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Glycan structure of Gc Protein-derived Macrophage Activating Factor as revealed by mass spectrometry

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ABSTRACT

Disagreement exists regarding the O-glycan structure attached to human vitamin D binding protein (DBP). Previously reported evidence indicated that the O-glycan of the Gc1S allele product is the linear core 1 NeuNAc-Gal-GalNAc-Thr trisaccharide. Here, glycan structural evidence is provided from glycan linkage analysis and over 30 serial glycosidase-digestion experiments which were followed by analysis of the intact protein by electrospray ionization mass spectrometry (ESI-MS). Results demonstrate that the O-glycan from the Gc1F protein is the same linear trisaccharide found on the Gc1S protein and that the hexose residue is galactose. In addition, the putative anti-cancer derivative of DBP known as Gc Protein-derived Macrophage Activating Factor (GcMAF, which is formed by the combined action of β-galactosidase and neuraminidase upon DBP) was analyzed intact by ESI-MS, revealing that the activating *E. coli* β-galactosidase cleaves nothing from the protein—leaving the glycan structure of active GcMAF as a Gal-GalNAc-Thr disaccharide, regardless of the order in which β-galactosidase and neuraminidase are applied. Moreover, glycosidase digestion results show that α-*N*-Acetylgalactosamindase (nagalase) lacks endoglycosidic function and only cleaves the DBP O-glycan once it has been trimmed down to a GalNAc-Thr monosaccharide—precluding the possibility of this enzyme removing the O-glycan trisaccharide from cancer-patient DBP *in vivo*.

1. Introduction

Human Vitamin D Binding Protein (DBP, also known as Gc Globulin or GcG) is a 458-amino acid protein with an unmodified mass of 51.2 kDa. DBP is found in blood plasma/serum at concentrations of 300–600 mg/L [1]. There are three common allelic variants of DBP within the human population: Gc1F, Gc1S and Gc2. Relative to the Gc1F protein, a D416E mutation defines the Gc1S protein, and a T420K mutation defines the Gc2 protein. Ten to thirty percent of circulating Gc1F and Gc1S DBP molecules carry an O-linked trisaccharide that has a mass consistent with the common sialylated core 1 O-glycan (i.e., NeuNAc-Gal-GalNAc-Thr/Ser) [2–6], which is known to be attached to T420 [4,7,8]. This monosaccharide composition of Gc1 DBP is consistent with that determined by Viau in 1983 [9]; but based on activity studies following application of glycosidases and/or lectin-based western blots, some believe that the Gc1S and/or Gc1F protein lacks sialic acid and contains mannose instead [10–17]. Likewise, the structure of this trisaccharide as linear [4,8] rather than branched appears to have been largely (but not completely [17]) accepted for the 1S allele product, but not the 1F protein—which some believe contains a dibranched structure [16,17]. Glycosylation of the Gc2 allele product at a site other than T420 (probably T418 [4]) is less controversial; about 5% of the Gc2 allele product [3–5] appears to carry a Gal-GalNAc-Thr disaccharide—a structure that is not debated [3–5,16,18].

Most of the controversy surrounding the glycan structure of DBP is derived from the glycosidase enzyme activities required to convert DBP to GcMAF, which were originally used to infer a structure for the O-glycan of DBP and GcMAF: GcMAF is a particular form of O-glycosylated DBP that results from the action of β-galactosidase and neuraminidase upon Gc1 allele products [18–20], or β-galactosidase alone upon Gc2 protein [18,20]. A few years after GcMAF was discovered, it was found that β-

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galactosidase and neuraminidase can be applied stepwise, in either order, to generate GcMAF [20]. The implication of a dibranched structure was taken at face value without further investigation, resulting in the widespread conclusion that the DBP trisaccharide on Gc1 allele produces must be dibranched with both the NeuNAc and Gal residues attached to GalNAc [11–14,21–25].

In addition to controversies about the structure of the DBP O-glycan, some controversy has remained over whether or not DBP is deglycosylated in cancer patients. Deglycosylation of DBP in cancer patients was accepted without question for over 10 years due to observation of a strong inverse correlation between patient serum α-*N*-Acetylgalactosamindase (a.k.a. nagalase) activity, which is elevated in cancer patients, and GcMAF "precursor activity" (i.e., the ability to generate GcMAF from patient DBP) [26,27]. This begat the long-accepted conclusion that DBP is deglycosylated in cancer patients [12,17,25]. Several years ago, however, we showed that DBP is not even slightly deglycosylated in cancer patients [5,6]. The lack of DBP deglycosylation in cancer patients has been accepted to some degree [28], but not completely [17].

