Glycoconjugate Journal 18, 391–400, 2001

Characterization of the sugar-binding specificity of the toxic lectins isolated from *Abrus pulchellus* seeds

Marcio V. Ramos[1]*, Alexandre H. Sampaio[2], Benildo S. Cavada[3], Juan J. Calvete[4], Thalles B. Grangeiro[1] and Henri Debray[5]*

[1]*Departamentos de Biologia*, [2]*Engenharia de pesca and* [3]*Bioquimica e Biologia Molecular from the Universidade Federal do Ceara, Campus do Pici, Cx. Postal 6033. Fortaleza-Ceara, Brasil, 60.451-970*, [4]*Intituto de Biomedicina de Valencia, C.S.I.C., Jaime Roig, 1146010, Valencia Spain*, [5]*Laboratoire de Biochimie et UMR N.8576 du CNRS, Université des Sciences et Technologies de Lille, 59655 Villeneuve D'Ascq CEDEX, France*

The sugar-binding specificity of the toxic lectins from *Abrus pulchellus* seeds was investigated by combination of affinity chromatography of glycopeptides and oligosaccharides of well-defined structures on a lectin-Sepharose column and measurement of the kinetic interactions in real time towards immobilized glycoproteins. The lectins showed strong affinity for a series of bi- and triantennary *N*-acetyllactosamine type glycans. The related asialo-oligosaccharides interact more strongly with the lectins. The best recognized structures were asialo-glycopeptides from fetuin. Accordingly, the kinetic interaction with immobilized asialofetuin was by far the most pronounced. Human and bovine lactotransferrins and human serotransferrin interacted to a lesser extent. The interaction with asialofetuin was inhibited by galactose in a dose dependent manner. Lactose, *N*-acetyllactosamine and lacto-*N*-biose exhibited similar degree of inhibition while *N*-acetylgalactosamine was a poor inhibitor. These results suggested that the carbohydrate-binding site of the *Abrus pulchellus* lectins was specific for galactose and possess a remarkable affinity for the sequences lactose [β-D-Gal-(1 $\rightarrow$ 4)-D-Glc], *N*-acetyllactosamine [β-D-Gal-(1 $\rightarrow$ 4)-D-GlcNAc] and lacto-*N*-biose [β-D-Gal-(1 $\rightarrow$ 3)-D-GlcNAc].

***Keywords:* *Abrus pulchellus*, lectin, plant toxin, protein-carbohydrate interaction, surface plasmon resonance**

Introduction

The seeds of *Abrus pulchellus* contain a lectin fraction with highly toxic properties. Similar to that found in the *Abrus precatorius* seeds, this toxic activity seems to be represented by a mixture of closely related isoforms of the lectin and may be purified by affinity chromatography [1]. Nonetheless, the collection of these isoforms exhibiting agglutinating and toxic activities has been shown to share common galactose-binding specificity [2–4]. As previously shown by SDS-PAGE, *Abrus pulchellus* lectins are composed of two polypeptide chains linked by disulfide bonds: a toxic A chain with a molecular weight of 29 000 Da which inactivates the 60S ribosomal subunit and inhibits protein synthesis in cells and a B chain with a molecular weight of 31 500 Da, which binds to glycosylated cell surface receptors with terminal non-reducing galactose residues, allowing the entry of the whole protein into the cell [1]. These properties associated with the fact that agglutinating or toxic effects are observed at concentrations as low as 30–100 $\mu g\,ml^{-1}$ requires further isolation of the isoforms in order to investigate some biological properties or applications for these proteins. Thus, *Abrus precatorius* toxins represent an excellent model to study anchorage and translocation of molecules on cell membrane and to construct chimerical immunotoxins composed of their B chain isolated from the toxic or agglutinating isoforms [5]. Since these toxins interact with a variety of different types of eukaryotic cells, it is worthy of investigation to determine the ability of *Abrus pulchellus* lectins to recognize different complex carbohydrate structures potentially representing cell surface receptors. Therefore, the aim of the present work is to extend previous preliminary results on the sugar specificity of *Abrus pulchellus* lectins obtained by inhibition with various sugars and glycoproteins of the hemagglutination induced by the lectins [4]. The fine sugar specificity of the lectins is here defined by studying the affinity of the immobilized lectins towards radiolabeled glycopeptides and oligosaccharides of known

*Corresponding authors: Marcio V. Ramos Tel./Fax: + 55852889818, E-mail: vramos@ufc.br and Henri Debray Tel.: + 33320434997; Fax: + 33320436555; E-mail: henri.debray@univ_lille1.fr

structure and by analysis of real-time interactions of the soluble lectins with immobilized glycoproteins containing well-defined glycans by surface plasmon resonance with a BiaCore 3000 apparatus.

Materials and methods

General

Carbohydrates galactose, lactose and *N*-acetyllactosamine and the glycoproteins porcine stomach mucin, fetuin, asialofetuin, porcine and bovine thyroglobulins, egg ovalbumin, α-1 acid glycoprotein and bovine ribonuclease B were purchased from Sigma chemical Co. (St. Louis, USA). Human lactotransferrin, human serotransferrin and bovine lactotransferrin were gifts from Dr. G. Spik and lacto-*N*-biose was a gift from Dr. G. Strecker (U.S.T.L., Villeneuve D'Ascq, France). Soybean agglutinin was isolated according to Sattsangi et al. [6] and jacalin according to Misquith et al. [7]. Sensor chips CM5, HBS buffer (10 mM Hepes/150 mM NaCl/0.05% BiaCore surfactant P20, pH 7.4) and amino coupling kit were supplied by BiaCore France.

Isolation and immobilization of the lectin

Seeds of *Abrus pulchellus* were collected in the state of Ceará, Brazil. The lectin fraction was obtained by affinity chromatography on a Sepharose-4B column as described by Ramos et al. [1]. Purity of the material was checked by 12.5% SDS-PAGE. The purified lectins were coupled to CNBr-activated Sepharose 4B (Pharmacia) following the procedure described by March et al. [8]. The amount of immobilized lectin was estimated to be 2.4 mg per ml of gel by subtracting the amount of unbound protein found in the supernatant and washing solutions after coupling.

