Biochimica et Biophysica Acta 1854 (2015) 20–30

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Structural analysis and binding properties of isoforms of tarin, the GNA-related lectin from *Colocasia esculenta*

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ARTICLE INFO

Article history:
Received 3 May 2014
Received in revised form 13 October 2014
Accepted 15 October 2014
Available online 23 October 2014

Keywords:
Tarin
Colocasia esculenta
Subunit isoform
Glycan microarray
Protein stability
Carbohydrate binding

ABSTRACT

The lectins, a class of proteins that occur widely in animals, plants, fungi, lichens and microorganisms, are known for their ability to specifically bind to carbohydrates. Plant lectins can be classified into 12 families including the *Galanthus nivalis* agglutinin (GNA)-related lectin superfamily, which is widespread among monocotyledonous plants and binds specifically to mannose, a behavior that confers remarkable anti-tumor, anti-viral and insecticidal properties on these proteins. The present study characterized a mitogenic lectin from this family, called tarin, which was purified from the crude extract from taro (*Colocasia esculenta*). The results showed that tarin is a glycoprotein with 2–3% carbohydrate content, composed of least 10 isoforms with pIs ranging from 5.5 to 9.5. The intact protein is a heterotetramer of 47 kDa composed of two non-identical and non-covalently associated polypeptides, with small subunits of 11.9 kDa and large subunits of 12.6 kDa. The tarin structure is stable and recovers or maintains its functional structure following treatments at different temperatures and pH. Tarin showed a complex carbohydrate specificity, binding with high affinity to high-mannose and complex N-glycans. Many of these ligands can be found in viruses, tumor cells and insects, as well as in hematopoietic progenitor cells. Chemical modifications confirmed that both conserved and non-conserved amino acids participate in this interaction. This study determined the structural and ligand binding characteristics of a GNA-related lectin that can be exploited for several different purposes, particularly as a proliferative therapeutic molecule that is able to enhance the immunological response.

1. Introduction

Plant lectins comprise a group of proteins with at least one non-catalytic domain and the ability to bind specifically and reversibly to simple or complex carbohydrates [1,2] through hydrogen bonds or Van der Waals interactions. Lectins may or may not show agglutinating activity or glycoconjugate precipitation [3].

Molecular and high-resolution techniques such as Nuclear Magnetic Resonance (NMR) and X-ray crystallography have been widely used to characterize plant lectins [4,5], revealing a heterogeneous group of proteins that vary widely in size, structure, molecular organization, and the constitution of their binding sites [6]. These proteins share many of the characteristics that define different groups of evolutionary and structurally related proteins, and consist of 12 families of plant lectins including the monocot mannose-binding lectin family, recently called the *Galanthus nivalis* agglutinin (GNA)-related lectin superfamily, which includes tarin [7]. The GNA-related family has attracted special attention because of the insecticidal, antiviral and antitumor activities of its members [8].

The lectins from the GNA-related lectin superfamily show considerable differences in their processing and post-translational modifications. In general, these proteins are synthesized as preproproteins in the endoplasmic reticulum, and after processing (removal of the signal peptide and proteolytic cleavage of the C-terminal peptide), can originate two types of protomers of one or two domains. The two-domain protomers (about 30 kDa) originate two monomers, with identical or similar masses, by proteolytic cleavage. The one-domain protomers (11–14 kDa) originate a single monomer. The resulting monomers (with masses ranging from 11 to 14 kDa) can combine to form a homo/hetero-dimer or tetramer [5,9].

Members of the GNA-related lectin family exhibit a unique β-prism fold and bind exclusively to mannose. Each monomer folds into a β-

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http://dx.doi.org/10.1016/j.bbapap.2014.10.013

prism consisting of three β-sheet subdomains (I, II and III), each formed by four antiparallel β-strands interconnected by Ω loops. Their specificity is determined by the presence of three binding sites, located in each of the three subdomains (I, II and III), which contain a conserved motif QXDXNXVXY that may or may not be functional [10,11].

In this study, a simple and reproducible strategy based on Cibacron blue chromatography, previously described, was used to purify a 12 kDa protein identified as the lectin from taro [*Colocasia esculenta* (L.) Schott], called tarin [12]. Taro is a monocotyledonous plant of the family Araceae, native to Asia; its large starchy underground corm is widely consumed in many countries [13]. Tarin is encoded by the *tar*1 gene and has a two-domain protomer of 28 kDa, which is proteolytically processed into two different monomers of about 12.5 kDa. The protein binds specifically to mannose, i.e., is a member of the GNA-related lectin superfamily [14–16].

The molecular mass of tarin is an open issue, since it has been reported to be 25 or 50 kDa [14–17]. The present study analyzed the structural characteristics of tarin, including its native mass, quaternary structure stability, the presence of isoforms in this preparation, and the presence of glycosylation. The binding specificity was also studied, using glycan microarray techniques together with modification of amino acids, to determine the participation of certain amino acids in mannose binding.

2. Materials and methods

2.1. Purification of tarin

Tubers of taro were purchased from a local market in Rio de Janeiro, Brazil. Crude extracts were prepared as described by Roy and colleagues [18]. One hundred grams of taro was homogenized in 0.2 M NaCl containing 1 g/L ascorbic acid (10 mL/g fresh weight) at pH 7.0 in a Waring blender. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 3000 ×*g* for 10 min. $CaCl_2$ (20 mM) was added to the supernatant, the pH adjusted to 9.0, and the suspension was allowed to stand overnight at 8 °C. Next, it was centrifuged at 3000 ×*g* for 10 min and the supernatant was pooled, adjusted to pH 4.0 and centrifuged at 3000 ×*g* for 10 min. The clear solution was adjusted to pH 7.5, allowed to stand overnight in an ice bath, and the precipitate formed was removed by centrifugation at 9000 ×*g* for 20 min. The crude extract was applied to a Cibacron Blue 3GA column (4% beaded agarose matrix) (1.6 × 20 cm) previously equilibrated with 10 mM Tris–HCl pH 7.5. The column was washed with the same buffer, and the bound protein was eluted with wash buffer containing 0.4 M NaCl. Flow rate was maintained at 1.7 mL/min, and 2 mL fractions were collected during the elution step until the absorbance at 280 nm dropped below 0.01/mL. The protein concentration was estimated by the method of Lowry [19], using bovine serum albumin (BSA) as a standard.

2.2. Agglutination and sugar inhibition evaluation

Hemagglutination assays were carried out using formaldehyde-treated rabbit erythrocytes (obtained from the Unit for Laboratory Animal Medicine at the University of Michigan) in a 96-well microtiter plate. Purified tarin solutions (30 μL) were serially diluted with phosphate-buffered saline (PBS) pH 7.2, followed by the addition of 30 μL of the 2% (v/v) rabbit erythrocyte suspension. The results were observed after 1 h and recorded as hemagglutination units (HU), which correspond to the smallest amount of protein that causes hemagglutination. For inhibition assays, a tarin solution (at least 4 HU, previously determined) was incubated for 10–15 min with a fixed concentration of various ligands, followed by the addition of 30 μL of 2% erythrocyte solution. Those ligands that were able to inhibit hemagglutination were serially diluted and the agglutination assays repeated to determine the minimum inhibitory concentration (MIC).

2.3. Determination of molecular mass of intact tarin by ESI-MS and MALDI-MS mass spectrometry

ESI-MS analysis was performed at the Protein Structure Facility at the University of Michigan, using a nanoAcuity/Qtof Premier instrument in positive ion mode. For denaturing conditions, tarin at 1.35 mg/mL was diluted (1:10) in 0.1% formic acid and 9 μL was injected into a protein microdesalting column. The LC gradient was 5% buffer B (0.1% formic acid in acetonitrile) to 90% B over 10 min. Flow rate was 30 μL per min. The mass accuracy is ±0.01%. Under non-denaturing conditions, the sample was analyzed by direct infusion of the 1.35 mg/mL sample, without dilution or modification of the buffer.

MALDI-TOF-MS analysis was performed on an Applied Biosystems 4700 Proteomics Analyzer 66 (TOF/TOF) using facilities provided by the Universidade de Aveiro. Purified non-digested protein in 10 mM Tris–HCl pH 7.5 or distilled water was deposited onto a probe plate, using α-cyano-4-hydroxycinnamic acid as the matrix.

2.4. Size exclusion chromatography with SEC-MALS

Tarin at 2.45 mg/mL was run on a WTC-030S5 SEC column attached to an ÄKTA HPLC system in-line with a Dawn Heleos II Multi-Angle Laser Light Scattering (MALS) instrument, an Optilab T-rEX differential refractometer, and a UV spectrometer. Chromatographic separation was achieved under isocratic conditions using phosphate buffered saline pH 7.4 as a mobile phase, at 10° C, and the flow rate was held at 0.350 mL/min. The output signals were imported into Astra 6 software (Wyatt Technology Corporation), using the Zimm model.