Here we present evidence based on glycan linkage analysis and serial glycosidic digestion followed by analysis of the intact DBP protein by electrospray ionization mass spectrometry (ESI-MS) for 1) the linearity of the DBP O-glycan in all Gc1 allele products, 2) its structure and monosaccharide composition as NeuNAc-(2-3)-Gal-(1-3)-GalNAc-Thr, 3) the inability of nagalase to act as an endoglycosidase (and thereby deglycosylate the DBP Gc1 trisaccharide or Gc2 disaccharide), and 4) the predominant glycan structure of active GcMAF as a Gal-GalNAc-Thr disaccharide—an unexpected finding that contradicts the commonly accepted notion that GcMAF bears a simple GalNAc-Thr monosaccharide [11,12,14–19,22,28–32].

2. Materials and methods

2.1. Materials

Vitamin D affinity-purified DBP and GcMAF were obtained from Immune Research Inc. (IRI, Sonora, CA). IRI isolated DBP from the blood plasma of an individual homozygous for the Gc1F allele using vitamin D affinity chromatography, then used immobilized neuraminidase and β-galactosidase from *E. coli* to activate the DBP to GcMAF according to the methods of Mohamad et al. [30]. The GcMAF preparation was tested for activity by IRI using a macrophage Fc-receptor mediated phagocytosis assay similar to that previously used by Yamamoto [33,34]; it was found to be active relative to a lipopolysaccharide reference. Custom-purified, salt-free lyophilized DBP was from Athens Research & Technology (Athens, GA). β-D-galactopyranosyl-(1-3)-D-galactose (a.k.a. Gal-β(1-3)-Gal) and 2-Acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactopyranose (a.k.a. Gal-β(1-3)-GalNAc) which were used as reference materials for linkage analysis were obtained from Carbosynth (San Diego, CA). *E. coli* β-galactosidase and neuraminidase were from Roche (Indianapolis, IN). All other glycosidic enzymes were from Prozyme (Hayward, CA). Cleavage specificities for all glycosidases are supplied as a footnote to Table 1. The activity units of each enzyme were used as defined by the supplier. 2-*O*-(*p*-Nitrophenyl)-α-D-*N*-acetylneuraminic acid was from Toronto Research Chemicals Inc (Toronto, Ontario, Canada). All other 4-nitrophenylglycoside substrates employed to confirm glycosidase activity were from Sigma-Aldrich (St. Louis, MO). Polyclonal rabbit anti-human DBP (GC-Globulin) antibodies (Cat. No. A0021) were obtained from DAKO (Carpinteria, CA). Hepes-buffered saline (HBS) was obtained from Biacore (Piscataway, NJ). Mass spectrometric immunoassay (MSIA) pipette tips for microscale immunoaffinity extraction and [high performance] liquid chromatography-mass spectrometry (LC-MS) solvents (highest purity available) were from ThermoFisher Scientific (Waltham, MA). All other chemicals were of the highest purity available from Sigma-Aldrich (St. Louis, MO).

2.2. ESI-MS

Analysis of intact DBP and GcMAF was carried out in a manner similar to that which we have previously described [3–5]. Briefly, a trap-and-elute form of sample concentration, rinse, and elution rather than traditional LC was used for these analyses. Affinity-purified DBP and GcMAF from IRI were supplied in 0.1 M sodium phosphate buffer, pH 7.1 at concentrations of 1 mg/mL and 0.1 mg/mL, respectively. Samples were brought to a final concentration of 2 μM in 0.1% trifluoroacetic acid and 4 μL was injected by a Spark Holland Endurance autosampler in microliter pick-up mode and loaded by an Eksigent nanoLC*1D at 10 μL/min (80/20 water/acetonitrile containing 0.1% formic acid, Solvent A) onto a capillary-sized protein concentration & desalting OPTI-TRAP™ (polymeric/reversed phase sorbent, part number 10-04814-TM, Optimize Technologies, Oregon City, OR) configured for unidirectional flow on a 6-port divert valve. Vitamin D affinity-purified DBP and GcMAF protein samples were desalted on the protein concentration & desalting trap by rinsing at 10 μL/min for 100 min to fully eliminate non-covalent phosphate adducts of the protein that were derived from the formulation buffer. All other samples were rinsed/desalted on-trap for just 2 min. Following on-trap rinsing/desalting, the divert valve position was automatically toggled and flow rate over the protein captrap cartridge changed to 1 μL/min solvent A (running directly to the ESI inlet) which was immediately ramped over 8 min of a linear gradient from 20% to 90% solvent B (100% acetonitrile).

For glycosidase digestion samples that were pre-extracted by pipette-tip based immunoaffinity extraction (described below), 10-μL samples were injected in the same manner, but were rinsed for 2 min prior to elution into the mass spectrometer by the same gradient.

DBP and its various proteoforms eluted into a Bruker MicrOTOF-Q (Q-TOF) mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the *m/z* range of 50–3000. ESI settings for the Agilent G1385A capillary nebulizer ion source were as follows: End Plate Offset −500 V, Capillary −4500 V, Nebulizer nitrogen 2 Bar, Dry Gas nitrogen 3.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 2 GHz. Spectra rate control was by summation at 1 Hz.