Affinity chromatography on lectin-Sepharose column

Samples of 400 µl of labeled glycopeptides (2×10^{-3} disintegration min^{-1}) or oligosaccharides (30×10^{-3} disintegration min^{-1}), were applied to the column of lectin-Sepharose 4B (0.5×18 cm) equilibrated at room temperature with 10 mM phosphate buffered saline (pH 7.4) containing 0.15 M NaCl (PBS). The column was then washed with PBS at a flow rate of 9.6 ml h^{-1} followed by the same buffer containing 150 mM galactose or 150 mM lactose or with 100 mM glycine buffer pH 2.6. Fractions of 1.6 ml were collected and aliquots counted in a Beckman LS-1800 scintillation counter. Recovery of total radioactivity applied to the column was usually higher than 90 per cent. Behavior of glycopeptides and related oligosaccharides on the lectin column was designed as unretained fractions (FNR) when eluted at the void volume of the column, retarded fractions (FR) when eluted after the void volume of the column with the starting buffer and eluted fractions (FE) when obtained after adding the sugar solution or decreasing of pH with glycine buffer.

Origin of glycopeptides and oligosaccharides

The structures tested for affinity to *Abrus pulchellus* lectins are shown in Table 1. Glycopeptides from bovine ribonuclease B (**1**–**5**), *N*-glycopeptides from fetuin (**14**) and from human α-1 acid glycoprotein, porcine and bovine thyroglobulins and egg ovalbumin (Table 2) were obtained after exhaustive hydrolysis of the proteins with pronase followed by gel filtration chromatography on a BioGel P2 column. Oligosaccharides

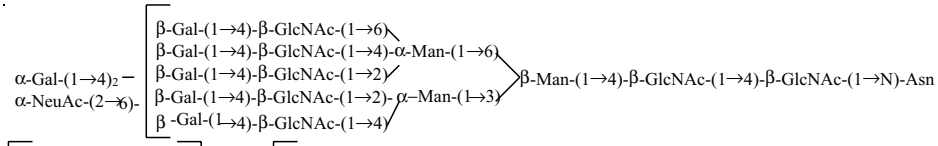
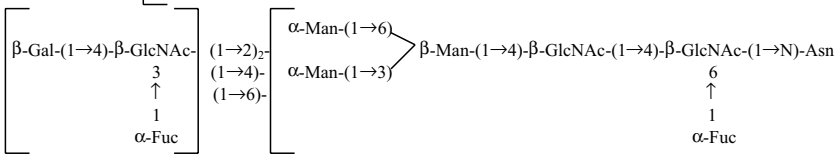
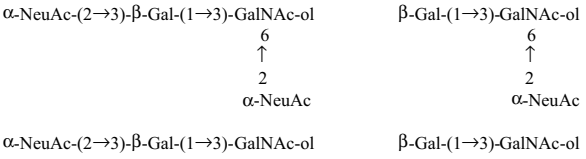
Table 1. Behaviour of glycopeptides and related oligosaccharides submitted to affinity chromatography on a *A. pulchellus* lectins-Sepharose column

Compound	*Structure*	*Elution profile*		
		FNR	*FR*	*FE*
1	α-Man-(1→2)-α-Man-(1→6) α-Man-(1→2)-α-Man-(1→3) ⟩α-Man-(1→6)⟩ β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn α-Man-(1→2)-α-Man-(1→2)-α-Man-(1→3)	+		
2	α-Man-(1→2)$_2$ - [α-Man-(1→6) α-Man-(1→3) ⟩α-Man-(1→6)⟩ β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn α-Man-(1→2)-α-Man-(1→3)]	+		
3	α-Man-(1→2) - [α-Man-(1→6) α-Man-(1→3) ⟩α-Man-(1→6)⟩ β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn α-Man-(1→2)- α-Man-(1→3)]	+		
4	α-Man-(1→6) α-Man-(1→3) ⟩α-Man-(1→6)⟩ β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn α-Man-(1→2)-α-Man-(1→3)	+		

Table 1. (*continued*)

Compound	*Structure*	*Elution profile*		
		FNR	*FR*	*FE*
5	α-Man-(1→6) α-Man-(1→3) > α-Man-(1→6) α-Man-(1→ 3) > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn	+		
6	α-NeuAc-(2→6)-β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→6) α-NeuAc-(2→6)-β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→3) > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn		+5	
7	α-NeuAc-(2→6)-β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→6) α-NeuAc-(2→6)-β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→3) > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn 6 ↑ 1 α-Fuc		+3	
8	β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→3) > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn		+18	
9	β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc(1→2)-α-Man-(1→3) > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc-(1→N)-Asn 6 ↑ 1 α-Fuc		+12	
10	α-NeuAc-(2→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) α-NeuAc-(2→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) > β-Man-(1→4)-GlcNAc	+		
11	α-NeuAc-(2→6)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) α-NeuAc-(2→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) > β-Man-(1→4)-GlcNAc	+		
12	β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) > β-Man-(1→4)-GlcNAc		+3	
13	β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-GlcNAc-(1→4) > β-Man-(1→4)-GlcNAc β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3)	+		
14	α-NeuAc -(2→3)$_2$- α -NeuAc -(2→ 6)- [β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) β-Gal-(1→3)-β-GlcNAc-(1→4)] > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc(1→N)-Asn		+15	
15	β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) > β-Man-(1→4)-β-GlcNAc-(1→4)-β-GlcNAc(1→N)-Asn β-Gal-(1→3)-β-GlcNAc-(1→4)			+
16	β-Gal-(1→4)-β-GlcNAc-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) > β-Man-(1→4)-GlcNAc β-Gal-(1→4)-β-GlcNAc-(1→4)		+1	
17	β-Gal-(1→4)-β-GlcNAc-(1→3)- [β-Gal-(1→4)-β-GlcNAc-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) β-Gal-(1→4)-β-GlcNAc-(1→4)] > β-Man-(1→4)-GlcNAc		+1	
18	β-Gal-(1→4)-β-GlcNAc-(1→3)$_2$- [β-Gal-(1→4)-β-GlcNAc-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) β-Gal-(1→4)-β-GlcNAc-(1→4)] > β-Man-(1→4)-GlcNAc		+2	
19	β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→6) β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→2)-α-Man-(1→3) β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→4) > β-Man-(1→4)-GlcNAc	+		