2.5. Heat treatment of tarin

The effect of temperature on tarin stability was evaluated according to Pan and colleagues [20], by heating aliquots of tarin (1 mg/mL) at 30, 40, 50, 60, 70, 80 or 90 °C for 30 min, cooling at 4 °C and assaying for hemagglutinating activity. Non-treated protein was considered as 100% active.

2.6. Effect of pH on tarin stability

pH pretreatment of tarin was performed according to Pan and colleagues [20]. Tarin solution (10 μL at 1 mg/mL) was incubated for 1 h with 90 μL of various buffer solutions. Aliquots of 50 μL of the treated protein were then mixed with 50 μL of 0.25 M Tris–HCl pH 7.5 containing 0.3 M NaCl to adjust the pH, and an aliquot of the mixture (30 μL) was serially diluted by adding 25 mM Tris–HCl pH 7.5 and 0.15 M NaCl, and tested for hemagglutinating activity. The following buffers were used: 1 N HCl (pH 1.0), 50 mM Glycine-HCl (pH 2.0–3.0), 50 mM acetate buffer (pH 4.0–5.0), 50 mM phosphate buffer (pH 6.0–7.0), 50 mM Tris–HCl buffer (pH 8.0–9.0) and 50 mM sodium bicarbonate buffer (pH 10.0–11.0).

2.7. Metal ion requirement for tarin activity

An aliquot (100 μL) of the tarin in elution buffer (548 μg/mL) was pre-incubated in the presence of 5 mM EDTA pH 7.2 for 1 h at room temperature. The tarin solution was serially diluted in PBS pH 7.2 containing 5 mM EDTA, and the agglutination activity was evaluated.

2.8. Two-dimensional electrophoresis (2-DE) fractionation

Tarin was fractionated using the Multiphor II system (Amersham Pharmacia Biotech, Uppsala, Sweden). Tarin (100 μg) in rehydration buffer (8 M urea, 2% CHAPS, 2% Immobilized pH gradient (IPG) buffer and trace of bromophenol blue) was incubated with the IPG strips (pH range 3.0–10) at room temperature for 18 h, after which, IEF was performed at 20 °C. After the first dimension was run, the strip was treated

with equilibration buffer (1.5 M Tris–HCl 6 M urea, 30% glycerol, 2% SDS, 0.01% bromophenol blue and 100 mg of dithiothreitol) and with blocking buffer (1.5 M Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, 0.01% bromophenol blue and 250 mg iodoacetamide). The second dimension was run in a polyacrylamide gel with a gradient of 12–14% with SDS (Excel-Gel XL SDS 12–14; Amersham Biosciences) at 15 °C. Spots revealed by 0.2% Coomassie blue staining were digested with trypsin (20 ng/μL), desalted using ZIPTip C18, mixed with the matrix solution (1:1 acetonitrile/water containing 0.3% formic acid) and deposited onto a probe plate using α-cyano-4-hydroxycinnamic acid as the matrix.

2.9. Carbohydrate content estimation

The neutral carbohydrate content of tarin was quantified by the phenol-sulfuric acid method [21]. In brief, tarin aliquots were treated with 62 μL of 80% phenol. Then, 5 mL of concentrated sulfuric acid was added rapidly, directly onto the liquid surface in order to obtain good mixing. The tubes were allowed to stand for 10 min, followed by shaking and placed in a water bath at 25–30 °C for 10 to 20 min before the absorbance was read at 490 nm. The content of carbohydrate-binding to tarin was determined through comparison to a standard glucose reference.

2.10. Chemical modification of amino-acid residues

The effects of chemical modification of amino-acid residues were evaluated based on the changes in tarin hemagglutinating activity.

Tryptophan residues were modified according to the method of Tawfik [22] using the Koshland reagent. Serine, tyrosine and lysine were modified according to the protocol of Sharma and colleagues [23] using phenylmethylsulfonyl fluoride (PMSF), N-acetylimidazole and sodium borohydride ($NaBH_4$) reagents, respectively.

To modify sulfhydryl groups, the method of Tawfik [24] was followed, using 5 mM 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB or Ellman's reagent).

Cysteine was also carboxymethylated following the method of Aitken and Learmonth [25], using β-mercaptoethanol and iodoacetamide. Arginine was modified according to the protocol of Tawfik [26], using p-hydroxyphenylglyoxal.

Aspartic and glutamic acids were modified according to the method of Tawfik [27], using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

2.11. Glycan microarray

Analysis was undertaken using the Core H of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/). Tarin was directly labeled with Alexa Fluor 488, scanned, and analyzed according to the protocol (Direct Binding Protocol and Scanning and Analysis Protocol) provided on the web page. The complete data can be viewed on the CFG website (search for *C. esculenta* at http://www.functionalglycomics.org/glycomics/publicdata/selectedScreens.jsp?sortBy=proteinName&sortOrder=asc).

3. Results and discussion

3.1. Tarin native molecular mass determination

Tarin was chromatographed on a Cibacron Blue 3GA column, and was eluted with 0.4 M NaCl added to Tris–HCl 10 mM pH 7.5 [12].

The purified tarin was run on SDS-PAGE in the presence or absence of β-mercaptoethanol and a single band was recovered, with an estimated molecular mass of ~12 kDa (Fig. 1A). This is in agreement with previous reports [14–16] showing that the tarin subunits are held together by non-covalent bonds.

The exact molecular mass of the subunits was determined by mass spectrometry (MALDI-MS and ESI-MS) under denaturing conditions (Fig. 1B). In both cases, two monomeric units with masses of 11,928.4 Da and 12,685.1 Da (MALDI-MS) or 11,924.7 Da and 12,680.0 Da (ESI-MS) were observed, although they were not resolved on SDS-PAGE. Tarin was also analyzed by non-denaturing ESI-MS through the direct infusion of the purified protein without dilution or modification of the buffer, in order to determine the native mass of the protein. Again, individual subunits with molecular masses of 11,924.7 Da and 12,681.6 Da were observed. There was no evidence of a heterodimer or higher-molecular-weight structure (results not shown), probably because the salt concentration in the original sample was high enough to separate its monomers. The mass spectrometry results reinforce the supposition that the subunits are held together by non-covalent bonds.

In an attempt to determine the native molecular mass of tarin, the purified protein was run on a gel filtration Sepharose CL-6B column in 50 mM Tris–HCl pH 7.5 with no salt, and the estimated native molecular mass of tarin was 26.6 kDa (data not shown). To prevent interactions between the protein and the resin matrix, the gel-filtration fractionation was repeated on a Superose-12 column equilibrated with PBS buffer pH 7.4 and 0.2 M mannose [17]. The molecular mass of tarin was estimated to be 30 kDa (data not shown). To reconcile the differences between the two gel-filtration procedures and to evaluate the possibility that the protein has a delayed elution pattern due to matrix interaction [28], tarin was loaded onto a silica-based size exclusion HPLC column with an in-line multi-angle light scattering detector coupled with a refractive index unit and UV detector. The peak was monodispersed, and the mean molecular mass was 47 kDa, as estimated by the Zimm methodology (Fig. 1C). Taken together, these data indicate that the tarin quaternary structure corresponds to a heterotetramer, as previously reported by Van Damme and colleagues [17], and differs from the structures reported by [14–16].

3.2. Tarin isoform analysis

In previous studies, the crude extract from taro was analyzed by two-dimensional electrophoresis, showing that the 12 kDa polypeptide band is composed of 5 major tarin subunit isoforms (pI ranging from 5.5 to 7.9). Four of them were identified as G1a, G1b, G1c and G1d by N-terminal sequencing [16,29]. N-terminal sequencing clustered the peptides in two groups, G1a/G1c and G1b/G1d, showing high intragroup identity, but sharing only 25% intergroup sequence similarity. They originated from the tarin precursor polypeptide with G1b/G1d at the N-terminus and G1a/G1c at the C-terminus [29]. Similarly, Monte-Neshich and colleagues [13] reported the existence of at least 10 isoforms, with pIs ranging from 5.5 to 9.5.

Since the affinity purification of tarin is not dependent on sugar binding, it was shown here that the tarin preparation is composed of a heterogeneous mixture of isolectins with similar molecular masses (47 kDa). This feature is a common characteristic of lectins from the GNA-related superfamily [30]. In contrast to previous studies that analyzed the crude extract, the present study reports the analysis, by 2D electrophoresis, of the purified tarin. This analysis resulted in 16 spots with apparent molecular masses of about 12 kDa and different pIs ranging from 6.5 to 9.5 (Fig. 1D). MALDI-TOF-TOF mass spectrometry and database searching for peptides generated by tryptic in-gel digestion of these spots confirmed, with a high degree of reliability, that at least 10 of them were tarin subunit isoforms (Table 1). Furthermore, MALDI-TOF spectra of three spots (labeled 1, 5 and 10 in Fig. 1D) showed masses corresponding to the N-terminal region of the G1a (2226.1189 Da, NIPFTDNLLFSGQVLYGDGR), G1b (2045.0631 Da, LGTNYLLSGQTLNTDGHLK) and G1d (2046.0502 Da, LGTNYLLSGQTLDTDGHLK) isoforms, and their identities were confirmed by MS/MS (Table 1). Our findings are similar to those of Monte-Neshich

and colleagues [13], which indicated the presence of multiple isolectins with about 12 kDa, and differ from the findings of other groups [16,29].