2.3. Glycan linkage analysis

Glycan linkage analysis (also known as methylation analysis [35]) was carried out as we have previously described [36,37].

2.3.1. Permethylation

Permethylation and subsequent clean-up procedures were adapted from the protocol of Goetz et al. [38], which was designed to permethylate and release O-linked glycans from pre-isolated glycoproteins. One hundred to five hundred micrograms of protein in 10 μL was added to a 1.5 mL polypropylene test tube. To the 10-μL sample was added 270 μL of dimethylsulfoxide (DMSO) and 105 μL of iodomethane. This solution was mixed thoroughly and placed onto a plugged 1-mL spin column containing approximately 0.7 g sodium hydroxide beads that had been preconditioned with acetonitrile followed by two rinses with DMSO. Samples were allowed to sit for 10–12 min with occasional stirring. Samples were then unplugged and spun in a microcentrifuge for 30 s at 4000 rpm (800 g) to retrieve the glycan-containing liquid. Samples were then transferred to a silanized 13 × 100 mm glass test tube. Three

Table 1
Treatment of DBP with various glycosidases (in serial) and resulting O-glycan glycoforms observed on the final intact protein by ESI-MS.

		Resulting relative abundance of DBP glycoforms			
	Glycosidase treatment(s)[a]	GalNAc	Gal-GalNAc	NeuNAc-Gal-GalNAc	Abbreviated interpretation of results
1	None		+	+++++	See Fig. 3
2	Neu		+++++		
3	Neu → ec β-Gal		+++++		Only bt β-Gal cleaves galactose (once it is exposed). Also suggests that Gal-GalNAc linkage is not 1–4 or 1-6
4	Neu → bt β-Gal	+++++			
5	Neu → sp β-Gal		+++++		
6	Neu → jb β-Gal		+++++		
7	Neu → α-Gal		+++++		Galactose is not α-linked
8	Neu → α-Mann		+++++		The hexose is not α-linked mannose
9	Neu → β-Mann		+++++		The hexose is not β-linked mannose
10	ec-β-Gal		+	+++++	
11	bt-β-Gal		+	+++++	Neu must be applied before bt β-Gal can cleave, suggesting the presence of a linear trisaccharide
12	sp-β-Gal		+	+++++	
13	jb-β-Gal		+	+++++	
14	Neu → ec β-Gal → α-Gal		+++++		ec β-Gal does not epimerize β-Gal to α-Gal
15	Neu → ec β-Gal → bt β-Gal	+++++			ec β-Gal does not affect the ability of bt β-Gal to cleave galactose
16	Neu → O-Glyc		+		The Gal-GalNAc disaccharide is likely Gal-GalNAc-α-Thr
17	Neu → ec β-Gal → O-Glyc		+		ec β-Gal does not affect the ability of O-Glyc to cleave Gal-GalNAc …
18	ec β-Gal → Neu → O-Glyc		+		… and order does not matter
19	Neu → bt β-Gal → Nag				Complete deglycosylation …
20	Neu → ec β-Gal → Nag		+++++		… but Nag does not act if Gal is not removed
21	Neu → ec β-Gal → bt β-Gal → Nag				ec β-Gal does not affect the ability of bt β-Gal to cleave Gal or of Nag to cleave GalNAc
22	Nag → Neu → O-Glyc		++++		Nag impedes the ability of O-Glyc to cleave Gal-GalNAc[b] – acting on both the disaccharide and trisaccharide
23	Neu → Nag → O-Glyc		++++		
24	Nag → Neu → bt β-Gal → Nag				
25	Nag		+	+++++	Nag does not act as an endo-glycosidase on the DBP O-glycan
26	Neu → Nag		+++++		
27	Nag → Neu → α-Gal		+++++		Nag does not facilitate cleavage of Gal by α-Gal
28	Neu → Nag → α-Gal		+++++		
29	Neu → Nag → ec β-Gal		+++++		Nag does not facilitate cleavage of Gal by ec β-Gal
30	Nag → Neu → ec β-Gal		+++++		
31	ec β-Gal → Neu → Nag		+++++		
32	Neu → Nag → ec β-Gal → O-Glyc		+++		ec β-Gal and Nag appear to competitively modify the O-glycan to make it susceptible and insusceptible to cleavage, respectively, by O-Glyc
33	Nag → Neu → ec β-Gal → O-Glyc		+++		
34	Neu → ec β-Gal → Nag → O-Glyc		+++		
35	ec β-Gal → Neu → Nag → O-Glyc		+++		

