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N-glycosylation potential of maize: The human lactoferrin used as a model

B. Samyn-Petit[1], V. Gruber[1], C. Flahaut[2], J.-P. Wajda-Dubos[1], S. Farrer[1], A. Pons[2], G. Desmaizieres[1], M.-C. Slomianny[2], M. Theisen[1] and P. Delannoy[2]*

[1] *Meristem Therapeutics, 8 rue des Frères Lumière, 63100 Clermont-Ferrand, France,* [2] *Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS n° 8576, Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq, France*

In order to determine the *N*-glycosylation potential of maize, a monocotyledon expression system for the production of recombinant glycoproteins, human lactoferrin was used as a model. The human lactoferrin coding sequence was inserted into the pUC18 plasmid under control of the wheat glutenin promoter. Maize was stably transformed and recombinant lactoferrin was purified from the fourth generation seeds. Glycosylation was analysed by gas chromatography, lectin detection, glycosidase digestions and mass spectrometry. The results indicated that both *N*-glycosylation sites of recombinant lactoferrin are mainly substituted by typical plant paucimannose-type glycans, with β1,2-xylose and α1,3-linked fucose at the proximal *N*-acetylglucosamine, and that complex-type glycans with Lewis^a determinants are not present in maize recombinant lactoferrin.

Keywords: **glycosylation, maize, human lactoferrin**

Introduction

Human proteins can be synthesized using different expression systems including bacteria, yeast, insect cells, mammalian cells or transgenic animals. Progress in biotechnology and plant transformation has elicited new developments for plant use as bioreactors for recombinant protein production. The plant system confers some advantages over other expression systems. Plants are well suited for industrial scale level and production costs are relatively low [1–3]. In addition, they can be considered as a safe production system with respect to lack of human and animal pathogens and other infectious agents. This is a great advantage of the plant system for the production of therapeutic proteins such as vaccines and antibodies. Direct administration of plant material as orally administered therapeutic molecules such as vaccines or antibodies has been proposed [4,5].

Several recent examples have shown that plants allow the production of complex mammalian proteins such as human haemoglobin [6], protein C [7], human collagen [8] and full-length antibodies [9,10] in an active form. Stability and biological activity of recombinant proteins are dependent on post-translational processing including proteolytic cleavage, proper folding with the assistance of chaperones, oligomerisation, disulfide bond formation and glycosylation. Plants and mammals differ in the biosynthesis of the glycan moiety [11,12]. They share the first steps of *N*-glycosylation with other eukaryotic organisms and are able to transfer in the endoplasmic reticulum, oligomannose-type glycans at specific *N*-glycosylation sites on nascent glycoproteins. On the other hand, plants lack several key enzymes involved in the processing and terminal glycosylation of *N*-glycans. Thus, *N*-glycans found in plants are usually truncated in comparison with mammalian complex-type *N*-glycans, lacking *N*-acetylneuraminic acid residues that are commonly found in human glycoproteins. Moreover, plant *N*-glycans strongly differ by the presence of β1,2-xylose and α1,3-fucose residues that substitute the $Man_3GlcNAc_2$ core. These oligosaccharide linkages are not found on mammalian *N*-linked glycans and represent potential immunogenic determinants [13,14].

Until now, plant glycosylation has been mainly studied in dicotyledons. The aim of our work is to determine the glycosylation potential of maize, a monocotyledon expression system. To date, avidin [15] has been produced in maize but

*To whom correspondence should be addressed: Philippe Delannoy, Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS n°8576, Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France. Tel.: +33 320 43 69 23; Fax: +33 320 43 65 55; E-mail: Philippe.Delannoy@univ-lille1.fr

the reference doesn't give any details on the glycosylation of this plant-based protein.

For that purpose, we have expressed human lactoferrin, a glycoprotein of the transferrin family and known to be substituted by two complex-type *N*-glycans [16]. After production and purification of the recombinant glycoprotein (maize rLf), we have analysed its carbohydrate composition by gas chromatography-mass spectrometry (GC-MS) and by lectin staining. Moreover, after reduction, alkylation and trypsin digestion, the oligosaccharides from maize rLf were released by *N*-glycosidase A treatment, purified and analysed by MALDI-TOF mass spectrometry.