Charge heterogeneity among GNA-related lectins is not unusual, since it was reported in four araceous GNA-related tuber lectins from *Arisaema consanguineum*, *Arisaema curvatum*, *Sauromatum guttatum* and *Gonatanthus pumilus* [31]. Another araceous tuber lectin, from *Arum maculatum,* also showed isoforms encoded by a family of genes [17].

Tarin microheterogeneity can be explained, at least partially, by the existence of allelic forms of the *tar1* gene. Southern blot analysis of the

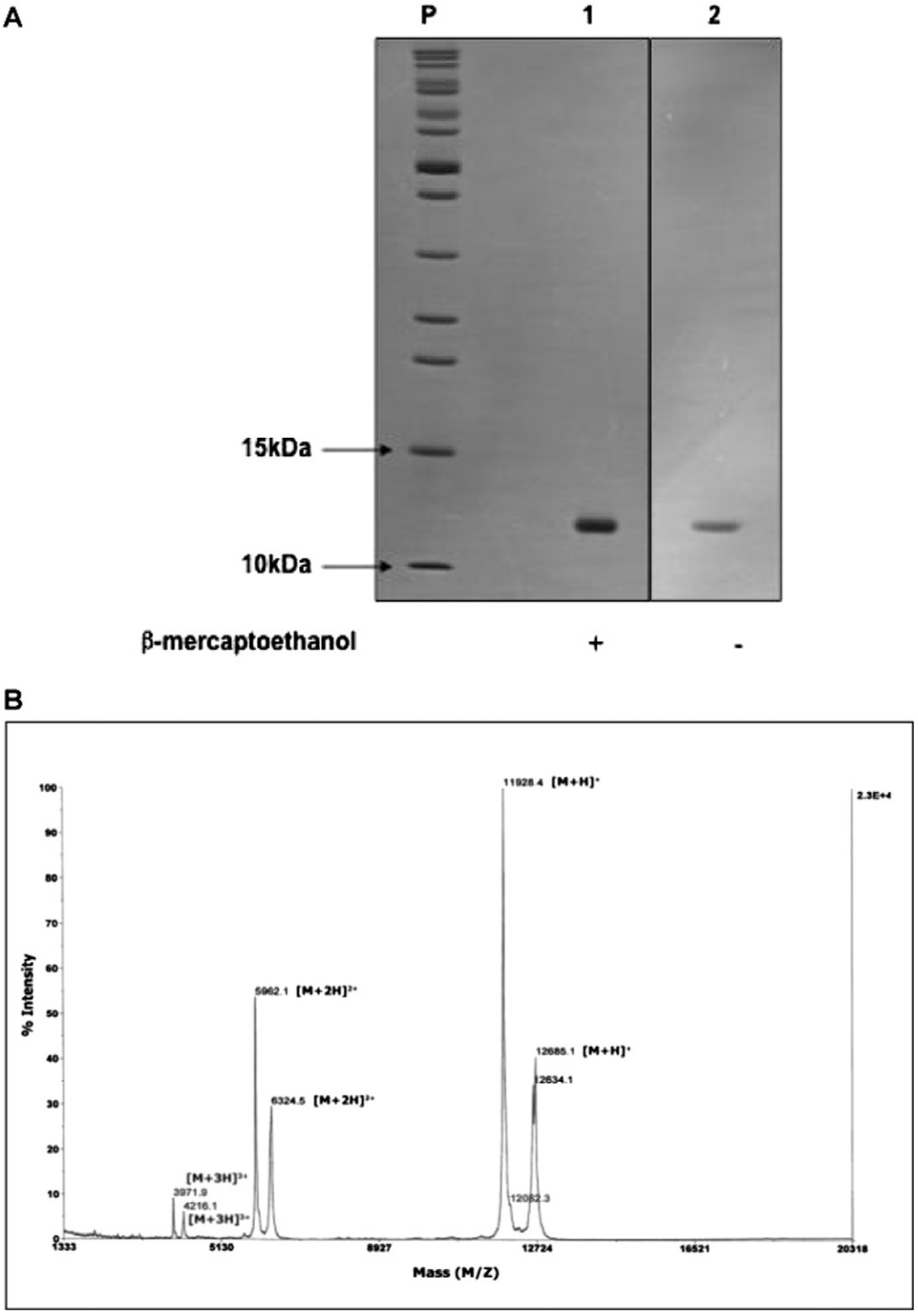
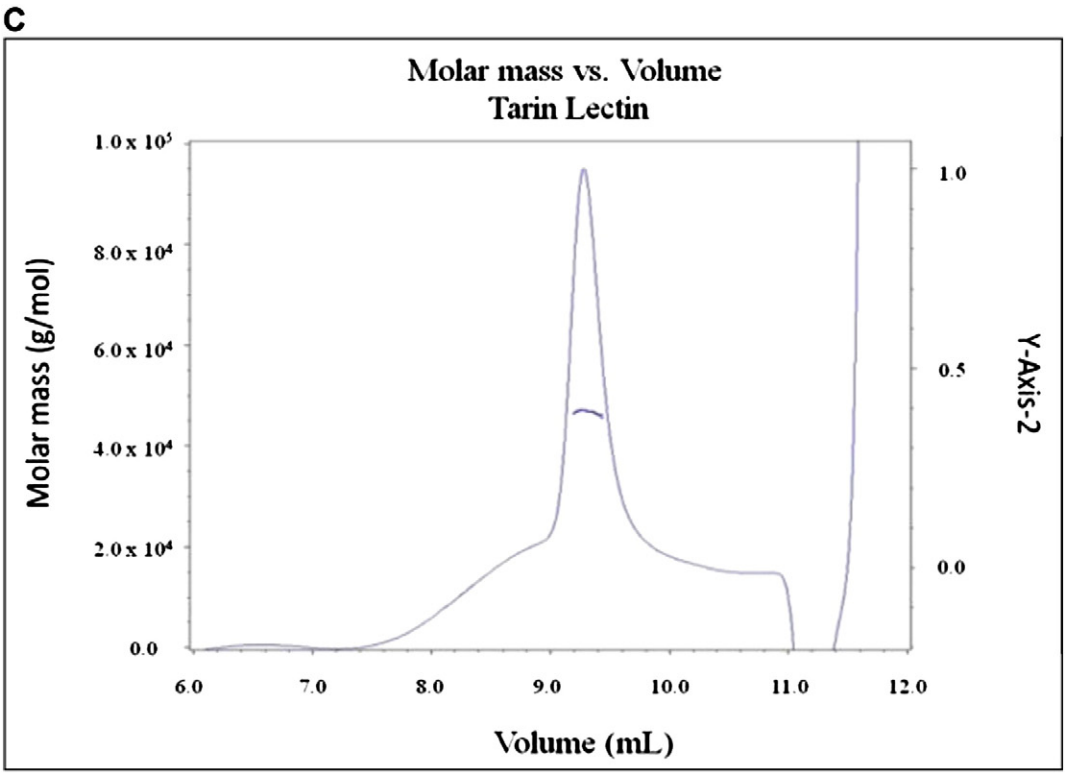


Fig. 1. Tarin identification and characterization. (A) The horizontal line represents a plot of the molar mass of tarin vs. time. The UV signal at 280 nm is overlaid, showing a peak at 9.3 min. The average molecular mass was calculated to be 47,130 Da. (B) 12.5% SDS-PAGE in the presence (lane 2) or absence (lane 3) of β-mercaptoethanol and molecular mass markers (lane 1). (C) Analysis of tarin in solution by MALDI-MS prior tryptic digestion. (D) Purified tarin (100 μg) was fractionated by 2-DE using an immobilized pH gradient (pH 3–10) in the first dimension and a polyacrylamide gradient gel (12–14%) containing SDS in the second dimension. Protein spots were stained with Coomassie blue. The spots identified by mass spectrometry are circled in red.