[a] Glycosidase Abbreviations and Nominal Expected Cleavage Specificity: Neu (Neuraminidase; α(2-3) > α(2-6) = α(2-8)), ec β-Gal (β-Galactosidase from *e. coli*; β(1-3) > β(1-4) > β(1-6)), bt β-Gal (β-Galactosidase from bovine testes; β(1-3,4)), sp β-Gal (β-Galactosidase from *s. pneumoniae*; β(1-4)), jb β-Gal (β-Galactosidase from jack bean; β(1-4,6)), α-Gal (α-Galactosidase; α(1-3,4,6)), α-Mann (α-mannosidase; α(1-2,3,6)), β-Mann (β-mannosidase; β(1-4)), O-Glyc (endo-α-*N*-Acetylgalactosaminidase/aka O-Glycanase; Gal-β-(1-3)-GalNAc-α-Thr/Ser), Nag (α-*N*-Acetylgalactosamindase/aka nagalase; GalNAc-α-R).
[b] But this was not due to enzyme inhibition—see Results and Discussion.

hundred microliters of acetonitrile was then added to the spin column to wash off all of the permethylated glycan. Spin columns were then centrifuged at 10,000 rpm (5000 g) for 30 s to collect the acetonitrile which was pooled with the rest of the sample. To the liquid sample was added 3.5 mL of 0.5 M NaCl followed by 1.2 mL of chloroform. Liquid/liquid extraction was performed 3 times, saving the chloroform layers, which were dried under a gentle stream of nitrogen.

Acid Hydrolysis, reduction, and acetylation procedures were adapted from Heiss et al. [39]:

2.3.2. Trifluoroacetic acid (TFA) hydrolysis

Three hundred twenty five microliters of 2 M TFA was added to each sample which was then capped tightly and heated at 121 °C

for 2 h. TFA was then removed by heat-assisted evaporation under a gentle stream of nitrogen.

2.3.3. Reduction of sugar aldehydes

A fresh 10 mg/mL solution of sodium borohydride in freshly prepared 1 M ammonium hydroxide was prepared and added (475 μL) to each test tube, mixed thoroughly and allowed to react for 1 h at room temperature. Residual borate was removed by adding 5 drops of methanol to each sample, drying under nitrogen, then adding 125 μL of 9:1 (v/v) methanol: acetic acid and drying again under nitrogen. Samples were then dried for about 30 min in a vacuum desiccator before proceeding.

2.3.4. Acetylation of nascent hydroxyl groups

Two hundred and fifty microliters of freshly made water-saturated acetic anhydride (16:234 (v/v) water: acetic anhydride) was added to each sample, which was mixed thoroughly to dissolve as much of the sample residue as possible. Next, 230 μL of concentrated TFA was added to each sample, which was then mixed, capped and incubated at 50 °C for 10 min.

2.3.5. Final clean-up

Two milliliters of dichloromethane was added to each sample along with 2 mL of water. Liquid/liquid extraction was carried out twice with water. The final organic layer was then dried in a silanized autosampler vial under nitrogen and reconstituted in 8 drops of acetone, mixed, capped and placed on the gas chromatograph-mass spectrometer (GC-MS) autosampler rack.

2.3.6. Gas chromatography-mass spectrometry

GC-MS was carried out on an Agilent A7890 gas chromatograph (equipped with a CTC PAL autosampler) coupled to a Waters GCT (Time-of-Flight) mass spectrometer. One microliter was injected at a split ratio of 10 onto an Agilent split-mode liner (Cat. No. 5183-4647) containing a small plug of silanized glass wool, maintained at 280 °C. Using helium as the carrier gas (0.8 mL/min, constant flow mode) samples were chromatographed over a 30 m DB-5ms GC column. The oven was initially held at 165 °C for 0.5 min followed by ramping at 10 °C/min to 265 °C then immediately ramping at 30 °C/min to 325 °C and holding for 3 min (15.5 min of total run time). The transfer line was maintained at 250 °C. Sample components eluting from the GC column were subjected to electron ionization (70 eV, 250 °C) and analyzed from 40 to 800 *m/z* with a "scan cycle" time of 0.2 s. The mass spectrometer was tuned and calibrated (to within 10 ppm mass accuracy) daily using perfluorotributylamine.

2.3.7. Data analysis

As we have previously described, initial identification of the final products resulting from methylation analysis preparation chemistry known as partially methylated alditol acetates (PMAAs) was made through the analysis of glycan standards and verified through comparison with the online electron ionization mass spectral library of PMAAs at the University of Georgia's Complex Carbohydrate Research Center: http://www.ccrc.uga.edu/databases/index.php#

EI mass spectra of relevant glycan standards are provided in Supplementary Material Fig. 1. Notably, sialic acid (i.e., NeuNAc) cannot be detected directly during this procedure as it is chemically unstable to one or more of the steps *following* permethylation [38]—probably acid hydrolysis; thus it can only be detected indirectly, vis-à-vis its linkage position to the next sugar residue.