Materials and methods

Materials

Heptafluorobutyric anhydride (HFBAA) was from Fluka (Buchs, Switzerland). Digoxygenin labelled lectins (*Concanavalin A* agglutinin (ConA), *Galanthus nivalis* agglutinin (GNA), *Ricinus communis* agglutinin (RCA-I), *Aleuria aurianta* agglutinin (AAA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA)), Blocking reagent, 4-nitro-blue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP), Recombinant peptide-*N*-glycosidase F (PNGase F) from *E. coli*, peptide-*N*-glycosidase A (PNGase A) from almonds and endoglycosidase H (endoH) were purchased from Roche Molecular Biochemicals (Meylan, France). Recombinant sialidase from *Clostridium perfringens*, DAB (3,3′-diamino-benzidine tetrahydrochloride), Soybean Trypsin Inhibitor (SBTI) and trypsin were from Sigma (St-Quentin Fallavier, France). Sequencing-grade modified trypsin was purchased from Promega (Zürich, Switzerland). 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was obtained from Molecular Probes. Inc (Leiden, Netherlands). 10% NP-40 solution was from Biolabs (Saint-Quentin en Yvelines, France). CNBr activated sepharose 4B was from Amersham Pharmacia (Orsay, France).

Isolation of hLf cDNA and vector construction

DNA manipulation and transformation of *E. coli* DH5α were performed according to standard protocols [17].

The human lactoferrin cDNA was isolated as a 2 kb EcoRI fragment from pBS-Lf12 according to Salmon et al. (1998) [18]. The human lactoferrin coding sequence was fused through restriction enzyme based cloning to the PCR amplified N-terminal signal sequence of the sweet potato sporamin precursor composed of its first 21 amino-acids as described previously [18]. The EcoRI fragment bearing the chimeric cDNA was placed under control of the wheat high molecular weight glutenin promoter [19] fused to the first intron of the rice actin gene [20] and the nopaline synthase terminator of *Agrobacterium tumefaciens* [21] in the pUC18 plasmid (GIBCO-BRL). The resulting plasmid was designated pLfm.

The plasmid pbar which carries the *Streptomyces hygroscopicus* bar gene [22] was used for selection of transformed plants. The bar gene is under control of the rice actin 1 promoter [23] and nopaline synthase terminator of *Agrobacterium tumefaciens* in plasmid pSP72 (Promega).

Transformation, production and purification of maize rLf

Maize transformation

An established callus line derived from a single immature embryo of the Hi-II maize germplasm [24] was transformed using particle bombardment-mediated transformation with a helium-powered particle acceleration device, PDS 1000 (BioRad). Tissue showing a friable type-II embryogenic morphology was sieved and cotransformed with the two plasmids pLfm and pbar according to the procedure of Tomes et al. [25]. Transformants expressing the bar gene were selected. Several callus-derived plants were generated, transferred to the greenhouse and crossed with an untransformed elite inbred maize variety to produce first generation seeds (T1). These T1 seeds were assayed for lactoferrin expression and the highest expressor events were retained and multiplied using self-pollinations and cross-pollinations with an untransformed elite inbred line. Fourth generation seeds (T4) were produced in a field trial following French regulations and were subjected to extraction, purification and analyses of the maize rLf.

Extraction and purification of maize rLf

T4 seeds were ground in liquid N_2 and macerated for 16 h at 4°C in 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl and 1 mM EDTA (seeds/extraction buffer ratio: 1/10). The homogenate was clarified using filtration through Miracloth and mixed with diatomacious earth for 30 min at room temperature. After filtration with Whatman paper, elution was performed with 50 mM sodium phosphate (pH 7.0) containing 1.8 M ammonium sulfate. The eluted fraction was filtered once more through Whatman paper and purified on a Phenyl Sepharose FF High Substituted column (Pharmacia) buffered with 50 mM sodium phosphate (pH 7.0) containing 1.8 M ammonium sulfate using a linear flow rate of 230 $cm \cdot h^{-1}$. Elution was performed in one step using 50 mM sodium phosphate (pH 7.0). The eluted fraction was applied to SP Sepharose HP column (Pharmacia) buffered in 50 mM sodium phosphate (pH 7.0) using a linear flow rate of 150 $cm \cdot h^{-1}$. Elution was performed with 50 mM sodium phosphate (pH 7.0) containing 1.5 M NaCl. Recombinant glycoprotein was dialyzed against 20 mM sodium phosphate (pH 7.0) overnight at 4°C and lyophilized. The powder was solubilized in water and the solution was applied to MonoS HR 5/5 column, first equilibrated with 20 mM sodium phosphate, pH 7.4 (to avoid the release of iron), and the elution was performed with NaCl gradient from 0 to 1 M, in 30 min and by using a linear flow rate of 1 $ml \cdot min^{-1}$.