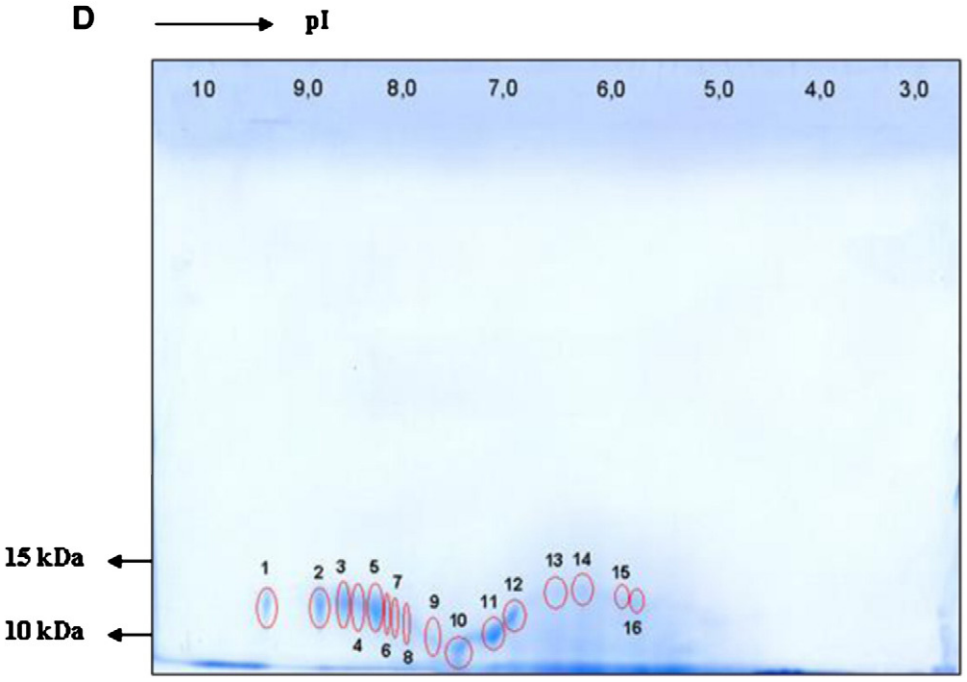


Fig. 1 (*continued*).

taro genomic DNA [29] and the primer extension analysis of *tar1* mRNA [16] revealed three different bands. These results, together with the occurrence of tarin subunits with nearly identical N-terminal sequences [29], reinforce the hypothesis that tarin is encoded by a small gene family. Alternatively, some tarin subunit isoforms could result from the heterogeneity in the oligosaccharide chain, as suggested by Hayes and Goldstein [32], or could be artifacts produced during the isolation procedures, by deamidation of asparagine or glutamine side chains [33].

3.3. Carbohydrate content analysis

Neutral carbohydrate content linked to the tarin primary structure was estimated to be approximately 2–3% of molecular mass, by the phenol-sulfuric acid method [21]. The low glycosylation of tarin is in agreement with the 0.47 to 1.47% carbohydrate contents of five monocot mannose-binding lectins from members of the family Araceae [31, 34]. Further studies are necessary to determine the glycosylation site and the oligosaccharide structure of tarin.

Table 1
Identification of 2D-Gel spots by mass spectrometry (MALDI-MS).

Spot[a]	Identification	UniProt accession no.	CI %	Protein score	Covered (%)
1	Lectin from *C. esculenta*	A5HMM7_COLES	100	138	19.20
	Globulin G1b (fragment)	Q9S8T5_COLES	99.59	92	79.16
2	Lectin from *C. esculenta*	A5HMM7_COLES	100	131	19.19
3	Lectin from *C. esculenta*	A5HMM7_COLES	100	153	19.19
4	Lectin from *C. esculenta*	A5HMM7_COLES	100	149	28.12
5	Lectin from *C. esculenta*	A5HMM7_COLES	100	252	51.34
	Globulin G1a (fragment)	Q9S8T3_COLES	99.626	93	58.82
6	Lectin from *C. esculenta*	A5HMM7_COLES	100	156	27.23
9	Lectin from *C. esculenta*	A5HMM7_COLES	100	135	19.19
10	Lectin from *C. esculenta*	A5HMM7_COLES	100	218	28.12
	Globulin G1d (fragment)	Q9S8T3_COLES	99.999	118	100
13	Lectin from *C. esculenta*	A5HMM7_COLES	100	198	28.12
14	Lectin from *C. esculenta*	A5HMM7_COLES	100	209	35.71

[a] Protein spot number follows the identification in 2D-Gels shown in Fig. 1D.

3.4. Effects of pH and temperature on tarin stability

Tarin retained 100% of its hemagglutinating activity when exposed to pHs ranging from 1 to 11 for 1 h (Fig. 2A) or to temperatures up to 70 °C for 30 min, and dropped to 37% and 25% of its initial activity after exposure to 80 °C and 90 °C, respectively (Fig. 2B). The stability of the tarin quaternary structure, evaluated by the ability to easily maintain or recover itself after exposure to extreme temperature and pH conditions, has also been reported for other monocot mannose-binding lectins from species of Araceae [31,35]. Tarin's stability over a wide pH range confers resistance to degradation within the insect digestive tract, increasing its potential against insects [18,36]. Tarin does not require metal ions for its hemagglutinating activity, since treatment with 5 mM EDTA for 1 h showed no effect (data not shown).

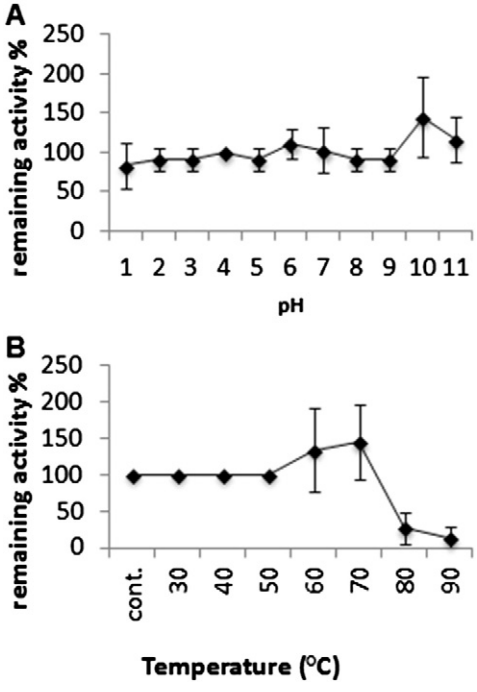


Fig. 2. Tarin stability following pH and heat treatments. Effect of pH (A) and temperature (B) on tarin hemagglutinating activity. Tarin was treated at different pHs for 1 h, and its activity was determined after the pH was readjusted to 7.5. Tarin was treated at different temperatures for 30 min, and the hemagglutinating activity was tested after the samples were cooled. Untreated-tarin hemagglutinating activity was considered to be 100% activity. Values are means of three independent experiments.

3.5. Chemical modification studies

Many monocot mannose-binding lectins share a common conserved motif, QXDXNXVXY, at the carbohydrate binding site, which confers the exclusive specificity on these lectins. This study analyzed the participation of some amino acids in the specificity of tarin. The effects of chemical modification of residues within the conserved motif on tarin activity are summarized in Table 2. Modification of arginine (R), lysine (K) and serine (S) had no effect on the hemagglutinating activity of tarin, indicating that these residues do not participate in the binding activity. Similarly, no change in tarin activity was observed after DTNB treatment, suggesting that the sulfhydryl groups are not involved in lectin activity. Modification of tryptophan (W) or aspartic/glutamic residues (D/E) resulted in partial losses of activity, indicating that these residues are probably involved in the binding activity.

Comparison of the four non-identical amino acid sequences of tarin found in the data bank resulted in the detection of two functional carbohydrate recognition sites (CRS) [36]. The presence of two aspartic residues in the first binding site (QD/NDCNLVLY) and one in the second (QGNDCNLVLY), in addition to the presence of the same amino acid in the vicinity, could explain the loss in activity when aspartic residues were modified.

Although no tryptophan residue is found in the consensus sequence of the binding site, some of this residue is located around it (W63/67, W95/99, W29, W84, W216). One of the tryptophan residues is very close to the CRS, suggesting that the residue is involved or participates in maintaining the conformation of the binding pocket.

Modification of tyrosine (Y) residues resulted in 75% loss of activity, suggesting that these residues participate in the binding activity. Tyrosine is one of the amino acids present in the conserved consensus

Table 2
Chemical modification of tarin residues.

Residue modified	Reagent used	Hemagglutinating activity (%)
Serine	PMSF	100
Lysine	$NaBH_4$	100
Tryptophan	HNB	75
Tyrosine	N-acetylimidazole	25
Sulfhydryl groups	DTNB	100
Cysteine	Iodoacetamide	100
Arginine	HPG	100
Aspartic/glutamic acid	EDC	75

PMSF	Phenylmethylsulfonylfluoride
$NaBH_4$	Sodium borohydride
HNB	2-Hydroxy-5-nitrobenzylbromide
DTNB	5-5′-Dithio-*bis* (2-nitrobenzoic acid) or Ellman's reagent
HPG	*p*-Hydroxyphenylglyoxal
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carboxiimide

sequence in both CRS. These residues seem to be crucial for maintaining the functionality of the binding site.