2.4. Glycosidic digestion of DBP and immunoaffinity purification for ESI-MS

Numerous serial digestion experiments were carried out as outlined in Table 1. In each case, 12.5–25 μL of a salt-free, 2 μg/μL solution of DBP was adjusted to the appropriate pH using concentrated reaction buffers supplied by the enzyme provider or made in-house using buffers and metal cofactors recommended by the supplier. The following volumes and activities of enzymes were typically added (whenever the specified enzyme was employed in a digestion series). No matter the typical amount or volume of enzyme added to a pre-existing volume, the final concentration in terms of activity units is specified: Neuraminidase (typically 6.25 μL of 5 mU/μL; final concentration 1 mU/μL), *E. coli* β-galactosidase (typically 5 μL of 2 U/μL; final concentration 330 mU/μL), bovine testes β-galactosidase (typically 8 μL of 5 mU/μL; final concentration 1.6 mU/μL), *S. pneumoniae* β-galactosidase (typically 20 μL of 2 mU/μL; final concentration 1 mU/μL), jack bean β-galactosidase (typically 2 μL of 25 mU/μL; final concentration 2.5 mU/μL), green coffee bean α-galactosidase (typically 1.5 μL of 100 mU/μL; final concentration 4.75 mU/μL), jack bean α-mannosidase (typically 12 μL of 180 mU/μL; final concentration 50 mU/μL), *H. pomatia* β-mannosidase (typically 11 μL of 88 mU/μL; final concentration 2.75 mU/μL), *S. pneumoniae* O-glycanase (typically 7.5 μL of 1.25 mU/μL; final concentration 0.33 mU/μL), α-*N*-Acetylgalactosaminidase (nagalase) (typically 12 μL of 20 U/μL; mU/μL; final concentration 4 U/μL).

Incubations were conducted within capped and parafilm-sealed polypropylene test tubes in an oven set at 37 °C. No liquid condensation at the top of the test tube vials was observed. Neuraminidase digestions proceeded for 2 h; all other enzymes were incubated overnight (i.e., at least 16 h). To ensure that all enzymes were active, after adding glycosidase, a 2.5-μL aliquot of the sample was added to 2.5 μL of a 0.8 mg/mL solution of the appropriate 4-nitrophenyl glucoside and incubated in parallel with the main sample. (For the enzyme inhibition experiment described below, quantitative measurements of 4-nitrophenol were made at 405 nm after an incubation period of 10 min) If the pH of the glycosidic digestion was outside of the neutral range, a negative control for this positive colorimetric control was made and consisted of equal volumes of reaction buffer and the 4-nitrophenyl glucoside. Once the incubation was complete the activity of the enzyme was verified in the positive control (i.e., 4-nitrophenyl glucoside sample) by the appearance of a deep yellow color. (If needed, the pH of the positive control sample was adjusted to 7–9 with 0.5 M NaOH prior to reading the color of the sample.) No results are reported for any incubations in which the positive control sample was negative (or in which the negative control for the positive control was positive—which never actually happened).

Following incubation with the final glycosidase, DBP was immunoaffinity extracted and analyzed by electrospray ionization-mass spectrometric immunoassay (ESI-MSIA) as we have previously described [5]. In brief, MSIA pipette tips that had been pre-activated with carbonyldiimidazole (CDI) [40] were derivatized with polyclonal DBP antibodies by repetitively flowing (aspirating and dispensing 750 times) 50 μL volumes of antibody solution (150 μL/well; 0.05 g/L in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffered saline, pH 4.7) through the tips. Antibody-linked tips were stored in HBS at 4 °C until the day of use at which time they were pre-rinsed (400 μL/well; 150 μL aspirate and dispense cycles; 10 cycles) with HBS then used to extract DBP from individual samples (25 μL of sample diluted with 75 μL of HBS) at room temperature (100 μL/well; 50 μL aspirate and dispense cycles; 750 cycles). Pipette tips were then ejected allowed to sit in their respective samples at room temperature until they were

individually (manually) washed (by drawing from a fresh reservoir of liquid and dispensing to waste) and eluted as follows: Five cycles of 200 μL of HBS, five cycles of 200 μL distilled water, five cycles 200 μL of 2 M ammonium acetate/acetonitrile (3:1 v/v), ten cycles of 200 μL of distilled water. Elution was accomplished by briefly air-drying the pipette frits then drawing 5.5 μL of 0.4% TFA (v/v), mixing over the pipette affinity capture frit for 20–30 s, and dispensing into a 96-conical well polypropylene autosampler tray. Frits were then washed with an additional 5.5 μL distilled water which was used to dilute the eluted sample. Ten microliters were injected into the LC-ESI-TOF-MS within 10 min of elution. LC-MS methods are described above.