Immunochemical analysis of maize rLf

hLf and maize rLf were analysed by 12.5% SDS-PAGE and electrotransferred to a nitrocellulose membrane for immunoblot analysis. The blot was washed in TBS for 30 sec, blocked in 4% milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 2 hours and washed three times with TBS. The membrane was then incubated overnight at 4°C with polyclonal rabbit anti-hLf antibodies (3.6 $mg \cdot ml^{-1}$), produced in our laboratory, at a dilution of 1/2000 in TBS-Tween 20 (0.05% v/w). After three washes with TBS-Tween 20 (0.05% v/w), the blot was incubated for 2 hours at 20°C with a solution of goat anti-rabbit IgG coupled to peroxydase at a dilution of 1/2000 in TBS-Tween 20 (0.05% v/w). After washing three times in TBS, the blot was stained with DAB.

Monosaccharide composition

Monosaccharide molar ratios were determined after methanolysis of 250 μg of glycoprotein during 24 hours in 0.5 M MeOH/HCl at 80°C, acylation with heptafluorobutyric anhydride and GC-EI/MS of the HFB derivatives of the O-methyl glycosides according to Zanetta et al. [26].

Detection of glycans with digoxigenin labelled lectins

Purified glycoproteins (2 and 5 μg) were resolved on a 4–20% SDS-PAGE and then blotted to nitrocellulose membrane (Gelman Sciences, Champs Sur Marne, France). Membranes were saturated in 2% polyvinylpyrrolidone (PVP) in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 2 hours and washed 3 times for 10 min in TBS. Then the blots were incubated 2 hours with the digoxigenin (dig) labelled lectins (1 μg/ml) diluted in TBS, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5. To control the lectin specificity, experiments were performed simultaneously with a specific competitor for each lectin (0.2 M mannose for ConA, 0.2 M α-methylmannoside for GNA, 0.2 M α-methylgalactopyranoside for RCA-I and 0.1 M L-fucose for AAA). In the case of sialic acid binding lectins, (SNA and MAA), negative controls were performed by treating the membranes with 0.5 U of *Clostridium perfringens* sialidase in 50 mM citrate buffer, pH 6.0 overnight at 37°C. The blots were then washed 3 times for 10 min in TBS, saturated in the Blocking reagent at 0.5% in TBS for 2 hours and incubated for 2 hours in presence of anti-digoxygenin alkaline phophatase-labelled Fab fragments (0.75 U/ml) (Roche Molecular Biochemicals) diluted in TBS buffer. Blots were washed 3 times in TBS buffer for 10 min and labelled glycoproteins were revealed by NBT-BCIP staining.

Susceptibility to tryptic proteolysis of hLf and maize rLf

One mg of hLf and maize rLf were solubilized in 526 μl and 333 μl of Tris/HCl (100 mM; pH 8.2), respectively, and saturated with iron at 20°C for 2 hours with Azari solution added to hLf and maize rLf to achieve a molar ratio of iron to hLf or maize rLf of 2.2 : 1 in the incubation mixture [27]. 300 μl of trypsin-Sepharose 4B (5 mg trypsin per ml of Sepharose) were then added to iron-saturated glycoproteins, the incubation was pursued with agitation for 20 hours at 37°C and stopped by adding a 10-fold molar excess of SBTI. Glycoproteins were analysed by 4–20% SDS-PAGE, Western-blotting and ConA staining as described above.

Deglycosylation treatment

Ten μg of purified glycoproteins (hLf and maize rLf) were solubilized either in 55 μl of ammonium bicarbonate buffer (20 mM; pH 8.0) for peptide-*N*-glycosidase F (PNGase F) treatment, or in 55 μl of sodium carbonate buffer (10 mM; pH 5.1) both containing 0.5% SDS and 1% β-mercapto-ethanol. The glycoprotein solutions were boiled for 10 min at 95°C and supplemented with 6.15 μl of 10% NP-40 solution. The glycoproteins were then treated with deglycosylation enzymes (PNGase F (0.4 U), Endo H (2 mU) and Endo F/PNGase F mixture (0.13 U)) at 37°C for 4 hours and analysed by 4–20% SDS-PAGE and Coomassie blue staining.

Glycopeptides

Reduction, alkylation and tryptic proteolysis

hLf (12.5 nmol) and maize rLf (12.5 nmol), were solubilized at a concentration of 1mg/ml in 100 mM Tris/HCl buffer (pH 8.0), denaturated, reduced and carboxamidomethylated as previously described [28]. After extensive dialysis against 100 mM Tris/HCl, 150 mM NaCl buffer (pH 8.0), sequencing-grade modified trypsin was added to a final enzyme-to-substrate ratio of 1/100 (w/w) and incubated overnight at 37°C. The proteolytic degradation was stopped by boiling for 10 min.

