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Short Communication

Non-canonical interactions between plant proteins and lectins cause false positives in lectin blots

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Lectins are proteins that specifically recognize and non-covalently bind to soluble carbohydrates or to the carbohydrate moieties of glycoproteins or glycolipids. Historically, lectin-blot analysis has been widely used as a tool for structural characterization of many mammalian glycoconjugates. In the present study, we demonstrate that the application of this technique to screen sugar moieties of plant proteins results in numerous false positives. Plants lack the enzyme machinery necessary to perform sialylation, however many bands appear upon probing of *N. benthamiana* L. leaf proteins with *Maacia amurensis* agglutinin (MAA) that recognizes specifically N-linked or core 2 O-linked glycans containing Neu5Ac/Gc-α2,3Galβ-1,4GlcNAc/Glc and O-linked glycans containing the trisaccharide Neu5Ac-3Galβ1-3GalNAc. The non-canonical binding is a direct result of sample preparation for SDS PAGE, because native proteins do not show an affinity to MAA-agarose resin. Moreover, inhibition with known hapten fails to prevent binding of MAA to plant proteins in lectin blots. We also provide evidence that interactions of a hydrophobic nature contribute, at least in part, to the non-specific binding, and that other lectins – *Sambucus nigra* agglutinin (SNA) and *Vicia villosa* agglutinin (VVA) – also bind non-specifically to plant proteins. In conclusion, lectin blot analysis of plant proteins should always be verified by probing the binding specificity with a known hapten inhibitor alongside appropriate mammalian glycoprotein controls. Alternatively, non-specific binding can be avoided if lectin affinity chromatography of the native plant proteins is performed prior to lectin blots analysis.

Keywords: plant proteins, lectin blot, MAA, SNA, VVA

Lectins are a diverse group of proteins isolated from different organisms that possess the ability to bind to specific carbohydrate structures without altering their properties. They have been widely used to study alterations on the surface of mammalian cells during physiological and pathological processes and to assist in purification and characterization of many mammalian glycoconjugates (Sharon, 2007). Surprisingly, although the vast majority of lectins are of plant origin, they have not been used extensively to gain insight into the carbohydrate structures attached to plant proteins (Shanmugham *et al*., 2006). Identification of such structures would assist

in better understanding of plant glycosylation potential, which remains an important barrier to using plants as bioreactors for production of pharmaceutically important protein therapeutics (Webster and Thomas, 2011). Among the existing lectin-based techniques, lectin blots are fast, easy to perform and do not require expensive and specific equipment or consumables (Gravel, 2002). Therefore, they represent an attractive experimental approach for initial screening and detection of existing glycan structures prior to investment of time for identifying the corresponding enzyme entities and pathways involved in their synthesis.

Studies performed with *Medicago sativa* L., Bright Yellow 2 tobacco cells (Paccalet *et al*., 2007) and *A. thaliana* L. (Castilho *et al*., 2008) have shown that the activity of the enzymes UDP-GlcNAc 2-epimerase (EC 5.1.3.14)/ManNAc kinase (EC 2.7.1.60) and Neu5Ac-9-P synthase (EC 2.5.1.57)/ Neu5Ac synthase (EC 4.1.3.19) directly involved in sialic acid biosynthesis in mammals and bacteria, are virtually non-existent. *N. bemthamiana* L. plants also lack these activities (our unpublished results). Work done by others (Takashima *et al*., 2006) and us (Daskalova *et al*., 2008) have also demonstrated that plant sialyltransferase-like proteins are either unable to transfer sialic acid, or their K_m values for CMP-Neu5Ac are extremely high, thus making this compound an unlikely *in vivo* substrate. In the present work we show that although plants lack enzymatic capability to synthesize sialic acid and sialylate glycoconjugates, numerous bands appear on lectin blots that are developed with sialic acid-specific lectins. We also demonstrate that other lectins, such as VVA that strongly recognizes terminal GalNAc residues attached to serine or threonine in a polypeptide, bind non-specifically to plant proteins in lectin blots despite that plants can not initiate mucin type O-glycosylation (Daskalova et al., 2010).