Table 1. (*continued*)

Compound	*Structure*	*Elution profile*		
		FNR	*FR*	*FE*
20		+		
21		+		
22		+		

10–**13**, **16**–**19**, **21** were isolated by Dr. G. Strecker (U.S.T.L., Villeneuve D'Ascq, France) from the urine of patients with various lysosomal diseases [9]. Glycopeptides from human serotransferrin and lactotransferrin, (**6**–**9**) and from turtle-dove ovomucoid (**20**) were kindly supplied by Prof. G. Spik (U.S.T.L., Villeneuve D'Ascq, France) [10]. *O*-glycans from fetuin (**22**) were obtained by reductive β-elimination of the purified glycoprotein according to Iyer and Carlson [11]. The homogeneity and structure of all glycans and glycopeptides were determined by ^{1}H-NMR spectroscopy, except for those representing mixture of glycopeptides (GP α-1 acid glycoprotein, GP porcine thyroglobulin and GP bovine lactotransferrin and GP egg ovalbumin). The asialo-forms of glycopeptides **6**, **7** and **14**, corresponding to glycopeptides **8**, **9** and **15** as well as those of α-1 acid glycoprotein, and bovine lactotransferrin were obtained by treatment with 0.1 N trifluoroacetic acid, 1 hour at 80°C followed by evaporation under N_2 atmosphere and two washes with methanol before solubilization in 400 µl of PBS.

Labeling of glycopeptides and oligosaccharides

The glycopeptides were radio-labeled with [^{14}C] acetic anhydride (10–30 mCi mmol^{-1}, Amersham) as previously described by Koide and Muramatsu [12]. Free-acetic acid labeled glycopeptides were obtained by gel filtration chromatography on a BioGel P2 (Bio-Rad) column equilibrated with distilled water. Oligosaccharides **10**–**13** and **16**–**19** were labeled at the reducing-terminal *N*-acetylglucosamine

Table 2. Behaviour of [^{14}C] labeled *N*-glycopeptides released from glycoproteins on the *Abrus pulchellus* lectins-Sepharose column

Origin of glycopeptides	*Characteristics of the fraction*	*Behavior on lectin-column*
α-1 acid glycoprotein	Mixture of tetra-, tri- and biantennary *N*-acetyllactosamine-type *N*-linked glycans with various degrees of sialylation and some with outer arm fucose residues and lactosamine repeats	FNR
Desialylated α-1 acid glycoprotein		FNR, FR + 9 and FR + 27
Ovalbumin	Mixture of oligomannosidic- and hybrid-type *N*-linked glycans	FNR
Porcine thyroglobulin	Mixture of oligomannosidic- and bi- and tri-antennary *N*-acetyllactosamine-type with core fucose *N*-linked glycans	FNR, FR + 4, FR + 12
Bovine lactotransferrin	Oligomannosidic- and sialylated *N*-acetyllactosamine-type *N*-linked glycans	FNR
Desialylated bovine lactotransferrin		FNR

residues by reduction with tritiated sodium borohydride (5–20 Ci mmol^{-1}, Amersham) according to Takasaki and Kobata [13].

Kinetic interaction with glycoproteins

The BIAcore 3000 apparatus (Pharmacia Biosensor) uses the surface plasma resonance (SPR) phenomenon occuring when surface plasmon waves are excited at a metal/liquid interface (the sensor surface), to monitor biomolecular binding events in real time, here between an immobilized glycoprotein and a flowing *Abrus pulchellus* lectins solution. Lectin binding to the glycoprotein of the sensor surface causes changes in the refractive index close to the surface which are detected as changes in the SPR signal, expressed in resonance units (RU) where one RU is equivalent to one picogram of protein per mm^2 on the sensor surface. These changes are measured continuously to form a sensorgram which provides a complete record of the progress of association and dissociation of the interactants. With knowledge of the assay principle and sample details, kinetic constants for association (K_A) and dissociation (K_D) can be also evaluated (for more details on the SPR methodology, see references 14–17).

The glycoproteins jacalin, asialofetuin, egg ovalbumin, human serotransferrin, bovine lactotransferrin, human lactotransferrin, ribonuclease B and ovomucoide (1 mg ml^{-1}) were bound to the dextran layer of the sensor chip using the amino coupling kit following the manufacturer instructions. For coupling, the glycoproteins were dissolved in 10 mM acetate buffer pH 4.5 or 10 mM citrate buffer pH 6.0. Blocking of non-reacting remaining groups was carried out with ethanolamine. The lectins (100 µg ml^{-1}) were injected on the cell surface of the sensor at a flow rate of 5 µl min^{-1} during 300 sec (association phase) and allowed to dissociate in equal time in HBS buffer. Intensity of interaction was determined as the difference between values of arbitrary resonance units (RU) at the beginning and the end of dissociation phase slighting the bulk contribution caused by passage of the sample under the cell surface. For inhibition assays, saccharides were injected at the end of dissociation phase and allowed to interact during 300 sec, followed by the running buffer (HBS). Inhibition was expressed as the percentage of lectins remaining bound on the asialofetuin sensor 300 seconds after the start of the inhibitor pulse = (Resonance units (RU) after 300 s passage of the inhibitor solution/Resonance units (RU) at the start of the inhibitor pulse) × 100.

All experiments were conducted at 25°C. Flow cell regeneration was achieved by two successive injections of 10 mM HCl and 10 mM NaOH to remove the remaining material still bound on the sensor surface. The immobilized glycoproteins were of the oligomannosidic-type (Soybean agglutinin and bovine ribonuclease B), oligomannosidic-type and hybrid-type (egg ovalbumin), oligomannosidic-type and *N*-acetyllactosamine-type (bovine lactotransferrin), *N*-acetyllactosamine-type (human sero- and lactotransferrins) or paucimannosidic-type glycans (jacalin) with the presence of β-1,2-xylose and α-1,3-fucose residues [18].