Enzymatic deglycosylation

The *N*-linked oligosaccharides were enzymatically released from the desalted glycopeptides of hLf or maize rLf. 1 nmol of glycopeptides were either treated with 0.5 U of PNGase F in 20 mM ammonium bicarbonate buffer, pH 8.0 or 0.05 mU of PNGase A in 10 mM sodium acetate buffer, pH 5.1. Incubations were performed overnight at 37°C. Prior to mass spectrometry analyses, peptides and oligosaccharides were desalted on C_{18} phase Sep-Pak cartridges (Waters, MA, USA) and on non-porous graphitized carbon columns (Alltech, IL, USA), respectively. Peptides were eluted with 80% acetonitrile, 0.1% trifluoroacetic acid (TFA) and oligosaccharides with 50% acetonitrile, 0.1% TFA. After freeze-drying, 10 pmol of peptides and 50 pmol of oligosaccharides were analysed by MALDI-TOF mass spectrometry.

MALDI-TOF

All mass spectra were acquired on a Voyager Elite (DE-STR) reflectron time-of-flight (TOF) mass spectrometer (Perspective

Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. Samples were analysed in delayed extraction mode using an accelerating voltage of 20 kV, a pulse delay time of 200 ns and a grid voltage of 66%. Detector bias gating was used to reduce the ion current below masses of 500 Da. Between 150 and 200 scans were averaged for each of the spectra shown.

Oligosaccharide samples were loaded onto the mass spectrometer target in 1 μl of water and mixed with 1 μl of 2.5-dihydroxybenzoïc acid (DHB) matrix (10 mg·ml^{-1} in 70/30: acetonitrile/water solution) and allowed to dry. Oligosaccharides were essentially observed as a $[M + Na]^+$ ions.

Results

Immunoblotting

Recombinant lactoferrin, purified from maize seeds, was analysed by 12.5% SDS-PAGE electrophoresis and compared to human milk lactoferrin used as a standard (Figure 1). The electrophoretic profile of maize rLf shows a single band with an apparent molecular weight of approximatively 85 kDa which was detected by Ponceau Red staining and with anti hLf antibodies. This result indicates the absence of contamination with other plant proteins and the absence of degradation products in maize rLf preparation.

Carbohydrate composition of hLf and maize rLf

Monosaccharide molar ratios of hLf and maize rLf were determined by GC-MS and are summarized in Table 1. The result obtained for hLf was similar to previous data [16], indicating that hLf is substituted by two di-antennary complex-type *N*-glycans [10]. Compared to milk hLf, the maize rLf shows several differences such as the presence of xylose residues, the absence of galactose and sialic acid residues and a 2.6-fold decrease in *N*-acetylglucosamine content. These data indicate that the carbohydrate composition of maize rLf is similar to that obtained for tobacco rLf [18], with all the characteristics of a plant glycosylation profile.

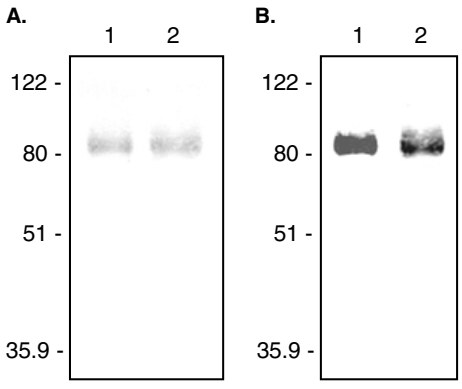

Figure 1. Immunodetection of recombinant lactoferrin. hLf (1) and maize rLf (2) were analysed by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. The blot was stained with Ponceau Red (A) and immunostained with rabbit anti-hLf primary antibodies and goat peroxydase-conjugated anti-rabbit IgG (B). The position of protein standards is shown on the left.

Table 1. Molar ratios of monosaccharides of hLf and maize rLf obtained by GC-MS. Molar ratios were calculated on the basis of 3 mol of mannose residue/mol of glycan

	Monosaccharide molar ratios					
	Fuc	*Gal*	*Man*	*GlcNAc*	*Xyl*	*Neu5Ac*
hLf	1.94 (1.4)[1]	2.37 (2.4)	3 (3)	4.7 (4.5)	0 (0)	0.95 (1.3)
Maize rLf	0.6	0.1	3	1.82	0.64	0
Tobacco rLf[2]	1.5	0.7	3	3.3	0.7	0

[1]Data into brackets are according to Spik et al. [16].
[2]Data from Salmon et al. [42]

Lectin analysis

A panel of lectins were used to further elucidate the carbohydrate composition of maize rLf. ConA and GNA, two mannose-specific lectins [29,30] revealed the presence of terminal α-linked mannose residues on both maize rLf and hLf (Figure 2). RCA_{120}, which is specific of β-linked galactose residues [31], confirmed the absence of galactose residues in maize rLf detected by GC-MS and revealed the non-sialylated antennae of complex-type *N*-glycans of hLf. SNA and MAA were used to reveal α2,6 and α2,3-linked sialic acid residues, respectively [32,33]. As shown in Figure 2, the binding of SNA indicates that hLf glycans are substituted by α2,6-linked sialic acids whereas no α2,3-linked sialic acids were detected with MAA. In contrast, neither SNA nor MAA were able to bind to maize rLf showing the total absence of sialic acids. The binding of AAA [34] clearly indicates the presence of α-L-fucose residues on maize rLf and hLf, and the lower signal observed for maize rLf is in correlation with the higher Fuc content of hLf detected by GC-MS.