3.6. Specificity of tarin–carbohydrate binding

The sugar specificity of tarin was evaluated by hapten inhibition of the hemagglutinating activity. The hemagglutinating activity of the lectin was inhibited by four glycoproteins (asialofetuin, asialoagalactofetuin, bovine submaxillary mucin and bovine thyroglobulin) of the 18 haptens tested (Table 3). The simple sugars or oligosaccharides, including mannose, and its oligomers did not show inhibitory activity, indicating that tarin has complex sugar specificity.

Glycan microarray analysis was performed through the Functional Glycomics Gateway (http://www.functionalglycomics.org/), using tarin at 0.2, 2.0, 20.0 and 200.0 μg/mL concentrations. The 30 highest average rank values, which rank the glycans bound to tarin according to their relative strength of binding in terms of total fluorescence, were calculated as described previously [37], excluding the fluorescence data from 200.0 μg/mL of tarin, to avoid evaluation of non-specific interactions (Table 4). The fluorescence signal increased roughly threefold for most glycans when the concentration of tarin was increased from 2.0 to 20.0 μg/mL. The data obtained at 20.0 μg/mL tarin are presented in Figs. 3 and 4. The glycan microarray results confirmed the complex sugar specificity exhibited by tarin in the hapten inhibition assays. The purified protein showed strong affinity toward the biantennary complex glycans, paucimannose and oligomannose glycans, and their fucosylated counterparts (Table 4).

The shortest tarin ligand in the microarray was the core pentasaccharide Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ (Table 4, glycans 49 and 50). The addition of two or three extra mannose residues to α-1,3/α-1,6 linked mannose in the pentasaccharide core increased the binding strength, as seen in glycans 213 and 212 (Fig. 3). Further extension of the triantennary complex with extra mannose residues weakened (glycan 208) or abolished (glycans 209 and 207) the tarin–carbohydrate interaction (Fig. 3). The chitobiose core and the asparagine present in the glycan stalk (glycan 49) likely contribute to the binding and specificity of tarin, since the protein did not bind to trimannose (Manα1-3(Manα1-6) Manα-Sp9, glycan 210). Additionally, the fucosylation at position 6 of the first N-acetyl glucosamine (Fig. 3, glycan 477) increased the binding strength. Interestingly, the replacement of asparagine linked to the pentasaccharide core (glycan 49) by glycine (glycan 50), which could relieve the steric hindrance at the binding site or allow additional interactions between the closest α-carboxyl group of glycine with the binding site, also increased the binding strength.

Glycans 212 and 213 (Table 4) can also be found as part of the gp120 glycoprotein of HIV-1 [38] and of the CA-125 glycoprotein antigen of epithelial ovarian cancer cells [39], respectively. Glycan 477 is found in insect glycoproteins [40]. Paucimannose (glycan 49) is commonly found in insects and nematodes [41] and in cancerous, but not in healthy tissues from humans [42].

Fig. 4 lists some selected complex glycans that showed high binding affinity. Glycans were covalently bonded to free asparagine and asparaginyl residue within a peptide. Most of them are biantennary glycans that have in common two identical antennae linked to Manα1-6 and Manα1-3 in the pentasaccharide core. These structures can be elongated by successive addition of GlcNAc (β1,2-linked to mannose) and/or Gal (β1,4 or β1,3-linked to GlcNAc) that can be capped with fucose, galactose or Neu5Ac. Structures with an additional fucose (α1,3-linked) to GlcNAc in the antenna or in the chitobiose core (α-1,6-linked) also showed affinity to tarin. However, given the complex nature of these glycans, the lack of intermediate structures in the microarray, and the difference in peptide sequences bound to glycans, there is insufficient information to establish the individual contribution of sugar alterations that could increase the binding strength and specificity of glycans to tarin.

Triantennary glycan 366- and tetra-antennary glycan 384-containing antennae, which are formed by the disaccharide Galβ-1,4GlcNAc (β1,2 or β1,4-linked to mannose), were also good ligands for tarin. Indeed, the binding of tetra-antennary glycan 384 (RFU 34,583 ± 1523) was similar to that of glycan 465 (33,668 ± 391), the best biantennary ligand for tarin at the concentration of 20 μg/mL.

It has been reported that the introduction of bisecting GlcNAc and/or core fucosylation can reduce the number of major glycan conformers, since they shift the conformational equilibrium and increase binding stringency, favoring a conformer-specific glycan receptor [43]. Fucosylation of the chitobiose stalk as seen in glycans 465, 475, 476 and in 350, was not able to determine the role of the chitobiose stalk fucosylation in glycan binding. Fucosylation increased the binding of tarin to glycan 475 threefold, in comparison to its unfucosylated form (glycan 323) (Fig. 4). On the other hand, fucosylated glycan 350 showed weaker binding than glycan 53, although they have similar structures. Because glycans 53 and 350 also differ in the amino acid extension of the chitobiose core (asparagine in glycan 53 and the tripeptide NST in glycan 350), it cannot be determined if the inhibitory effect was due to the fucosylation or to the NST peptide.

Interestingly, the terminal sequence in the antenna of glycan 465 is equal to Lewis^y-type tetrasaccharide (Fucα1-2Galβ1-4(Fuca α1-3) GlcNAc) which is found in N-linked, O-linked or lipid-linked terminal glycan structures [44]. The Lewis^y epitope is a relatively rare sequence in humans. It is found in small amounts in normal epithelial cells, including those in the stomach and colon [45], and is also found in N-glycans of human seminal plasma [46], human sperm [47], hematopoietic progenitor cells [48], and in peripheral blood granulocytes [49]. Le^y expression is increased in most epithelial-derived cancer cells, including colon, stomach, ovary, breast, pancreas, prostate and lung, and is generally considered to be a cancer-associated antigen [50]. Also, glycan 358 characterizes the antigen H2 (CD173-Fucα1-2Galβ1-4GlcNAcβ1-), which can be found in leukemic cells and in hematopoietic progenitor cells [48]. These findings explain the proliferative properties, reported

Table 3
Hemagglutination-inhibition test.

Haptens tested	Minimum concentration required for inhibition (MIC) μg/mL
1 — Fetuin 1.3 mg/mL	NI
2 — Asialofetuin 1 mg/mL	31.3
3 — Asialogalactofetuin 1 mg/mL	62.5
4 — Bov. Sub. Mucin 1 mg/mL	125.0
5 — Asialomucin 1 mg/mL	NI
6 — Bov. Thyroglobulin 2 mg/mL	250.0
7 — Yeast Mannan 2 mg/mL	NI
8 — Mannose 100 mM	NI
9 — Me-α-man 100 mM	NI
10 — M-α-2M 50 mM	NI
11 — Glc 200 mM	NI
12 — Glc NAc 100 mM	NI
13 — M3	NI
14 — M5	NI
15 — Me-α-Gal 100 mM	NI
16 — LacNAc 100 mM	NI
17 — GalNAc 100 mM	NI
18 — L-Fucose 200 mM	NI

NI No inhibition
Bov. Sub. Mucin bovine submaxillary mucin
Me-α-man Methyl-α-D-Mannopyranoside
M-α-2M Mannose-α-2-mannose
Glc Glucose
GlcNAc N-Acetyl-D-Glucosamine
Me-α-Gal Methyl-α-D-Galactopyranoside
LacNAc N-Acetyl-D-Lactosamine
GalNAc N-Acetyl-D-Galactosamine
M3 Trimannoside
M5 Pentamannoside
Agglutination inhibition was evaluated using 2% rabbit cells treated with trypsin.

Table 4
Tarin specificity by glycan microarray.

Glycan	Glycan structure	Mean rank
High-mannose N-glycans		
212	Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	37.7
477	Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp19	30.9
213	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	27.3
50	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp13	17.4
49	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	8.1
Complex N-glycans		
465	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6(Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-6AA	97.9
475	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-6AA	83.8
384	Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-4)Manα1-3(Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp21	75.3
321	Galβ1-3GlcNAcβ1-2Manα1-3(Galβ1-3GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp19	38.8
476	Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6(Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-6AA	38.4
385	GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Sp21	37.8
366	Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-4)Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Sp21	36.7
400	Galα1-4Galβ1-4GlcNAcβ1-2Manα1-3(Galα1-4Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-LVANKT	33.9
399	Galα1-4Galβ1-3GlcNAcβ1-2Manα1-3(Galα1-4Galβ1-3GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp19	30.6
350	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	20.6
351	Galβ1-3GlcNAcβ1-2Manα1-3(Galβ1-3GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	18.4
466	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-6(Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp19	17.9
52	GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp13	16.8
360	Galα1-3Galβ1-4GlcNAcβ1-2Manα1-3(Galα1-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	16.3
348	Galβ1-4GlcNAcβ1-2Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	14.2
53	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	13.8
393	Galβ1-4GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Sp12	12.5
424	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-2Manα1-3(Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	12.4
323	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	10.5
358	Fucα1-2Galβ1-4GlcNAcβ1-2Manα1-3(Fucα1-2Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	10.0
349	GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	9.9
54	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-N(LT)AVL	9.9
368	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-2Manα1-3(Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	9.9
371	Galα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-2Manα1-3(Galα1-3(Fucα1-2)Galβ1-3GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	9.0
361	Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	7.5

in a previous study by our research group, where tarin was shown to stimulate the in vitro and in vivo proliferation of mouse hematopoietic cells, particularly the B cell population [12].