3. Results and Discussion

3.1. Linkage pattern and monosaccharide composition of Gc1 allele products

Vitamin D affinity-purified DBP from a donor homozygous for the 1F allele product (Fig. 1) was subjected to glycan linkage analysis (also known as methylation analysis—a classic glycan analysis technique [35]) as we [36,37] and others [39] have previously described. In brief, the intact protein is first permethylated. During this process, O-linked glycans are non-reductively eliminated [38] and all exchangeable protons are replaced with methyl groups.

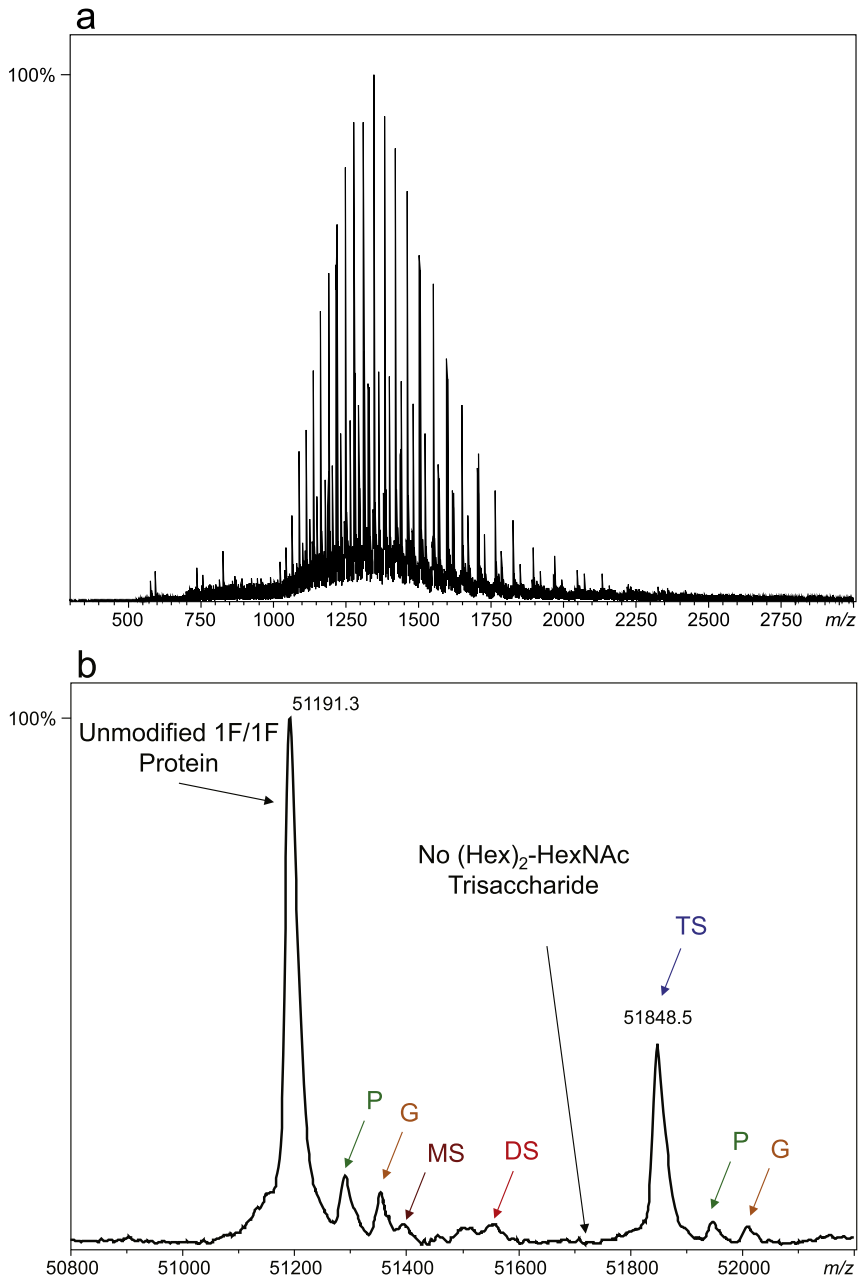


Fig. 1. Raw mass spectrum (a) and charge deconvoluted ESI mass spectrum (b) of the vitamin D affinity-purified DBP from a plasma donor homozygous for the Gc1F allele product. These spectra indicate the purity of the protein preparation, the homozygous nature of the source plasma (cf. reference [3]), and the lack of evidence for a [dibranched], mannose-containing $(Hex)_2$-HexNAc-Thr trisaccharide. Modified forms of DBP observed include a non-covalent phosphate adduct (P), a non-enzymatically glycated form (G), a GalNAc-only monosaccharide form (MS), a Gal-GalNAc disaccharide form (DS), and a NeuNAc-Gal-GalNAc trisaccharide form (TS). Calculated MH^+ masses for all forms are provided in Supplementary Material Table 1.