Analysis of N- and C-lobes of hLf and maize rLf

As previously described, the mild trypsin digestion of iron-saturated lactoferrin generates a limited number of peptides, mainly 51-kDa and 39-kDa fragments which are identical to the C-terminal and N-terminal lobes of the glycoprotein, respectively [35,36], both containing one potential site of *N*-glycosylation.

Iron-saturated hLf and maize rLf were digested with immobilized trypsin and the generated fragments were analysed by SDS-PAGE and ConA staining. As shown in Figure 3, the digestion profile of maize rLf was very similar to that of hLf. In particular, the 51-kDa and 39-kDa fragments obtained from

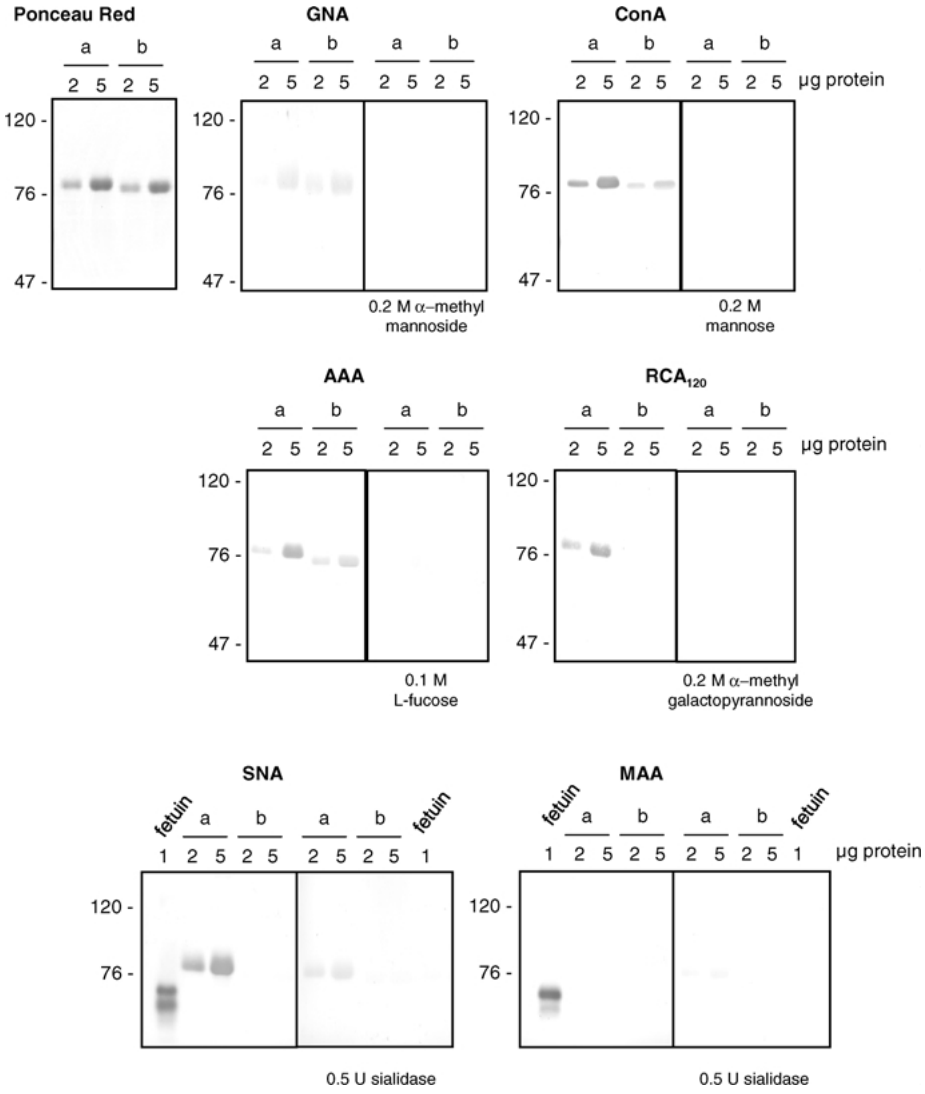

Figure 2. Lectin analysis of human and maize lactoferrin glycosylation. hLf (a) and maize rLf (b) were resolved by 12.5% SDS-PAGE and revealed by a panel of digoxigenin-labelled lectins To control the lectin specificity, the incubations were performed simultaneously with a specific competitor for each lectin. In the case of sialic acid binding lectins (SNA and MAA), fetuin was used for positive control, and treating the membranes with *C. perfringens* sialidase performed negative controls. The positions of protein standards (in kDa) are shown on the left of the blots.

maize rLf were revealed by ConA, indicating that the two glycosylation sites were occupied by *N*-glycans.