Biantennary glycan 475/A2F and glycan 323/A2 (unfucosylated), two N-linked glycans that are commonly found in mammals, showed the terminal carbohydrate sequence Neu5Aca2–6Galβ1–4GlcNAc β

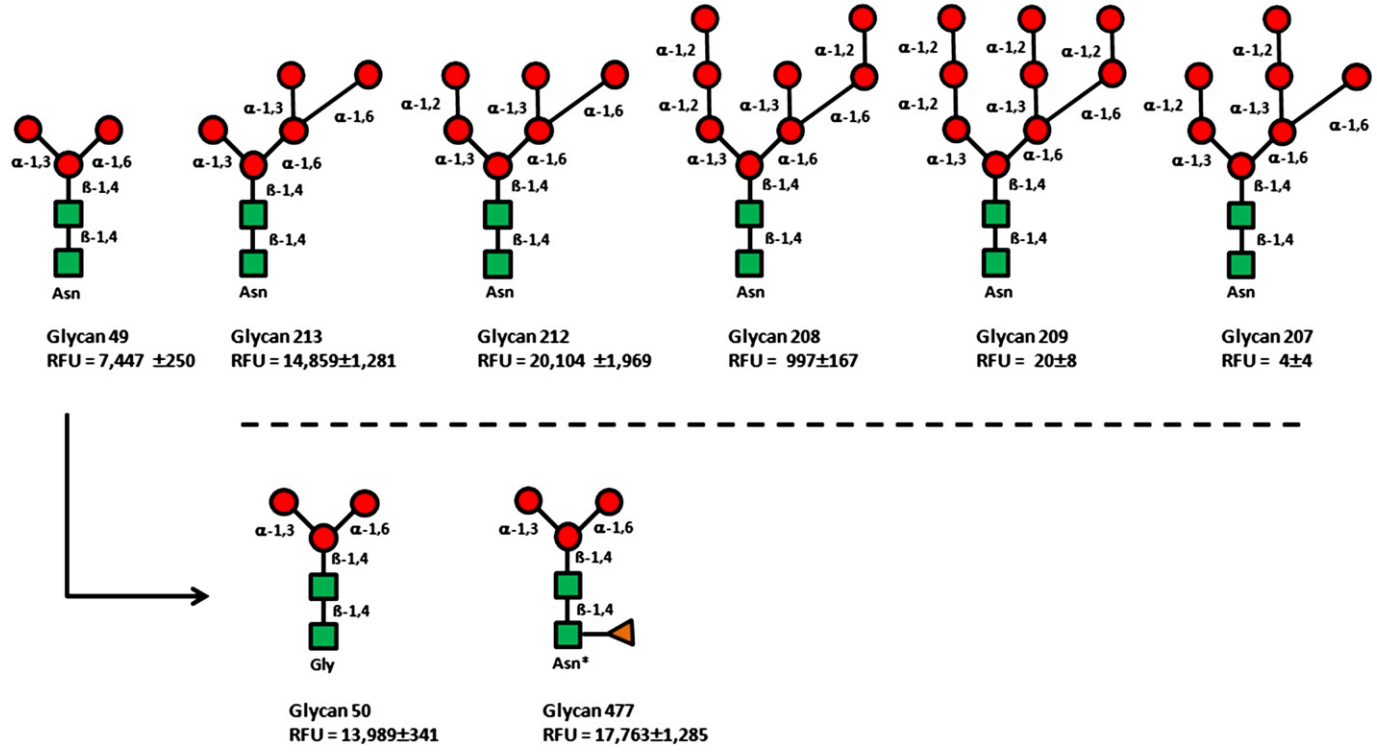


Fig. 3. High-mannose N-linked glycan-bound tarin in a glycan microarray. Structures of high-mannose oligosaccharide-bound tarin in the glycan microarray. Glycan number is the same as in Table VII. Green square — N-acetylglucosamine; red circle — mannose; orange triangle — fucose. RFU (relative fluorescence unit) was obtained from a glycan microarray assay at 20 μg/mL tarin. Some glycan structures that do not bind to tarin are presented for comparison. Asn* (glycan 477) is asparagine in dipeptide EN or NK.

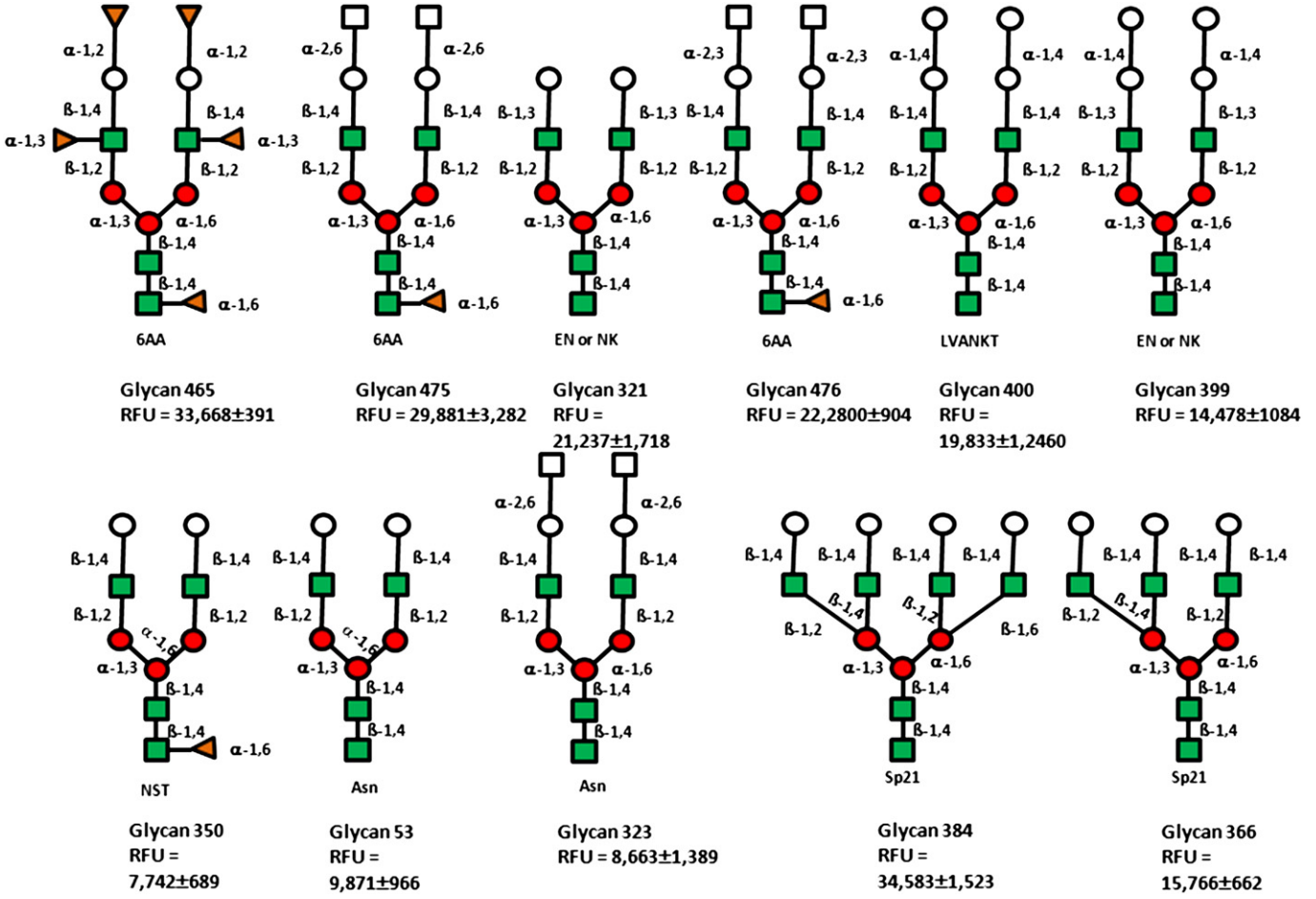


Fig. 4. Structures of complex glycan-bound tarin in the glycan microarray. Glycan number is the same as in Table VII and in the supplementary data. Green square — N-acetylglucosamine; red circle — mannose; orange triangle — fucose; white circle — galactose; white square — N-acetylneuraminic acid. RFU (relative fluorescence unit) was obtained from a glycan microarray assay at 20 μg/mL tarin. Some glycan structures that do not bind to tarin are presented for comparison.

1,2-linked to mannoses in the pentasaccharide core. This sequence binds with high affinity to all human influenza A and B viruses [51], and glycan 323 can protect mice from experimental influenza infection [52]. Glycan 323 was able to bind to *Colchicum autumnale* agglutinin and *Tulipa* hybrid lectin in a glycan microarray assay [53].