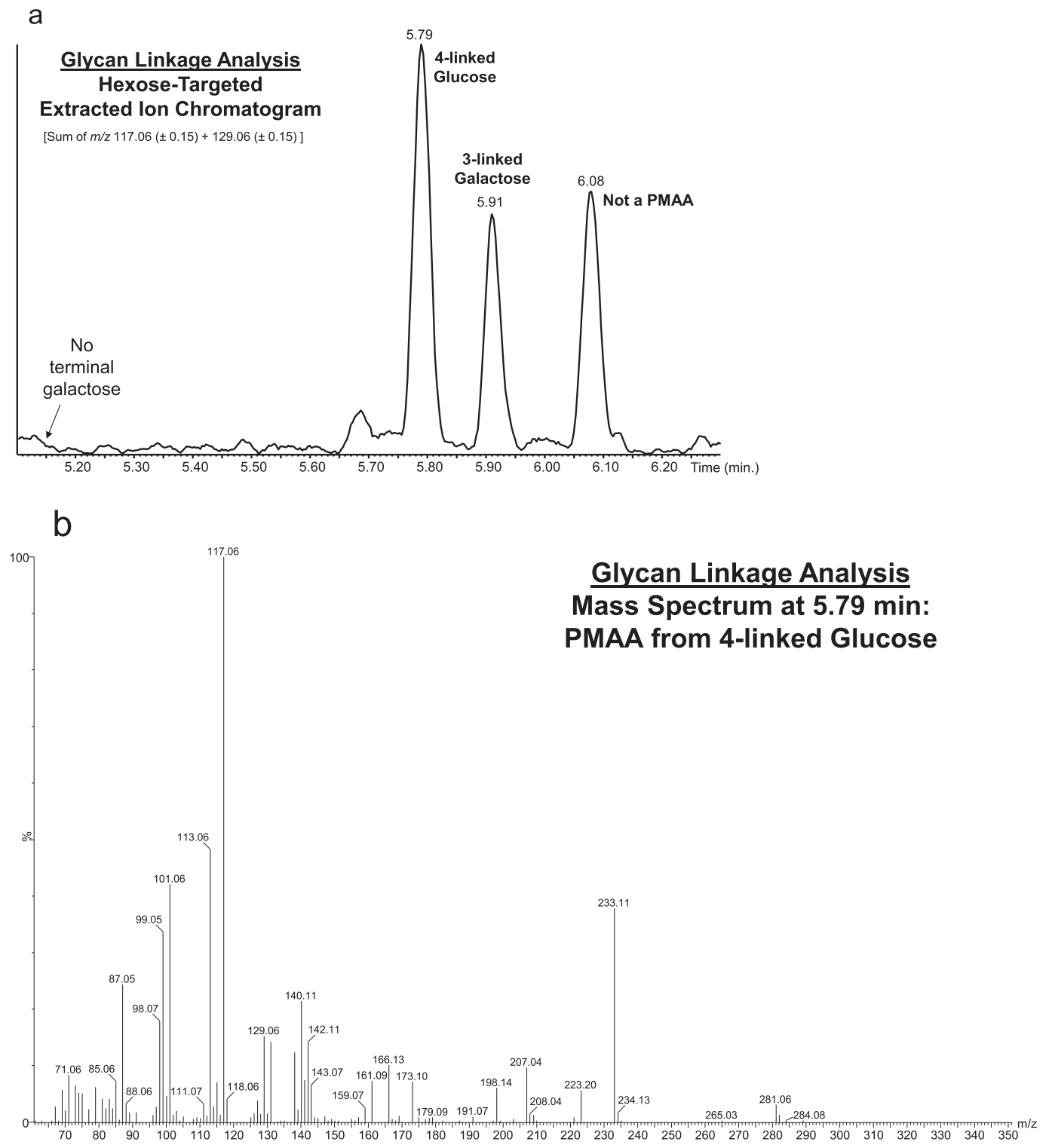


Fig. 2. Glycan linkage analysis of the vitamin D affinity-purified DBP from a plasma donor homozygous for the 1F allele product (Fig. 1). Hexose-targeted extracted ion chromatogram (a) and EI mass spectra (b–c) of the partially methylated alditol acetates (PMAA). As explained in the main text, 4-linked glucose (b) is not observed in O-linked glycans and is most likely a contaminant from glycosphingolipids or cellulose; 3-linked galactose (c) was the only other hexose identified and corresponds to the expected hexose residue from a linear DBP O-glycan. No terminal-galactose was detected at the expected retention time of 5.14 min [36]. EI mass spectra of 4-linked glucose and 3-linked galactose standards are provided in Supplementary Material Fig. 1.