Deglycosylation treatments

In order to further analyze the nature of glycans substituting hLf and maize rLf, both glycoproteins were treated by several deglycosylation enzymes. As shown in Figure 4, hLf was sensitive to PNGase F but resistant to endo H, confirming that the glycan moiety of hLf is mainly of complex-type *N*-glycans. By contrast, maize rLf was completely resistant to both PNGase F and endo H treatment, and also resistant to the action of PNGase F/endo F mixture that hydrolyses *N*-glycans of the oligomannose and complex-type [37]. This result seems to indicate that rLf glycans are mainly substituted by an α1,3-linked fucose at the proximal *N*-acetylglucosamine.

Isolation and characterization of hLf and maize rLf oligosaccharides

HLf and maize rLf have been denaturated, reduced and carboxamidomethylated and finally hydrolysed by trypsin. The hLf and maize rLf trypsic digests have been next submitted to PNGase F and PNGase A digestions respectively. Liberated oligosaccharides have been separated from peptides, purified and analysed by MALDI-TOF.

MALDI mass measurements of glycans liberated from hLf by PNGase F (Figure 5A) indicated three major

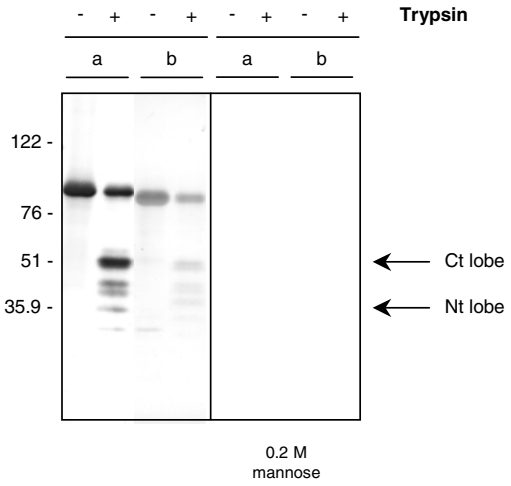

Figure 3. ConA staining of mild trypsin digested human and maize lactoferrins. Iron-saturated hLf (a) and maize rLf (b) were incubated with immobilized trypsin for 20 h (+) and analysed by SDS-PAGE and ConA staining. To control the lectin specificity, incubation was performed simultaneously with 0.2 M mannose. The position of protein standards is shown on the left.

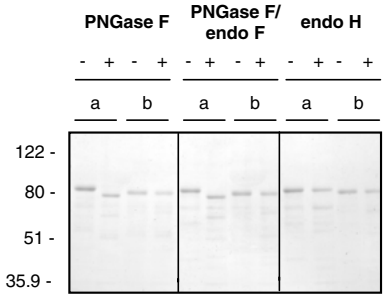

Figure 4. Deglycosylation of human and maize lactoferrins. hLf (a) and maize rLf (b) were treated by deglycosylation enzymes (PNGase F (0.4 U), endo H (2 mU) and endo F/PNGase F mixture (0.13 U)) and analysed by 4–20% SDS-PAGE and Coomassie blue staining. The position of protein standards (in kDa) is indicated on the left.

oligosacharides exhibiting $[M+Na]^+$ ions at m/z 1663.71, 1810.20 and 1956.25, respectively, that are consistent with the structures $Hex_5HexNAc_4$, Hex_5(dHex)HexNAc4 and $Hex_5(dHex)_2HexNAc_4$ (Hex = hexose). Three minor oligosaccharides exhibiting $[M+Na]^+$ions at m/z 2102.20, 2321.41 and 2467.01, respectively, and consistent with the structures $NeuAcHex_5(dHex)HexNAc_4$, $Hex_6(dHex)_2HexNAc_5$ and $Hex_6(dHex)_3HexNAc_5$ were also detected. These structures are in agreement with those described by Matsumoto et al. [38] which correspond to di-antennary *N*-glycans, mono- or difucosylated, monofucosylated and monosialylated, and di-antennary *N*-glycans, di- or trifucosylated, elongated with one *N*-acetyllactosaminic disaccharide. Similar results were obtained after PNGase A digestion of hLf (data not shown).