Materials and Methods

Protein extraction and SDS-PAGE

Total proteins from *N. benthamiana* L. leaves were isolated using Sigma Plant Total Protein Extraction Kit (Sigma, St. Louis, MO, USA). SDS samples were prepared using 5x SDS sample buffer (0.1 M TrisHCl, pH 6.8, 50% (v/v) glycerol, 3.7 M β-mercaptoethanol, 10% (w/v) SDS and 0.05% (w/v) bromophenol blue) and boiling for 5 min. Proteins were loaded on 4-20% gradient gels (BioRad, Hercules, CA, USA) along with Kaleidoscope Prestained Standards (BioRad) and blotted onto 0.45 μm pore size Immobilon-P PVDF membrane.

Total soluble proteins from *N. benthamiana* L. leaves for lectin chromatography were isolated by extraction with cold 50 mM Tris HCl buffer pH 7.4 containing 0.1 M NaCl, 1 mM $MgCl_2$, 3 mM DTT, and 1 mM PMSF. The homogenate was strained through cheesecloth and centrifuged at 10 000 × g for 15 min at 4°C. The supernatant was saturated with ammonium sulfate to a final concentration of 75% (w/v) and incubated overnight at 4°C. Precipitated proteins were collected by centrifugation at 15 000 × g for 45 min at 4°C. The pellet was dissolved in a small volume of 50 mM Tris HCl buffer pH 7.4 containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0. 1 M NaCl and passed through PD10 columns (Amersham Biosciences, Piscataway, MA),

Lectin blots and lectin chromatography

Blots were probed with biotin-conjugated *Maackia amurensis* agglutinin (MAA), *Sambucus nigra* agglutinin (SNA) or *Vicia villosa* agglutinin (VVA, EY Labs, San Mateo, CA, USA). Membranes were blocked with 1% (w/v) gelatin in TBST buffer (0.05 M TrisHCl, 0.15 M NaCl, 0.05% (v/v) Tween 20, pH 7.5) for 30 min, then washed twice for 10 min with TBST and once for 10 min with TBS buffer 1 (0.05 M TrisHCl, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5).

Blots were incubated for 1 h with 5 µg/ml lectin in TBS buffer 1 without or in the presence of 5 mM EDTA, or 50 mM lactose, or 0.5 mM 8-anilino-1-naphtalenesulfonic acid (ANS, Sigma). After three washes with TBST buffer, for 10 min each, blots were incubated for 1 h with avidin-conjugated alkaline phosphatase (EY Labs) and washed again as specified above. Visualization was performed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chromogenic system (Sigma Fast BCIP/NBT tablets).

Lectin chromatography was performed with *Maackia amurensis* immobilized lectin gel (EY Laboratories, San Mateo, CA) according to manufacturer's recommendations. Binding was performed for 2 h at room temperature or overnight at 4°C while shaking. The non-bound proteins were then collected and the column was washed with 10 column volumes of starting buffer (50 mM Tris HCl buffer pH 7.4 containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0. 1 M NaCl). Bound proteins were eluted with 20 mM ethylenediamine. Aliquots of non-bound, bound proteins and washes were analysed by lectin blot analysis described above.

Results and Discussion

We probed proteins isolated from leaves of *N. benthamiana* L. with *Maacia amurensis* agglutinin (MAA), a mixture of leucoagglutinin (MAL) that binds with strongest affinity to Neu5Ac/Gc-α2, 3Galβ-1,4GlcNAc/Glc structure, and hemagglutinin (MAH) whose preferred substrate is the trisaccharide Neu5Ac-3Galβ1-3GalNAc. Illustration of a typical MAA blot of soluble leaf proteins is presented in Fig. 1A, first panel. The presence of numerous bands suggests that sialylation is a conventional posttranslational modification in plants.

Indeed, in 2003, identification of sialylated proteins in *A. thaliana* L. was reported (Shah *et al*., 2003). However, this finding was highly controversial. These results have not been reproduced (Seveno *et al*., 2004), and it was also found by HPLC–electrospray tandem mass spectrometry that sialic acid concentrations in plants are in the range of inadvertent contamination (Zeleny *et a*l., 2006). Later, several studies have demonstrated that plants do not have enzymatic potential to synthesize sialic acid and sialylate glycoconjygates (Takashima *et al*., 2006; Paccalet *et al*., 2007; Castilho *et al*., 2008; Daskalova *et al*., 2008). In the light of these results, we further explored the specificity of the binding of *N. bethamiana* L. leaf proteins to MAA. Bovine fetuin, a glycoprotein that contains N-linked glycans with terminal Neu5Ac-2-3Galβ1-4GlcNAc, Neu5Ac-α2-6Galβ1-4GlcNAc, Galβ1-4GlcNAc residues and O-linked glycans with Neu5Acα2-3Galβ1-3±(Neu5Acα2-6)GalNAc structures (Green *et al*., 1988) was run in parallel as a positive control.