Results

Three distinct elution profiles were obtained when different glycopeptides and related oligosaccharides were applied to the immobilized *A. pulchellus*-lectins-Sepharose column. Under the experimental conditions, the unretained fractions (FNR), with structures lacking interaction with *A. pulchellus* lectins were recovered at the void volume of the column (fractions 5–6). The structures exhibiting weak interaction and eluting as retarded fractions (FR) (fractions 9–24), were named according to the number of fractions eluted after the void volume (FR + number of additional fractions). Structures with strong interaction with the lectins, named eluted fractions (FE), were only recovered after adding lactose or galactose in PBS or 100 mM glycine buffer pH 2.6.

The immobilized *Abrus pulchellus* lectins did not interact with oligomannosidic- type glycans represented by structures **1**–**5** (Table 1). According to the obtained results, *Abrus puchellus* lectins interacted with glycopeptides or oligosaccharides which possess unmasked *N*-acetyllactosamine sequences β-Gal-(1 → 4)-β-GlcNAc (structures **8**–**9**, **12**–**13**, **16**–**18**). In contrast with some galactose-specific lectins such as *Erythrina* [19], *Butea frondosa* [20], *Ricinus communis* [21] or *Vatairea macrocarpa* [22], the affinity between *A. pulchellus* lectins and *N*-acetyllactosamine-type oligosaccharides was not increased with the number of unmasked *N*-acetyllactosamine sequences present in the carbohydrate structures.

The *A. pulchellus* lectins did not show any affinity for a penta-antennary glycopeptide from turtle-dove ovomucoid (structure **20**) which possesses two unmasked *N*-acetyllactosamine sequences, two *N*-acetyllactosamine sequences substituted each at O-4 of galactose by an α-D-galactose residue and one such sequence substituted at O-6 by an *N*-acetylneuraminic acid residue, perhaps because of steric hindrance introduced by these substituting residues. Substitution of the *N*-acetyllactosamine sequences by sialic acid residues diminished (structures **6**–**7**, FR + 5 and FR + 3) or completely abolished (structures **10**–**11**, FNR) the affinity of the *A. pulchellus* lectins for these kind of structures, as compared to the unsubstituted ones. Moreover, the lectins also exhibited a better affinity for a biantennary glycopeptide (structure **6**, FR + 5) compared to the related biantennary oligosaccharide (structure **12**, FR + 3). This could be explained by the fact that in the glycopeptide, the glycan-amino linkage leads to a structure more rigid than those of the related oligosaccharide [18].

It was noteworthy that the presence of an α-fucose residue at O-6 of the *N*-acetylglucosamine residue involved in the *N*-glycosylamine linkage strongly decreased the interaction between the glycopeptide and the lectins (structures **6**, **8**, FR + 5 and FR + 18 versus **7**, **9**, FR + 3 and FR + 12). Substitution of the β-linked mannose residue by an additional

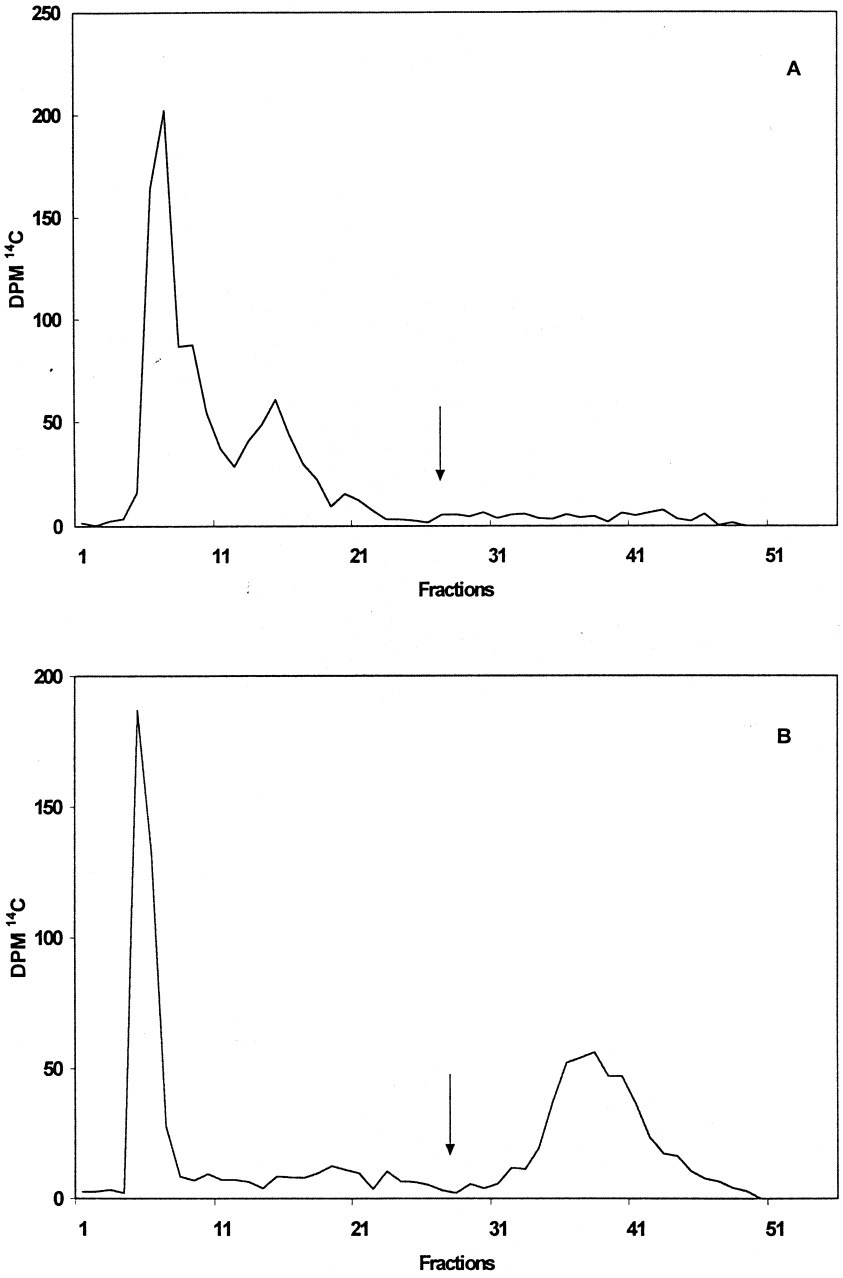


Figure 1. Affinity chromatography of [^{14}C]-glycopeptides from fetuin on *Abrus pulchellus* lectins-Sepharose column. Sialylated glycopeptides (Panel A) and acid treated glycopeptides (Panel B). Fractions of 1.6 ml, flow rate 9.6 ml h^{-1}. Arrows indicate the addition of 100 mM glycine buffer pH 2.6.