Oligosaccharides from maize rLf were only detected by MALDI-TOF after PNGase A deglycosylation. As indicated in Figure 5B, three major oligosaccharides exhibiting $[M+Na]^+$ ions at m/z 1048.83, 1064.81 and 1210.75, respectively, and consistent with the structures $Hex_2(dHex)(Pen)HexNAc_2$, $Hex_3(Pen)HexNAc_2$ and $Hex_3(dHex)(Pen)HexNAc_2$ (Pen = pentose) were observed. We also observed two minor oligosaccharides exhibiting $[M+Na]^+$ ions at m/z 1413.68, and 1616.61 consistent with the structures $Hex_3(dHex)(Pen)HexNAc_3$ and $Hex_3(dHex)(Pen)HexNAc_4$, this last structure corresponding to plant complex-type *N*-glycans $GlcNAc_2(Xyl\beta 1\text{-}2)Man_3(Fuc\alpha 1\text{-}3)GlcNAc_2$. This result indicated that maize rLf glycans are not of the complex-type but are essentially of the paucimannose-type. In addition, the resistance to PNGase F digestion confirmed our previous observation (Figure 4) and indicated that the pentasaccharidic core is mainly substituted by $\alpha 1$-3 linked fucose, with a major oligosaccharide $(Xyl\beta 1\text{-}2)Man_3(Fuc\alpha 1\text{-}3)GlcNAc_2$.

Discussion

The present paper reports for the first time the *N*-glycosylation pattern of the recombinant human glycoprotein expressed in maize, a monocotyledon expression system allowing full-scale commercial production. Elucidation of protein glycosylation is not only necessary to understand glycoprotein structures and functions in plants but is also crucial for biopharmaceutical production. The choice of human Lf as a model was based on its biological properties. Lf is a natural defence iron-binding protein that has been found to possess anti-bacterial, anti-fungal, anti-viral, anti-neoplastic and anti-inflammatory activity and is considered as a novel therapeutic with broad spectrum potential [38].

Human lactoferrin was also an interesting model to determine the glycosylation potential in maize because data were available on glycosylation of native Lf and recombinant Lf produced in other expression systems. *N*-glycosylation of milk derived human lactoferrin has been extensively studied [16, 39], showing that hLf contains two *N*-acetyllactosaminic-type *N*-glycans, more or less fucosylated and sialylated. Moreover, *N*-glycosylation of human Lf produced in several different expression systems, including mammalian cells [40], lepidopteran cells [41], tobacco [42], and transgenic mice [43], has been investigated.

To date, no data were available on *N*-glycosylation of proteins expressed in maize. Our preliminary studies on the native maize rLf using GC-MS allowed us to establish its monosaccharide composition. Two main monosaccharides are present on the glycoprotein: mannose and *N*-acetylglucosamine, associated with fucose and xylose (Table 1). The monosaccharide composition was confirmed using a panel of lectins, and the mild trypsin proteolysis of the iron-saturated rLf has indicated that both glycosylation sites (Asn_{138} and Asn_{479}) were occupied. The resistance of the native rLf to PNGase F/Endo F deglycosylation indicated that the glycans were mainly

