycophorin) covalently attached to different glycoproteins, strongly suggests that the receptors for plant RIPs may be widespread in nature. Firstly, this toxin, and others previously studied, all interact with *N*-linked and *O*-linked glycan structures. Secondly, it is well established that these glycan structures occur on a large number of cell surface glycoproteins. Therefore, we can suggest that these structures may be present in different cells surfaces. Accordingly, RIPs type II usually invade all Eukariotic cells disregarding their origin.

TABLE II

Haemagglutinating activity inhibition of the *Abrus pulchellus* seed toxin by galactose and derivatives

Carbohydrate	MM	
	Rabbit (2%)	Human ORh$^+$ (2%)
D(+)Galactopyranoside[a]	NI	16.7
2-amino-2-deoxy-D-galctopyranoside[a]	NI	NI
D-Galacturonic acid[a]	NI	NI
Metyl-a-D-galactopyranoside[a]	33.3	2.1
Metyl-b-D-galactopyranoside[a]	NI	NI
2-acetamido-2-deoxy-D-galactopyranoside[b]	NI	33.3
r-Nitrophenyl-a-D-galactopyranoside[a]	NI	NI
r-Nitrophenyl-b-D-galactopyranoside[a]	NI	NI
O-Nitrophenyl-b-D-galactopyranoside[a]	NI	NI
b-D-Gal-[1® 4]-a-D-Glc[a]	33.3	4.16
L(+)Rhamnopyranose[a]	NI	16.7
O-a-D-galactopyranosyl-[1® 6]-a-D-glucopyranosyl-b-D-fructofuranoside[c]	33.3	NI
6-Deoxi-L-galactopyranose[a]	NI	NI
6-Deoxi-D-galactopyranose[a]	NI	NI

NI: not inhibitory even at the following concentrations – *a*: 33.3 mM, *b*: 16.6 mM and *c*: 6.6 mM . NI: not inhibitory.