The presence of a known hapten inhibitor, lactose, at a concentration of 50 mM was able to abolish the binding of MAA to fetuin. In contrast, all bands from the plant sample remained unaffected (Fig. 1A, second panel). This result implies that not only structures that act as primary substrates for MAA, but also those defined as weak, or secondary substrates (Geister and Jarvis, 2011) are not present on plant leaf proteins.

The vast majority of lectins, including MAA, require metal ions, normally Ca^{2+} and Mn^{2+} (Imberty *et al*., 2000) for binding activity. The absence of those ions could lead to large conformational changes in the ion binding loop that further cause substrate drifting (Kaushik *et al*., 2009). In our experiments, addition of EDTA at a concentration of 5 mM during incubation of the blots with MAA completely inhibited MAA binding to fetuin, without causing a strong effect on MAA binding to plant leaf proteins (Fig. 1A, third panel).

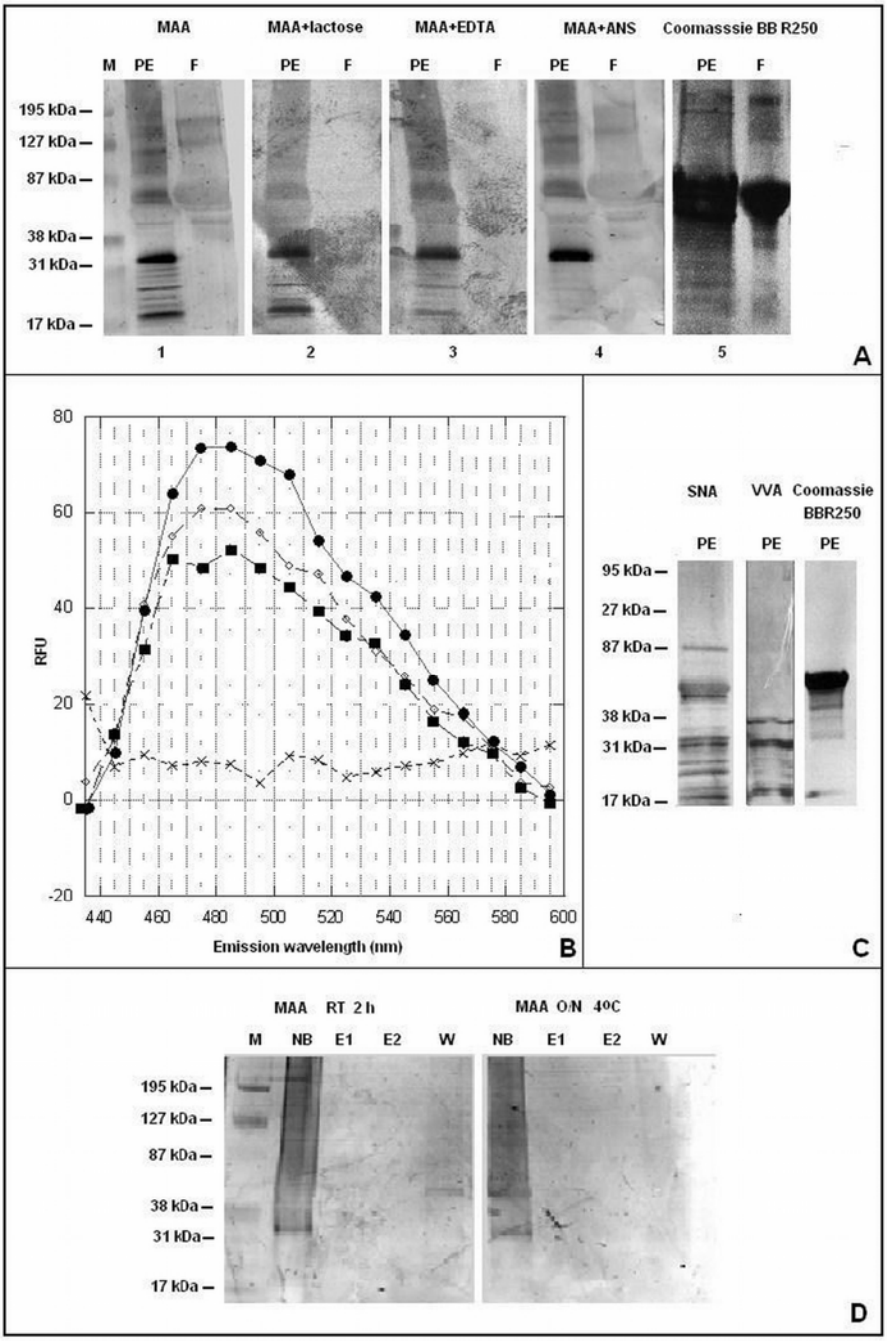

Figure 1. Probing of *N. benthamiana* L. soluble proteins with lectins. A – Probing of plant extracts (PE) and fetuin (F) with MAA alone (first panel), MAA and 50 mM lactose (second panel), MAA and 5 mM EDTA (third panel), MAA and 0.5 mM ANS (fourth panel). Coomassie Brilliant Blue R250 staining is presented in the fifth panel; **B** – Emission spectra (excitation at 388 nm) of ANS (0.5 mM) alone (-x-) or in the presence of 1 mg/ml MAA (-■-), MAA coupled to biotin (-○-), or SNA (-●-); **C** – probing of plant extracts with SNA (first panel) and VVA (second panel). Coomassie Brilliant Blue R250 staining is presented in the third panel; **D** – MAA lectin blots of plant extract after MAA lectin chromatography – proteins were incubated with the resin for either 2 h at room temperature or overnight at 4°C: NB – nonbound proteins, E – eluted bound proteins, W – wash.

We hypothesized that the observed non-canonical binding results from denaturation of the proteins during SDS PAGE sample preparation, which in turn could enhance hydrophobic interactions with MAA molecules. The three-dimensional x-ray crystal structure of the complex between MAL and sialyllactose reveals the presence of numerous hydrophobic patches on the protein surface (Imberty *et al*., 2000) that could take part in this type of binding. Indeed, incubation of MAA with ANS, a fluorescent dye that binds to the hydrophobic regions of a protein (Stryer, 1968; Saucier *et al*., 1985), resulted in formation of a protein-dye complex, as evidenced by the appearance of a broad emission peak with a maximum at 470-480 nm upon excitation at 388 nm. Biotin, to which MAA was coupled, did not contribute significantly to MAA hydrophobicity (Fig. 1B).