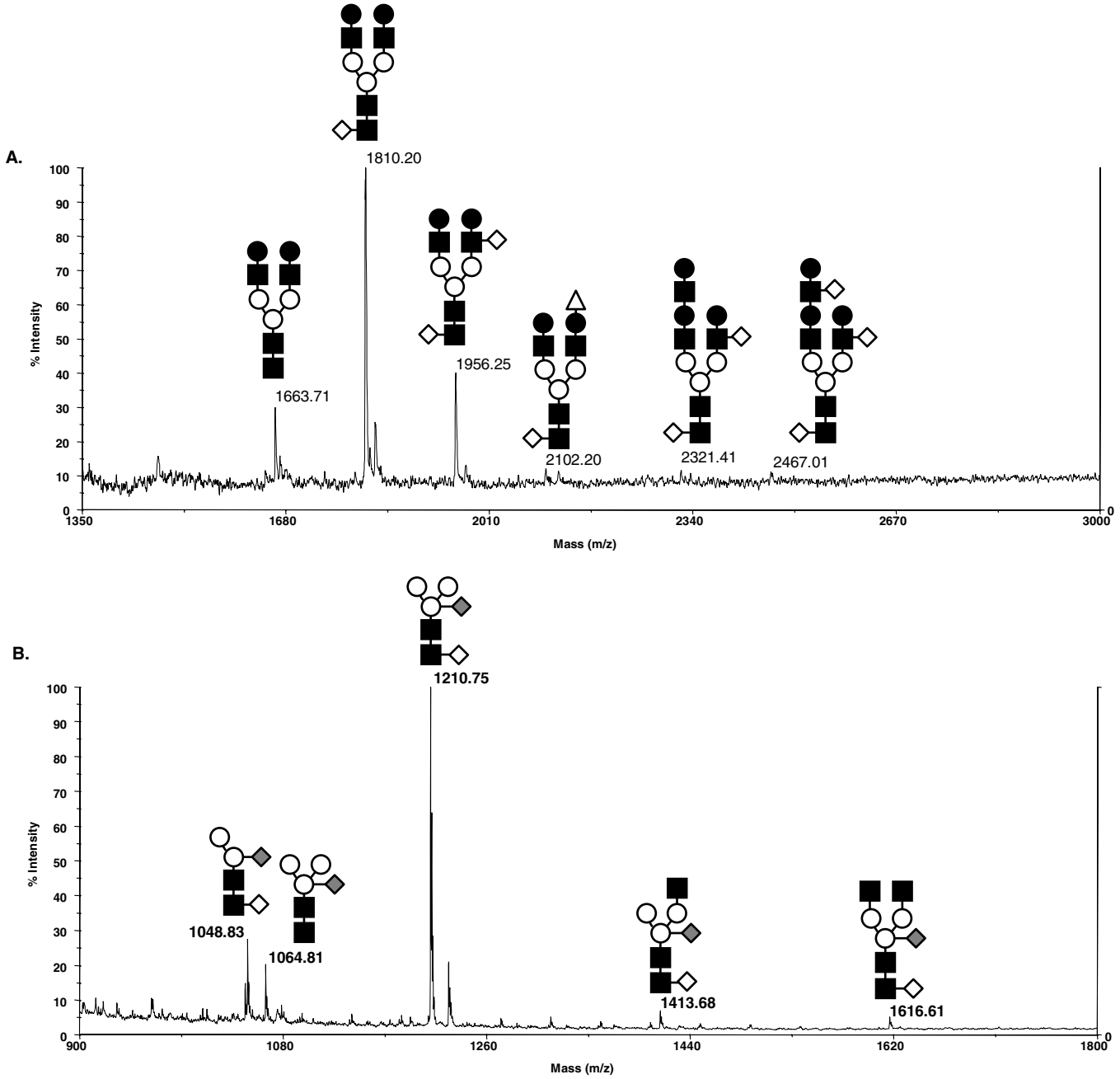

Figure 5. MALDI-TOF mass spectra of human lactoferrin and maize lactoferrin *N*-glycans. hLf (A) and maize rLf (B) spectra were recorded in positive ion reflective mode using 2,5-dihydroxybenzoic acid as matrix. ○ Mannose; ■ *N*-acetylglucosamine; ● galactose; –◇ α1,3-fucose; ◇– α1,6-fucose; △ sialic acid; ◆ xylose.

substituted by α1,3-linked fucose at the proximal *N*-acetylglucosamine. Furthermore, MALDI-TOF-MS analysis have confirmed the presence of a mixture of paucimannose plant-based structures, 80% of which being fucosylated and xylosylated, with a main glycoform (Xylβ1-2)Man$_3$(Fucα1-3)GlcNAc$_2$ and several minor compounds, corresponding to degradation forms of complex-type *N*-glycans (Figure 5). We have also detected little amounts of GlcNAc$_2$(Xylβ1-2)Man$_3$(Fucα1-3)GlcNAc$_2$, showing that the UDP-GlcNAc: *N*-acetylglucosaminyltransferase I and II are, at least weakly, expressed in the maize. However, probably due to the absence of key enzymes such as galactosyltransferases, the processing of maize *N*-glycans is directed to the degradation pathway, leading to the accumulation of truncated *N*-glycans. Wilson et al. have recently demonstrated that the Lewis^a-type structure (Galβ1-3[Fucα1-4]GlcNAc-R), firstly described in plants in sycamore *N*-glycans [44], is very widespread among plants, including mono- and dicotyledons [45]. Our results indicated that such

complex-type glycans do not exist in maize rLf. The lack of complex type structures with Lewis[a] determinants has also been reported for other *magnoliophytae* including monocotyledons (coconut) and dicotyledons (cauliflower, mung bean, papaya, peanut) [45]. This observation seems to indicate that the glycosylation potential is not correlated to the classes of flowering plants. At the end of the maturation, maize rLf *N*-glycans have typical structures that differ from those found in hLf by the absence of galactose and sialic acid and the presence of β1,2-xylose and α1,3-fucose residues.

Like lepidopteran cell glycosylation, plant-specific *N*-glycosylation could represent a limitation for the use of certain recombinant glycoproteins of human origin produced in transgenic plants. Attempts to improve the glycosylation capacity of this plant expression system are currently undertaken, to allow the production of complex glycoproteins with higher therapeutic efficacy and security.

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