β-(1 $\rightarrow$ 4)-GlcNAc residue (structure **13**) abolished the affinity of *A. pulchellus* lectins for these kind of structures, as compared to the unsubstituted one (structure **12**, FR + 3). The presence of one or two poly-*N*-acetyllactosamine sequences on a tetra-antennary oligosaccharide did not alter significantly the interaction of these structures with the lectins (structures **17**–**18**, FR + 1, FR + 2 versus **16**, FR + 1) while four repetitions of this sequence abolished the interaction (structure **19**) indicating that each binding-site can accommodate only one β-Gal-(1 $\rightarrow$ 4)-β-GlcNAc sequence.

The elution profiles of native and desialylated fetuin glycopeptides are shown in Figure 1. According to Green et al. [23], bovine fetuin contains a great number of *N*-acetyllactosamine-type glycans differing in the number of peripheral branches (17% of biantennary and 83% of triantennary glycans), in extent of sialylation, *N*-acetylneuraminic acid linkage (α-2,3 versus α-2,6) and linkage (β-1,4 versus β-1,3) of galactose residues. The tri-antennary glycopeptides **14**–**15** represent two of the major *N*-linked glycans from bovine fetuin. Panel A of Figure 1 shows that,

when this complex mixture of sialylated glycopeptides is applied on the *A. pulchellus* lectins-Sepharose column, a non-retained fraction and a retarded fraction (FR + 8) are obtained. However, when the desialylated glycopeptides are applied on the column (panel B), the previously retarded fraction is now strongly bound to the column and must be eluted with galactose, lactose or glycine buffer. This data fully agrees with that previously described for *Abrus precatorius* lectins [24]. This behavior of native or desialylated fetuin glycopeptides can be compared to that of native or desialylated glycopeptides from α-1 acid glycoprotein (Table 2), which are also complex mixtures of bi-, tri- and tetra-antennary *N*-acetyllactosamine-type glycans [25]. None of these native or desialylated glycopeptides are bound to the column. This could be due to the fact that the glycopeptides from α-1 acid glycoprotein, as well as those from the *N*-acetyllactosamine-type glycopeptides from bovine lactotransferrin [26] or porcine thyroglobulin [27], do not contain a Gal-β-(1 → 3)-β-GlcNAc-(1 → 4) moiety but an *N*-acetyllactosamine unit attached to the α-1,3 linked core mannose residue. This oligosaccharide determinant is present in some tri-antennary glycans from bovine fetuin (structures **14**–**15**, FR + 15 and FE, respectively) and may represent an important structural feature in the interaction of oligosaccharides with *A. pulchellus* lectins.

The behavior of different mixtures of *N*-glycopeptides on a *A. pulchellus* lectins-Sepharose column is shown in Table 2. Native glycopeptides isolated from α-1-acid glycoprotein, bovine lactotransferrin or egg ovalbumin did not show any interaction with the lectins. Some desialylated glycopeptides from α-1-acid glycoprotein and porcine thyroglobulin with bi-, tri- and tetra-antennary *N*-acetyllactosamine-type glycans were eluted as retarded fractions according to their affinity for the lectins.

None of the reduced *O*-linked glycans released from fetuin by reductive β-elimination (structures **22**, FNR) and containing the Gal-β-(1 → 3)-GalNAcol sequence showed affinity for the immobilized lectins.

In order to extend the results obtained by the analysis of the behavior of well-defined oligosaccharides and glycopeptides on an immobilized *A. pulchellus* lectins-sepharose column, surface plasmon resonance experiments were carried out using glycoproteins with well identified glycan moieties. As expected, asialofetuin was the most reactive among the assayed glycoproteins (Figure 2). Other glycoproteins such as human lacto- and serotransferrin and bovine lactotransferrin also reacted with the lectins but to a much lesser extent while other glycoproteins such as egg ovomucoid and ovalbumin, soybean and jacalin agglutinins did not react.

The binding specificity of *A. pulchellus* lectins towards glycans of the *N*-acetyllactosamine-type was then further investigated. Addition of D-galactose led to a faster dissociation of the complex between soluble lectins and immobilized asialofetuin in a dose-dependent manner (Figure 3). At 10 mM concentration, dissociation rate was increased by 42%.

Under the same experimental conditions, 10 mM concentration of lactose [β-D-Gal-(1 → 4)-D-Glc], *N*-acetyllactosamine [β-D-Gal-(1 → 4)-D-GlcNAc] and lacto-*N*-biose [β-D-Gal-(1 → 3)-D-GlcNAc] possessed similar inhibition power with dissociation rates increased by 45.7, 43.2 and 42.9%,

