Biochimica et Biophysica Acta 1850 (2015) 1815–1821

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Effects of selective cleavage of high-mannose-type glycans of *Maackia amurensis* leukoagglutinin on sialic acid-binding activity

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ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form 7 May 2015
Accepted 12 May 2015
Available online 21 May 2015

Keywords:
Maackia amurensis lectin
N-linked glycan
High-mannose-type
Activity

ABSTRACT

Background: *Maackia amurensis* leukoagglutinin (MAL) is a glycoprotein and sialic acid-binding lectin that is used widely in the detection and characterization of sialoglycoconjugates and human cancer cells. However, its *N*-linked glycan structure and role have yet to be determined.
Methods: The *N*-linked glycans were analyzed using high-performance liquid chromatography with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and the secondary structure was investigated using circular dichroism analysis. A hemagglutination assay was performed. Furthermore, surface plasmon resonance analysis, and fluorescence microscopy and fluorescence-activated cell-sorting analysis were conducted to assess the sialoglycoprotein-binding ability and its usefulness in the detection of human breast cancer MCF-7 cells, respectively.
Results: Analysis of the *N*-linked glycan structure of MAL confirmed the presence of eight glycans, comprising two α1,3-fucosylated paucimannosidic-type and six high-mannose-type glycans. Glycan analysis of MAL that had been treated with peptide *N*-glycosidase F (de-M-MAL) revealed that while the two α1,3-fucosylated paucimannosidic glycans remained attached following the treatment, the six high-mannose-type glycans had been completely cleaved from the original MAL. There were almost no secondary structural changes between MAL and de-M-MAL; however, the lectin activities exhibited by MAL, such as hemagglutination and binding to a sialoglycoprotein, were completely absent in de-M-MAL, and the ability to detect human breast cancer MCF-7 cells was 77% lower in de-M-MAL than in MAL.
Conclusion: The high-mannose-type glycans in intact MAL are closely associated with its lectin activities.
General significance: This is the first report of the *N*-linked glycan structure of MAL and the effect of high-mannose-type glycans on lectin activities.

1. Introduction

Lectins, which are proteins or glycoproteins that reversibly bind to specific carbohydrates [1], have been found in various organisms such as viruses, fungi, bacteria, animals, and plants [2]. Certain lectins are known to bind sialic acid, receptors for which are known to exist for viruses, peptide hormones, toxins, and cell surfaces [3]. Sialic acid-binding lectins may thus provide useful tools for detection, isolation, characterization, regulation, and biochemical studies of sialoglycoconjugates, and may represent targets for drug discovery [3,4]. Sialic acid-binding lectins are found ubiquitously in microorganisms and eukaryotes [4]; however, only a few sialic acid-binding lectins from plants have been reported, such as *Sambucus nigra* L. agglutinin, *Sambucus canadensis* lectin, prickly lettuce (*Lactuca scariole*) agglutinin, and *Maackia amurensis* agglutinin (MAA) [4]. Of these, MAA is widely used as a positive control with which to compare the binding constants of newly found sialic acid-binding lectins [5], has diagnostic potential for non-small-cell lung cancer [6], has been shown to inhibit melanoma cell growth [7], and can be used to detect human breast cancer cells [8].

MAA has been isolated from *M. amurensis* seeds, and found to comprise two isolectins: *M. amurensis* hemagglutinin (MAH) and *M. amurensis* leukoagglutinin (MAL) [10,11]. MAH is a tetramer that is composed of a 33-kDa subunit, while MAL is a dimer that is composed of a disulfide-containing 70-kDa subunit [12]. At least three *N*-linked glycans are attached to each monomer of MAL [13], and MAL is used in histology to stain α2,3-linked sialosides [9]. The *N*-linked glycan structure of MAL has yet to be determined.