When ANS was included during the incubation of plant protein blots with MAA, the intensities of many bands decreased, while MAA binding to fetuin was not affected (Fig. 1A, fourth panel). It is important to note that the non-canonical interactions between blotted plant proteins and lectins are not restricted to MAA only. *Sambucus nigra* agglutinin, another sialic acid-specific lectin that has an affinity for Neu5Ac-α2,6-Gal/GalNAc, and, to a lesser extent, Neu5Ac-α2,3-Gal residues, also forms complexes with ANS (Fig. 1B). *N. benthamiana* L. leaf proteins bind non-specifically to SNA in lectin blots, too (Fig. 1C, first panel).

However, native tobacco soluble proteins do not exhibit any affinity to MAA-agarose resin (Fig. 1D) or SNA-agarose resin (data not shown) when incubated either for 2 h at room temperature or overnight at 4°C. These results also indicate that it is the denaturation of the proteins during SDS PAGE sample preparation that causes the non-specific interactions.

The non-canonical binding of plant proteins to lectins in lectin blots is not restricted to sialic acid-specific lectins. Recently, we demonstrated that plants do not have the ability to transfer N-acetylgalactosamine (GalNAc) to the hydroxyl group of serine and threonine amino acid residues of acceptor proteins (Daskalova *et al*., 2010). Nonetheless, false positive binding was detected if plant proteins were directly subjected to lectin blot analysis with *Vicia villosa* agglutinin (Fig. 1C, second panel). However, none of the native proteins bound to VVA-agarose resin (Daskalova *et al*., 2010).
In conclusion, use of lectin blots for structural characterization of plant protein can be misleading. Verification should be performed to clarify for specificity of binding. We recommend incubation with known hapten inhibitors and a positive mammalian glycoprotein control. Alternatively, lectin affinity chromatography with native plant proteins should be performed prior to lectin blotting.

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