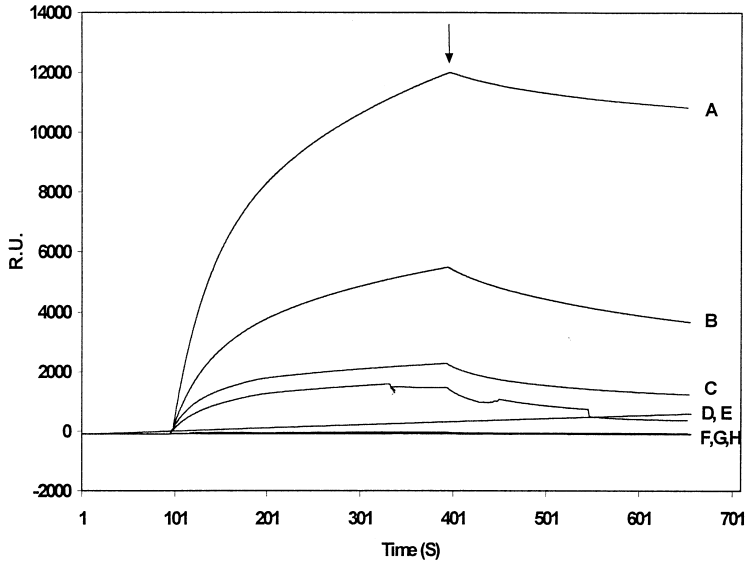


Figure 2. Kinetic interactions of soluble *Abrus pulchellus* toxic lectins with immobilized glycoproteins measured in real time by surface plasmon resonance technology. Experimental conditions are described in Materials and Methods. The upward and downward parts of the curves correspond to the association (circulating buffer containing the lectins) and the dissociation (circulating buffer devoid of lectins) phases, respectively. Arrow indicates end of injection and beginning of dissociation phase. Glycoproteins: asialofetuin (A), human lactotransferrin (B), bovine lactotransferrin (C), human serotransferrin (D), ovomucoid (E), Ovalbumin (F), soybean agglutinin (G) and jacalin (H).

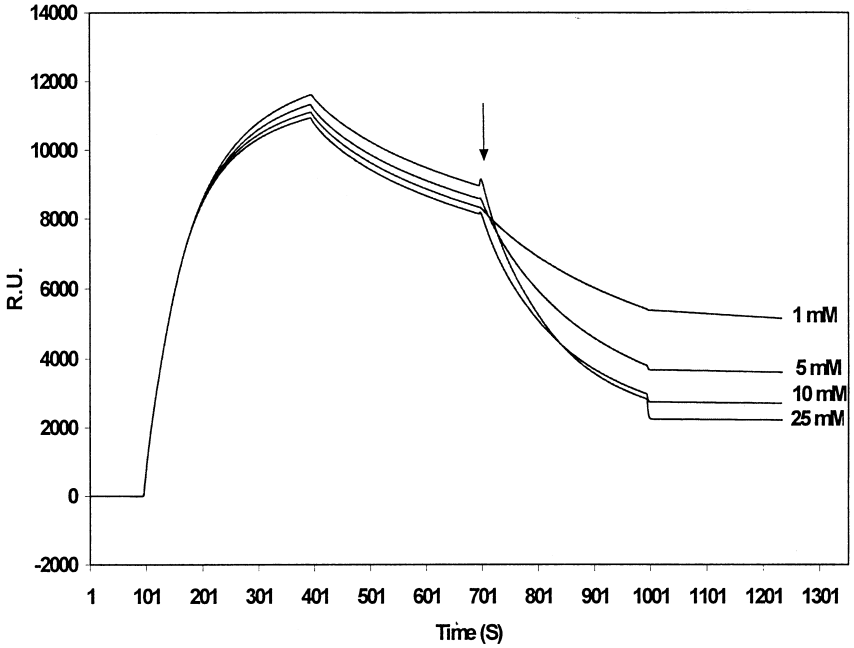


Figure 3. Inhibition of interaction between soluble *Abrus pulchellus* toxic lectins and asialofetuin by D-galactose at different concentrations. Arrow indicates end of dissociation phase in HBS and the beginning of dissociation phase in presence of the sugar. Inhibition is expressed as the percentage of lectins remaining bound on the asialofetuin sensor 300 seconds after the start of the inhibitor pulse = (Resonance units (RU) after 300 s passage of the inhibitor solution/Resonance units (RU) at the start of the inhibitor pulse × 100).

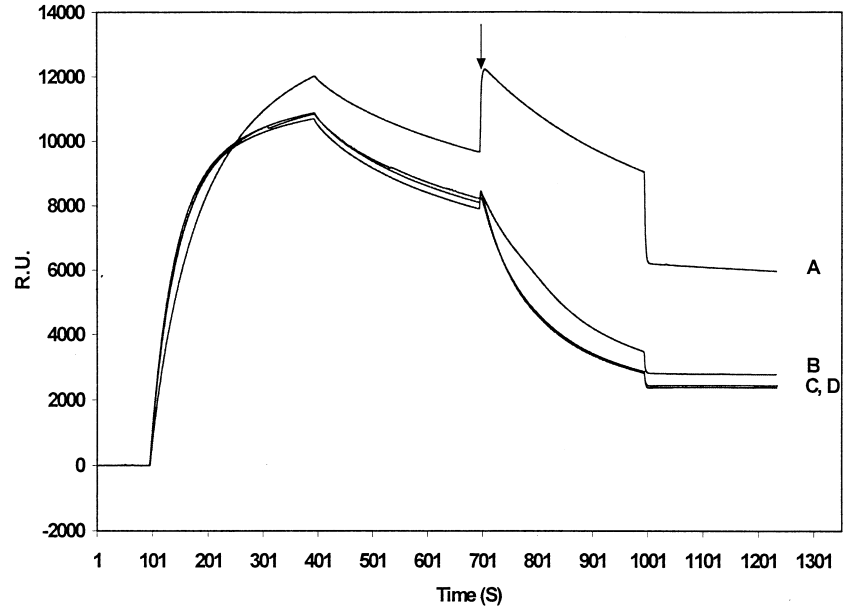


Figure 4. Inhibition of the interaction between soluble *Abrus pulchellus* toxic lectins and asialofetuin by 100 mM *N*-acetylgalactosamine (A), and lactose (B), *N*-acetyllactosamine (C) and lacto-*N*-biose at 10 mM (D), in HBS. Arrow indicates end of dissociation phase in HBS and the beginning of dissociation phase in presence of the sugar. Inhibition is expressed as the percentage of lectins remaining bound on the asialofetuin sensor 300 seconds after the start of the inhibitor pulse = (Resonance units (RU) after 300 s passage of the inhibitor solution/Resonance units (RU) at the start of the inhibitor pulse × 100).