The *N*-linked glycans play a crucial role in eukaryotes; for example, they assist protein folding, possess targeting information, recognize

Abbreviations: 2-AB, 2-aminobenzamide; CD, circular dichroism; de-M-MAL, peptide *N*-glycosidase F-treated *Maackia amurensis* leukoagglutinin; Endo H, Endo-β-*N*-acetylglucosaminidase H; FITC, fluorescein isothiocyanate; GlcNAc, *N*-acetylglucosamine; GU, glucose units; HPLC, high-performance liquid chromatography; MAA, *M. amurensis* agglutinin; MAH, *M. amurensis* hemagglutinin; MAL, *M. amurensis* leukoagglutinin; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Man, mannose; PBS, phosphate-buffered saline; PBST, PBS with Tween 20; PNGase A, peptide *N*-glycosidase A; PNGase F, peptide *N*-glycosidase F; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; TFMS, trifluoromethanesulfonic acid

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http://dx.doi.org/10.1016/j.bbagen.2015.05.011
0304-4165/

proteins, and are involved in cell-to-cell adhesion [14]. Plant *N*-linked glycans are classified as a high-mannose (Man)-type, with the structure of $(Man)_{5-9}$*N*-acetylglucosamine $(GlcNAc)_2$, a complex type, with a terminal GlcNAc or larger antennae that are β1,2-linked to the α1,6- or α1,3-Man of the core structure, and paucimannosidic-glycans, with an α1,3-fucose and/or a β1,2-xylose linked to the core pentasaccharide $Man_{3-2}GlcNAc_2$ [15].

It has been reported that *N*-linked glycans-containing lectins are harbored by some plants, such as soybean (*Glycine max*) [16], runner beans (*Phaseolus vulgaris*) [17], castor-oil beans (*Ricinus communis*) [18], breadfruit tree (*Artocarpus integrifolia*) lectin [19], and the bark of golden rain shrub (*Laburnum anagyroides*) [20]. However, with the exception of the hemagglutination activity of tomato lectin, which is totally lost after *N*-linked glycans are chemically removed using trifluoromethanesulfonic acid (TFMS) [21], and the hemagglutination activity of snail lectin, which is destroyed by *O*-glycan removal via β-elimination using alkaline borohydride treatment [22], the roles of glycans in these lectins have not been reported in detail.

Peptide *N*-glycosidase F (PNGase F) is an amidase that cleaves between the innermost GlcNAc and Asn residues of high-Man-, hybrid-, and complex-type *N*-linked glycans on glycoproteins without the accompanying protein degradation caused by chemical deglycosylation. This process was accompanied by deamidation of the glycan-attached Asn and its conversion into Asp. However, α1,3-fucosylated and/or β1,2-xylosylated glycans cannot be cleaved with PNGase F [15,23]. These PNGase-F-resistant *N*-linked glycans, together with the three aforementioned types of glycans, can be cleaved by glycoamidase A (*N*-glycosidase A, or PNGase A) from sweet almond [24].

In this study, PNGase A was used to analyze the *N*-linked glycan structures of MAL using high-performance liquid chromatography (HPLC) with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). PNGase F was used to cleave high-Man-type *N*-linked glycans of MAL (PNGase F-treated MAL, de-M-MAL). The resulting cleavage efficiency was confirmed by glycan analysis of de-M-MAL using PNGase A. The glycans released from MAL by PNGase F were also analyzed. In addition, the structural effect of *N*-linked glycans on intact MAL was investigated by comparing the secondary structures of MAL and de-M-MAL in solution. The role of *N*-linked glycans on the lectin activities of MAL were then examined by comparing the hemagglutination and sialoglycoprotein-binding abilities, as well as the ability to detect sialic acid-expressed human breast cancer MCF-7 cells of both MAL and de-M-MAL by submitting them to a hemagglutination assay, surface plasmon resonance (SPR) analysis against a sialoglycoprotein (fetuin), and fluorescence microscopy and fluorescence-activated cell sorting analysis, respectively.