The above results for tarin sugar-binding specificity are in agreement with those reported by Van Damme and colleagues [53], who showed that the majority of two-domain monocot-mannose binding lectins are able to strongly interact with both high-mannose and complex N-glycans. The presence of multiple isolectins in our preparation may account for the complex specificity shown by tarin and shared by other lectins of this family, as mentioned previously [30].

However, Van Damme and colleagues [53] reported the occurrence of multiple independent domain duplication/tandem events among most of the known two-domain GNA-related lectins. The sequence of the amino acids that compose the binding sites of two-domain lectins is less conservative than in the single-domain lectins. As a result, two-domain lectins exhibit dual specificity toward different targets, including both high-mannose and complex N-glycans, typical animal glycans, which supports the idea that these proteins evolved for purposes of defense.

The four primary sequences of tarin deposited in the data bank contain two functional binding sites. The second binding site differs from the first in one amino acid substitution (D52/G171). However, the four entries share the same sequence in the second binding site [36]. Except for one of the sequences (A5HMM7_COLES) where the aspartic residue is replaced by asparagine in the first binding site, all the other amino acids are identical. Although the composition of their binding sites is strongly conserved, they all have different pIs as revealed by ProtParam analysis, which suggests that each entry represents a specific tarin isoform. The complex specificity exhibited by tarin glycan binding is not an exclusive behavior of this protein, but rather is a common characteristic shared by most of the two-domain lectins of this family. Therefore, it is more likely that the complex specificity results from a process of divergence than from a heterogeneous mixture of isolectins.

Our results for tarin binding specificity provide a molecular basis for tarin's insecticidal activity [18,54] and its anti-metastatic activity against breast and prostate-cancer cell lines [55], and point to possible new applications for this biotechnologically versatile protein.

4. Conclusions

Treatment of a tarin preparation in different pHs and temperatures showed that it is a stable glycoprotein whose activity does not depend on metal ions, and is probably encoded by a multigene family, as evidenced by the 2-DE results. The results of the mass spectrometry (ESI and MALDI) indicate that the tarin monomers are held together by non-covalent bonds to form its biological unit, a tetramer of 47 kDa. Binding studies of tarin confirmed its preference for complex and high-mannose glycans including specific antigens, which concords with its remarkable antitumor, antiviral and insecticidal properties. This detailed study of purified tarin indicates that it is a stable protein that can be exploited for a variety of purposes.

List of abbreviations

PMSF Phenylmethylsulfonylfluoride
$NaBH_4$ Sodium Borohydride
HNB 2-Hydroxy-5-Nitrobenzylbromide
DTNB 5-5′-Dithio-*bis* (2-nitrobenzoic acid) or Ellman's reagent
HPG *p*-Hydroxyphenylglyoxal;
EDC 1-Ethyl-3-(3-dimethylaminopropyl) carboxiimide
Me-α-man Methyl-α-DMannopyranoside;
M-α-2M Mannose-α-2-mannose
Glc Glucose
GlcNAc N-Acetyl-D-glucosamine
Me-α-Gal Methyl-α-DGalactopyranoside
LacNAc N-Acetyl-D-Lactosamine
GalNAc NAcetyl-D-Galactosamine
M3 Trimannoside
M5 Pentamannoside
2-DE Two-dimensional electrophoresis
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PBS Phosphate-buffered saline
ESI-MS Electrospray ionization mass spectrometry
MALDI-TOF Matrix-assisted laser desorption/ionization time of flight
LC-MS Liquid chromatography mass spectrometry
MALS Multi-angle light scattering
IPG Immobilized pH gradient
IEF Isoelectric focusing
GNA-lectin *Galanthus nivalis* lectin

Competing interests

None declared.

Authors' contributions

PRP — as a PhD student, was involved in the entire study, the design, and interpretation of the data; HCW — participated in the interpretation of data and helped to draft the manuscript; MAV — helped to draft the manuscript and participated in the design; JLM — participated in the design of the study and interpretation of data; JAS — participated in the design of the study and interpretation of data; IJG — participated in the design of the study and interpretation of data; VMFP — participated in the design and coordination and helped to draft the manuscript; JTS — participated in the design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Marcus A. Nadruz Coelho from the Jardim Botânico do Rio de Janeiro for confirming the identification of the taro and Jorginaldo William Oliveira from RFA herbarium (Universidade Federal do Rio de Janeiro) for helping with the exsiccate. Patricia R. Pereira was supported by a scholarship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/fulbright (CAPES/Fulbright). Also, we thank Dr. Daniel Southworth from the University of Michigan Life Sciences Institute for his advice on and the use of the SEC-MALS system.

References

[1] W.J. Peumans, E.J. Van Damme, Lectins as plant defense proteins, Plant Physiol. 109 (1995) 347–352.
[2] C.R. Carlini, M.F. Grossi-de-Sa, Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides, Toxicon 40 (2002) 1515–1539.
[3] I.M. Vasconcelos, J.T. Oliveira, Antinutritional properties of plant lectins, Toxicon 44 (2004) 385–403.
[4] W.J. Peumans, E.J. Van Damme, A. Barre, P. Rouge, Classification of plant lectins in families of structurally and evolutionary related proteins, Adv. Exp. Med. Biol. 491 (2001) 27–54.
[5] E.J.M.V. Damme, W.J. Peumans, A. Barre, P. Rougé, Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles, Crit. Rev. Plant Sci. 17 (1998) 575–692.
[6] N. Sharon, Lectins: past, present and future, Biochem. Soc. Trans. 36 (2008) 1457–1460.
[7] C.-y. Li, L. Meng, B. Liu, J.-k. Bao, Galanthus nivalis agglutinin (GNA)-related lectins: traditional proteins, burgeoning drugs? Curr. Chem. Biol. 3 (2009) 323–333.
[8] E.J.M. Van Damme, N. Lannoo, W.J. Peumans, Chapter 3 plant lectins, in: K. Jean-Claude, D. Michel (Eds.), Advances in Botanical Research, vol. 48, Academic Press, 2008, pp. 107–209.
[9] A. Barre, E.J. Van Damme, W.J. Peumans, P. Rouge, Structure–function relationship of monocot mannose-binding lectins, Plant Physiol. 112 (1996) 1531–1540.
[10] G. Hester, H. Kaku, I.J. Goldstein, C.S. Wright, Structure of mannose-specific snowdrop (*Galanthus nivalis*) lectin is representative of a new plant lectin family, Nat. Struct. Biol. 2 (1995) 472–479.
[11] E.J.M. Van Damme, A.K. Allen, W.J. Peumans, Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (*Galanthus nivalis*) bulbs, FEBS Lett. 215 (1987) 140–144.
[12] P.R. Pereira, E.M. Del Aguila, M.A. Vericimo, R.B. Zingali, V.M. Paschoalin, J.T. Silva, Purification and characterization of the lectin from taro (*Colocasia esculenta*) and its effect on mouse splenocyte proliferation in vitro and in vivo, Protein J. 33 (2014) 92–99.
[13] D.C. Monte-Neshich, T.L. Rocha, R.L. Guimarães, E.F. Santana, M.E. Loureiro, M. Valle, M.F. Grossi de Sá, Characterization and spatial localization of the major globulin families of taro (*Colocasia esculenta* L. Schott) tubers, Plant Sci. 112 (1995) 149–159.
[14] M. Carneiro, C.A. Rodrigues, L.A.B. De Castro, M.C. Da Silva, M.V. Coutinho, Isolation characterization of the major albumin from *Colocasia esculenta* Corms, Plant Sci. 67 (1990) 39–46.
[15] M. Hirai, K. Nakamura, T. Imai, T. Sato, cDNAs encoding for storage proteins in the tubers of taro (*Colocasia esculenta* Schott), Idengaku Zasshi 68 (1993) 229–236.
[16] I.C. Bezerra, L.A. Castro, G. Neshich, E.R. de Almeida, M.F. de Sa, L.V. Mello, D.C. Monte-Neshich, A corm-specific gene encodes tarin, a major globulin of taro (*Colocasia esculenta* L. Schott), Plant Mol. Biol. 28 (1995) 137–144.
[17] E.J. Van Damme, K. Goossens, K. Smeets, F. Van Leuven, P. Verhaert, W.J. Peumans, The major tuber storage protein of araceae species is a lectin. Characterization and molecular cloning of the lectin from *Arum maculatum* L, Plant Physiol. 107 (1995) 1147–1158.
[18] A. Roy, S. Banerjee, P. Majumder, S. Das, Efficiency of mannose-binding plant lectins in controlling a homopteran insect, the red cotton bug, J. Agric. Food Chem. 50 (2002) 6775–6779.
[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[20] S. Pan, J. Tang, X. Gu, Isolation and characterization of a novel fucose-binding lectin from the gill of bighead carp (*Aristichthys nobilis*), Vet. Immunol. Immunopathol. 133 (2010) 154–164.
[21] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[22] D. Tawfik, Modification of tryptophan with 2-hydroxy-5-nitrobenzylbromide, in: J. Walker (Ed.), The Protein Protocols Handbook, Humana Press, 2009, pp. 871–872.
[23] S. Arishya, N. Tzi Bun, W. Jack Ho, L. Peng, Purification and characterization of a lectin from *Phaseolus vulgaris* cv. (Anasazi beans), J. Biomed. Biotechnol. 2009 (2009).
[24] D. Tawfik, Modification of sulfhydryl groups with DTNB, in: J. Walker (Ed.), The Protein Protocols Handbook, Humana Press, 2009, pp. 873–874.
[25] A. Aitken, M. Learmonth, Carboxymethylation of cysteine using iodoacetamide/iodoacetic acid, in: J. Walker (Ed.), The Protein Protocols Handbook, Humana Press, 2002, pp. 455–456.
[26] D. Tawfik, Modification of arginine side chains with p-hydroxyphenylglyoxal, in: J. Walker (Ed.), The Protein Protocols Handbook, Humana Press, 2002, pp. 475–476.
[27] D. Tawfik, Amidation of carboxyl groups, in: J. Walker (Ed.), The Protein Protocols Handbook, Humana Press, 2009, pp. 865–866.
[28] S.G. De-Simone, C.C. Netto, F.P. Silva Jr., Simple affinity chromatographic procedure to purify beta-galactoside binding lectins, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 838 (2006) 135–138.
[29] L.A. de Castro, M. Carneiro, C. Neshich Dde, G.R. de Paiva, Spatial and temporal gene expression patterns occur during corm development, Plant Cell 4 (1992) 1549–1559.
[30] E.J.M. Vandamme, I.J. Goldstein, W.J. Peumans, A comparative-study of mannose-binding lectins from the Amaryllidaceae and Alliaceae, Phytochemistry 30 (1991) 509–514.
[31] S. Shangary, J. Singh, S.S. Kamboj, K.K. Kamboj, R.S. Sandhu, Purification and properties of four monocot lectins from the family Araceae, Phytochemistry 40 (1995) 449–455.
[32] C.E. Hayes, I.J. Goldstein, An alpha-D-galactosyl-binding lectin from *Bandeiraea simplicifolia* seeds. Isolation by affinity chromatography and characterization, J. Biol. Chem. 249 (1974) 1904–1914.
[33] N.E. Robinson, Protein deamidation, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5283–5288.
[34] J. Singh, S.S. Kamboj, R.S. Sandhu, S. Shangary, K.K. Kamboj, Purification and characterization of a tuber lectin from *Alocasia indica*, Phytochemistry 33 (1993) 979–983.