Table 3. Comparison of the carbohydrate-binding specificity of *A. pulchellus* toxic lectins with other well-studied toxic galactose-binding plant lectins

Proteins	*Main structural inhibitory motif recognized*
A. pulchellus lectins	β-D-Gal-(1 → 4)-D-Glc β-D-Gal-(1 → 4)-D-GlcNAc β-D-Gal-(1 → 3)-D-GlcNAc
A. precatorius agglutinin[1]	β-Gal-(1 → 3)-GalNAc > β-Gal-(1 → 3/4)-GlcNAc α-Gal-(1 → 4)-β-Gal-(1 → 4)-GlcNAc
A. precatorius toxin (abrin a,b)[2]	α-Gal-(1 → 4)-β-Gal-(1 → 4)-GlcNAc
Ricinus communis agglutinin[3]	β-Gal-(1 → 4)-GlcNAc >> β-Gal-(1 → 4)-Glc α-Gal-(1 → 4)-β-Gal-(1 → 4)-GlcNAc
R. communis toxin (ricin)[2]	α-Gal-(1 → 4)-β-Gal-(1 → 4)-GlcNAc
Sambucus nigra toxic agglutinin V[4]	GalNAc >> Gal
Sambucus nigra toxic agglutinin I[5]	α-NeuAc-(2 → 6)-Gal/GalNAc
Viscum album toxic agglutinin I[6]	α-Gal-(1 → 4)-Gal/α-Gal-(1 → 3)-Gal >> α-NeuAc-(2 → 3/6)-β-Gal-(1 → 4)-GlcNAc
V. album toxic agglutinin II[6]	D-Gal/D-GalNAc

[1]Wu et al. [38], [2]Wu et al. [3], [3]Sharma et al. [41], [4]Van Damme et al. [36], [5]Van Damme et al. [40], [6]Debray et al. [39].

respectively (Figure 4). Interestingly, only a very weak inhibition occurred in the presence of *N*-acetyl-D-galactosamine and even at 100 mM concentration, dissociation rate was slightly increased by 16.6%.

Discussion

The seeds of fabaceae *Abrus pulchellus* contain a highly toxic lectin fraction, comparable to the classic RIP (ribosome inactivating proteins) type 2 toxins. Previous reports comparing some biochemical and biological properties of the *A. pulchellus* and *A. precatorius* lectins confirmed the similarities between these closely related proteins [1,4]. This implies the occurrence of highly toxic and poorly agglutinating toxins (abrins) and poorly toxic but highly agglutinating lectins (Abrus agglutinin) [28,29]. Type 2 RIPs consist of two polypeptide chains, both necessary for the toxin molecule to act: an A chain with an enzymatic site catalyzing the depurinization of a precise rRNA sequence in ribosomes rendering them inactive to protein synthesis [30,31] and a B chain with lectin properties, which binds to glycosylated receptors with terminal non-reducing galactose residues on the cell surface, allowing the entry of the whole protein [32,33]. Independently of their structural arrangement, anchorage of RIPs on the cell surface to trigger toxic or haemagglutinating effects must be mediated by the carbohydrate-binding activity [33].

The monosaccharide-binding specificity of lectins with type 2 RIP activity is invariably directed towards D-galactose and *N*-acetyl-D-galactosamine residues. These monosaccharides in the non-reducing terminal position of glycoconjugates are widely found in membrane glycoproteins and glycolipids present on cell surfaces. Detailed investigation of the fine sugar specificity of toxic lectins like those from *A. precatorius, Ricinus communis, Viscum album* and the recently characterized *Sambucus nigra* toxic lectins, demonstrated the extended nature of the galactose-binding site and strengthened the similarities among this group of proteins (Table 3). As a rule, toxins and their related lectins are capable of interacting with the same glycoconjugates differing significantly in their affinity rates. Differences in the potential to agglutinate or kill cells persisted for a long time as a controversy to classify these proteins as true lectins. In the early nineties, Citores and co-workers [35] demonstrated the enzymatic activity of the A chain from *Abrus precatorius* and *Ricinus communis* agglutinins and later Van Damme et al. [36] demonstrated the unusual structure of a RIP type 2 from *Sambucus nigra*. These two findings suggested that lectins with RIP properties should be classified by their functions and not by their structural arrangement. Finally, these and other toxic lectins were grouped and designated chimerical lectins, according to the classification proposed by Peumans and Van Damme [37]. These facts emphasize the remarkable similarities shared by this family of proteins.

According to our results, the galactose-binding *A. pulchellus* lectins recognize the sequences [β-D-Gal-(1 → 4)-D-Glc], [β-D-Gal-(1 → 4)-D-GlcNAc] and [β-D-Gal-(1 → 3)-D-GlcNAc] to the same extent. Substitution of the *N*-acetyllactosamine sequence by an *N*-acetylneuraminic acid residue linked in α-(2 → 6) decreases the affinity of the lectins. The first sequence may also be weakly recognized in the presence of a

N-acetylneuraminic acid branched in α-(2 → 6). It is noteworthy that the Thomsen-Friedenreich antigen [β-D-Gal-(1 → 3)-D-GalNAc], corresponding to the desialylated *O*-glycans from fetuin, are not recognized by the lectins. These results disagree with those found by Wu et al. [38] who identified this epitope as the best ligand for the *A. precatorius* agglutinin. This difference cannot be explained in terms of sensitivity of the used methods and should therefore be further investigated. Comparing these results with those summarized in Table 3 suggests that the carbohydrate-binding site of these toxic lectins possesses a unique specificity for the monosaccharide galactose. Nonetheless, a structural adaptability has allowed the enlargement of this site in order to accommodate more complex galactosylated carbohydrate sequences found on a large variety of glycoconjugates. In particular, the Gal-β-(1 → 3)-β-GlcNAc-(1 → 4) sequence found in some triantennary glycans from bovine fetuin (structures **14**–**15** of Table 1) may represent an important structural determinant in the interaction of oligosaccharides with *A. pulchellus* lectins.

In conclusion, more fine structural and functional investigations on these type 2 ribosome-inactivating proteins during the last decade have demonstrated that toxic lectins represent a very heterogeneous structural group of proteins with conserved carbohydrate-binding properties [35,36,39,40,41]. This suggests that the biological activities of these proteins are depending more on their carbohydrate-binding sites than on their structural organizations.