2. Materials and methods

2.1. Preparation of de-M-MAL

MAL was purchased from EY Laboratories (San Mateo, CA, USA). The de-M-MAL was produced by digesting the *N*-linked glycans of MAL (0.2 mg) with 100 U of PNGase F (molecular mass, 36 kDa; 200 μl; Sigma, St. Louis, MO, USA) at 37 °C for 18 h in 20 mM ammonium bicarbonate (pH 8.0). The hydrolyzed glycoprotein (de-M-MAL) and the *N*-linked glycans released from MAL were separated using centrifugal filter devices with a molecular mass cutoff of 10 kDa (Millipore, Milford, MA, USA). The de-M-MAL was further purified using a strong anion-exchange column (6.4 × 30 mm; Resource Q, GE Healthcare Life Sciences, Uppsala, Sweden) to remove the enzyme. The column was equilibrated with 50 mM sodium phosphate buffer (pH 7.0), and the de-M-MAL was eluted with a 0–1 M NaCl linear gradient at 1.0 ml/min. The collected fractions were dialyzed against distilled water, and then lyophilized and stored at −20 °C.

2.2. Glycan analysis

The *N*-linked glycan structures of MAL were determined by combining HPLC and MALDI-TOF MS analyses according to the methods described in our recent papers [25,26]. Briefly, *N*-linked glycans were released using 0.1 mU of PNGase A (Seikagakukogyo, Tokyo, Japan) and purified with a graphitized carbon cartridge. The reducing ends of released glycans were derivatized with 2-aminobenzamide (2-AB; Sigma). Excess fluorophore was removed using microcrystalline cellulose (Sigma). Purified 2-AB-labeled-glycans were separated by HPLC using a TSK-gel Amide 80 column (4.6 × 250 mm; Tosoh, Tokyo, Japan). Fluorescence was observed using emission and excitation wavelengths of 420 and 330 nm, respectively. The retention times of the separated glycans were converted to glucose units (GU) by comparison with a 2-AB-labeled glucose homopolymer ladder (Ludger, Oxfordshire, UK). The glycan structure was assigned by matching the GU value to reference GU values in "GlycoBase" (http://glycobase.nibrt.ie/glycobase/show_nibrt.action). The MALDI-TOF MS (positive mode) data of the glycans were obtained using an UltraFlex III system (Bruker Daltonik, Bremen, Germany).

2.3. Electrophoretic analyses

The molecular masses of MAL and de-M-MAL were analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli using a 10% acrylamide slab gel [27]. The gel was stained with Coomassie blue R-250. Molecular mass markers (10–250 kDa) were purchased from Bio-Rad (Hercules, CA, USA).

2.4. Circular dichroism analysis

The far-UV circular dichroism (CD) spectrum was measured using a Chirascan plus CD spectrometer (Applied Photophysics, Leatherhead, UK). The spectra of MAL and de-M-MAL from 190 to 260 nm were analyzed in a 0.02-cm quartz cuvette at a temperature of 25 °C. Secondary structure contents were analyzed using CDNN CD spectra deconvolution software.

2.5. Hemagglutination assay

Hemagglutination assay data were assessed using mouse erythrocytes. Each sample was twofold serial diluted in saline and incubated with an equal volume of 3% (v/v) heparinized mouse erythrocytes for 24 h at 4 °C. The minimum concentration of lectin that caused agglutination was defined as 1 unit, and the hemagglutination titer was defined as the reciprocal of the highest dilution at which agglutination of erythrocytes could be observed. Experiments were conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Chung-Ang University (Approval no. 13-0011).

2.6. SPR analysis

All experiments were performed at 25 °C using a ProteOn XPR36 biosensor (Bio-Rad) and GLC sensor chip (Bio-Rad). After equilibration of the system equilibrium using phosphate-buffered saline (PBS) with Tween 20 (PBST; 20 mM sodium phosphate, 0.15 M NaCl, and 0.005% Tween 20, pH 7.4), the sensor chip was activated using activation reagents [0.25 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] in 0.063 mM *N*-hydroxysulfosuccinimide. Fetuin was injected into 10 mM sodium acetate buffer (pH 4.5), followed by an injection of 1 M ethanolamine-HCl (pH 8.5) to block the reaction spot. Fetuin was immobilized at 254 resonance units on the surface of the sensor chip. The kinetic response data of both the lectin and fetuin were analyzed in PBST at a flow rate of 50 μl/min. MAL or de-M-MAL were diluted twofold and then injected for 150 s.