[35] G.G. Bhat, K.N. Shetty, N.N. Nagre, V.V. Neekhra, S. Lingaraju, R.S. Bhat, S.R. Inamdar, K. Suguna, B.M. Swamy, Purification, characterization and molecular cloning of a monocot mannose-binding lectin from *Remusatia vivipara* with nematicidal activity, Glycoconj. J. 27 (2010) 309–320.
[36] A. Das, Characterization of a highly potent insecticidal lectin from *Colocasia esculenta* tuber and cloning of its coding sequence, Am. J. Plant Sci. 04 (2013) 408–416.
[37] D.F. Smith, X. Song, R.D. Cummings, Use of glycan microarrays to explore specificity of glycan-binding proteins, Methods Enzymol. 480 (2010) 417–444.
[38] T. Mizuochi, M.W. Spellman, M. Larkin, J. Solomon, L.J. Basa, T. Feizi, Carbohydrate structures of the human-immunodeficiency-virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese-hamster ovary cells, Biochem. J. 254 (1988) 599–603.
[39] N. Kui Wong, R.L. Easton, M. Panico, M. Sutton-Smith, J.C. Morrison, F.A. Lattanzio, H.R. Morris, G.F. Clark, A. Dell, M.S. Patankar, Characterization of the oligosaccharides associated with the human ovarian tumor marker CA125, J. Biol. Chem. 278 (2003) 28619–28634.
[40] M. Tiemeyer, S.B. Selleck, J.D. Esko, Arthropoda, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials of Glycobiology, Cold Spring Harbor (NY), 2009.
[41] H. Schachter, Paucimannose N-glycans in *Caenorhabditis elegans* and *Drosophila melanogaster*, Carbohydr. Res. 344 (2009) 1391–1396.
[42] B. Zipser, D. Bello-DeOcampo, S. Diestel, M.-H. Tai, B. Schmitz, Mannitou monoclonal antibody uniquely recognizes paucimannose, a marker for human cancer, stemness, and inflammation, J. Carbohydr. Chem. 31 (2012) 504–518.
[43] W. Nishima, N. Miyashita, Y. Yamaguchi, Y. Sugita, S. Re, Effect of bisecting GlcNAc and core fucosylation on conformational properties of biantennary complex-type N-glycans in solution, J. Phys. Chem. B 116 (2012) 8504–8512.
[44] I.O. Potapenko, V.D. Haakensen, T. Luders, A. Helland, I. Bukholm, T. Sorlie, V.N. Kristensen, O.C. Lingjaerde, A.L. Borresen-Dale, Glycan gene expression signatures in normal and malignant breast tissue; possible role in diagnosis and progression, Mol. Oncol. 4 (2010) 98–118.
[45] S. Zhang, H.S. Zhang, C. Cordon-Cardo, V.E. Reuter, A.K. Singhal, K.O. Lloyd, P.O. Livingston, Selection of tumor antigens as targets for immune attack using immunohistochemistry: II. Blood group-related antigens, Int. J. Cancer 73 (1997) 50–56.
[46] P.C. Pang, B. Tissot, E.Z. Drobnis, H.R. Morris, A. Dell, G.F. Clark, Analysis of the human seminal plasma glycome reveals the presence of immunomodulatory carbohydrate functional groups, J. Proteome Res. 8 (2009) 4906–4915.
[47] P.C. Pang, B. Tissot, E.Z. Drobnis, P. Sutovsky, H.R. Morris, G.F. Clark, A. Dell, Expression of bisecting type and Lewis^x/Lewis^y terminated N-glycans on human sperm, J. Biol. Chem. 282 (2007) 36593–36602.
[48] Y. Cao, A. Merling, U. Karsten, R. Schwartz-Albiez, The fucosylated histo-blood group antigens H type 2 (blood group O, CD173) and Lewis Y (CD174) are expressed on CD34+ hematopoietic progenitors but absent on mature lymphocytes, Glycobiology 11 (2001) 677–683.
[49] M. Dettke, G. Palfi, H. Loibner, Activation-dependent expression of the blood group-related Lewis Y antigen on peripheral blood granulocytes, J. Leukoc. Biol. 68 (2000) 511–514.
[50] D.H. Dube, C.R. Bertozzi, Glycans in cancer and inflammation—potential for therapeutics and diagnostics, Nat. Rev. Drug Discov. 4 (2005) 477–488.
[51] A.S. Gambaryan, A.B. Tuzikov, V.E. Piskarev, S.S. Yamnikova, D.K. Lvov, J.S. Robertson, N.V. Bovin, M.N. Matrosovich, Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl(N-acetyllactosamine), Virology 232 (1997) 345–350.
[52] A.S. Gambaryan, A.B. Tuzikov, A.A. Chinarev, L.R. Juneja, N.V. Bovin, M.N. Matrosovich, Polymeric inhibitor of influenza virus attachment protects mice from experimental influenza infection, Antivir. Res. 55 (2002) 201–205.
[53] E.J. Van Damme, S. Nakamura-Tsuruta, D.F. Smith, M. Ongenaert, H.C. Winter, P. Rouge, I.J. Goldstein, H. Mo, J. Kominami, R. Culerrier, A. Barre, J. Hirabayashi, W.J. Peumans, Phylogenetic and specificity studies of two-domain GNA-related lectins: generation of multispecificity through domain duplication and divergent evolution, Biochem. J. 404 (2007) 51–61.
[54] S.C.M. Leal-Bertioli, A.V. Pascoal, P.M. GuimarÃEs, M.F.G. De SÁ, R.L. GuimarÃEs, D.C. Monte, D.J. Bertioli, Transgenic tobacco plants expressing Tarin 1 inhibit the growth of *Pseudomonas syringae* pv. tomato and the development of *Spodoptera frugiperda*, Ann. Appl. Biol. 143 (2003) 349–357.
[55] N. Kundu, P. Campbell, B. Hampton, C.Y. Lin, X. Ma, N. Ambulos, X.F. Zhao, O. Goloubeva, D. Holt, A.M. Fulton, Antimetastatic activity isolated from *Colocasia esculenta* (taro), Anti-Cancer Drugs 23 (2012) 200–211.