Acknowledgments

This work was supported by grants from International Foundation for Science, grant N. F/3070-1 (M.V.R.), program CAPES/COFECUB, CNPq, PADCT, FUNCAP, CNRS and Bio-Tools Ecological Foundation. We are indebted to the service commun d'imagerie cellulaire de l'Université de Lille II for providing access to the BiaCore Apparatus.

References

1 Ramos MV, Mota DM, Teixeira CR, Cavada BS, Moreira RA. *Toxicon* **36**, 477–84 (1998).
2 Yamasaki N, Absar N, Funatsu G. *J Biochem* **105**, 708–11 (1989).
3 Wu AM, Song SC, Wu JH, Pfuller C, Chow LP, Lin JY. *Biochim Biophys Acta* **1243**, 124–8 (1995).
4 Ramos MV, Teixeira CR, Bomfim LR, Madeira SVF, Moreira RA. *Mem Inst Oswaldo Cruz* **94**, 185–8 (1999).
5 Pastan I, Kreitman RJ. *Adv Drug Deliv Rev* **31**, 53–88 (1998).
6 Sattsangi PD, Sattsangi S, Grossman HH. *J Chem Educ* **59**, 977–9 (1982).
7 Misquith S, Rani PG, Surolia A. *J Biol Chem* **269**, 30393–401 (1994).
8 March SC, Parikh I, Cuatrecasas P. *Anal Biochem* **60**, 149–52 (1974).
9 Strecker G, Montreuil J. *Biochimie* **61**, 1199–246 (1979).
10 Spik G, Coddeville B, Mazurier J, Bourne Y, Cambillau C, Montreuil J. *Adv Exp Med Biol* **357**, 21–32 (1994).
11 Iyer RN, Carlson DM. *Arch Biochem Biophys* **142**, 101–5 (1971).
12 Koide N, Muramatsu TJ. *J Biol Chem* **249**, 4897–904 (1974).
13 Takasaki S, Kobata A. *J Biochem* **76**, 783–9 (1974).
14 Zeng X, Murata T, Kawagishi H, Usui T, Kobayashi K. *Carbohydr Res* **312**, 209–17 (1998).
15 Satoh A, Matsumoto I. *Anal Biochem* **275**, 268–70 (1999).
16 Canziani G, Zhang W, Cines D, Rux A, Willis S, Cohen G, Eisenberg R, Chaiken I. *Methods* **19**, 253–9 (1999).
17 Rich RL, Myszka DG. *Curr Opin Biotech* **11**, 54–61 (2000).
18 Montreuil J. In *Methods on glycoconjugates. A Laboratory Manual*, Verbert A (ed), Harwood Academic, pp. 1–23 (1995).
19 Debray H, Montreuil J, Lis H, Sharon N. *Carbohydr Res* **151**, 359–70 (1986).
20 Ayouba A, Debray H, Rougé P. *Glycoconjugate J* **9**, 141–7 (1992).
21 Debray H, Pierce-Cretel A, Spik G, Montreuil J. In *Lectins, Biology, Biochemistry, Clinical Biochemistry*, Bog-Hansen TC, Spengler GA (eds), Volume 3, Berlin, Walter de Gruyter, pp. 335–50 (1983).
22 Ramos MV, Bonfim LR, Cavada BS, Alencar NMN, Grangeiro TB, Debray H. *Prot Pept Letters* **7**, 241–8 (2000).
23 Green ED, Adelt G, Baenziger JU, Wilson S, Van Halbeek H. *J Biol Chem* **263**, 18253–68 (1988).
24 Sandvig K, Olsnes S, Pihl A. *Eur J Biochem* **88**, 307–13 (1978).
25 Fournet B, Montreuil J, Strecker G, Dorland L, Haverkamp J, Vliegenthart JFG, Binette JP, Schmid K. *Biochemistry* **17**, 5206–14 (1978).
26 Coddeville B, Strecker G, Wieruszeski JM, Vliegenthart JF, Van Halbeek H, Peter-Katalinic J, Egge H, Spik G. *Carbohydr Res* **236**, 145–64 (1992).
27 De Waard P, Kooveraar A, Kamerling JP, Vliegenthart JF. *J Biol Chem* **266**, 4237–43 (1991).
28 Lin JY, Lee TC, Hu ST, Tung TC. *Toxicon* **19**, 41–51 (1981).
29 Hegde R, Maiti TK, Podder SK. *Anal Biochem* **194**, 101–9 (1991).
30 Endo Y, Kunio T. *J Biol Chem* **262**, 812–30 (1987).
31 Liu CH, Tsi CC, Lin SC, Wang LI, Hsu CI, Hwang MJ, Lin JY. *J Biol Chem* **275**, 1897–901 (2000).
32 Barbieri L, Batelli MG, Stirpe F. *Biochim Biophys Acta* **1154**, 237–82 (1993).
33 Sousa M, Roberts LM, Lord JM. *Eur J Biochem* **260**, 355–61 (1999).
34 Ohba H, Toyokawa S, Hoshino T, Itoh K, Yamasaki N. *Biosci Biotechnol Biochem* **61**, 737–9 (1997).
35 Citores L, Ferreras JM, Iglesias R, Carbajales ML, Arias FJ, Jimenez P, Rojo MA, Girbes T. *FEBS Lett* **329**, 59–62 (1993).
36 Van Damme EJM, Barre A, Rougé P, Van Leuven F, Peumans WJ. *Eur J Biochem* **235**, 128–37 (1996a).
37 Peumans WJ, Van Damme EJM. *Plant Physiol* **109**, 347–52 (1995).
38 Wu AM, Lin SR, Chin LK. *J Biol Chem* **267**, 19130–9 (1992).
39 Debray H, Montreuil J, Franz H. *Glycoconjugate J* **11**, 550–7 (1994).
40 Van Damme EJM, Barre A, Rougé P, Van Leuven F, Peumans WJ. *Eur J Biochem* **237**, 505–13 (1996b).
41 Sharma S, Bharadwaj S, Surolia A, Podder SK. *Biochem J* **333**, 539–42 (1998).

Received 18 July 2001; revised 20 November 2001;
accepted 28 November 2001