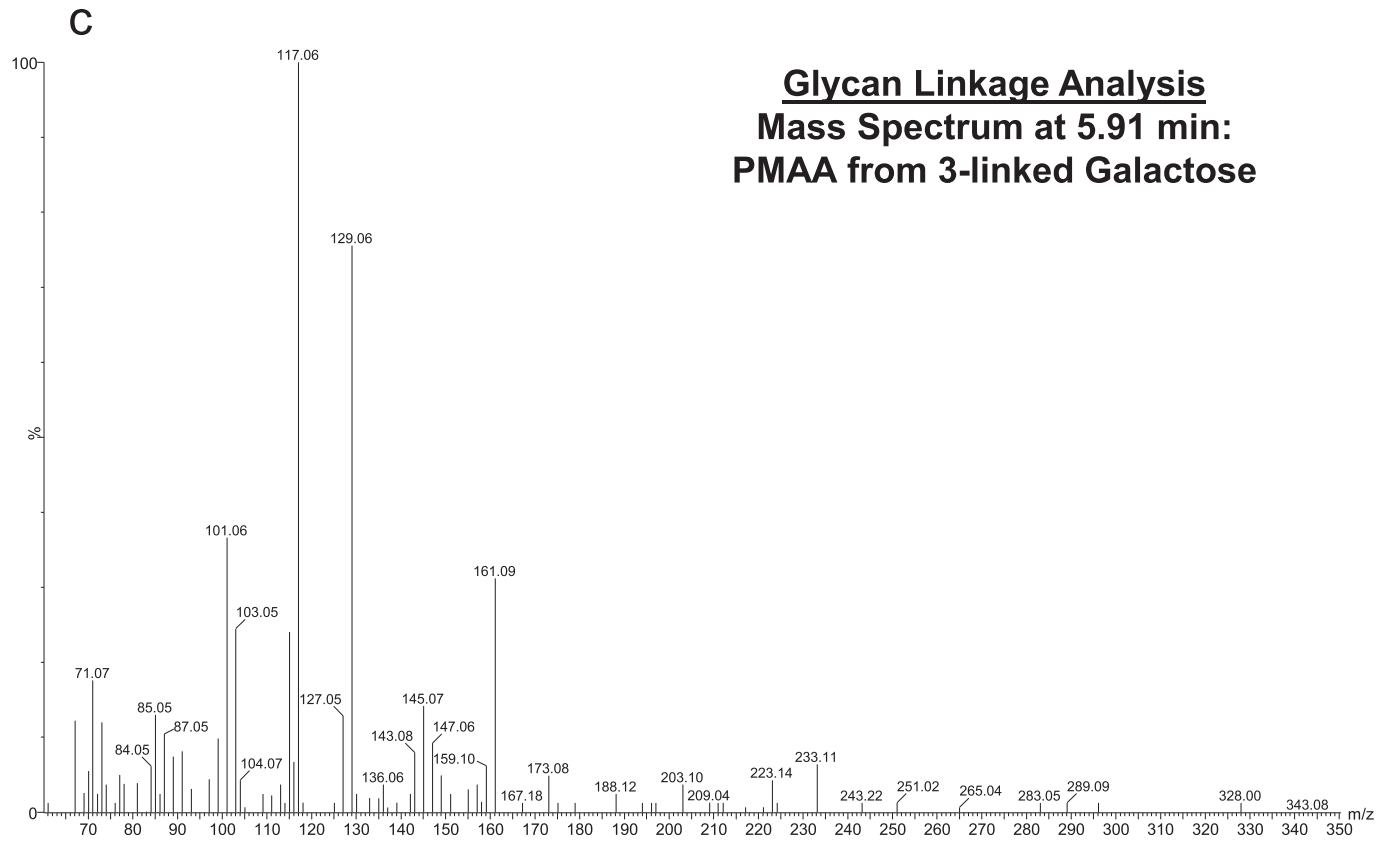


Fig. 2. (*continued*).

The permethylated glycan is then hydrolyzed into monosaccharides, which possess free (non-permethylated) hydroxyl groups at positions at which there was formerly a linkage between sugar residues. The monosaccharides are then reduced and acetylated to mark linkage positions. Once acetylation is complete the final GC-MS-ready molecules are referred to as partially methylated alditol acetates (PMAAs). It should be noted that in the original glycan polymer all carbohydrate residues are linked at their 1-position, meaning that all PMAAs end up acetylated at the 1-position. Likewise, all PMAAs derived from pyranose rings (6-membered rings) are also acetylated at their 5-positions. (PMAAs derived from furanose rings (5-membered rings) are acetylated at the 4-position, but these are uncommon in mammalian glycans and none were observed in this study.) In total, the permethylation, hydrolysis, reduction, and acetylation processes result in PMAAs that retain the monosaccharide identity, ring size, and linkage pattern information of the residue as it existed in the original glycan. During analysis by GC-MS, each unique PMAA produces a distinct retention time and EI-mass spectrum.

Two major chromatographic peaks representative of hexose residues were produced by PMAAs derived from vitamin D affinity-purified DBP (Fig. 2a). Their retention times and spectra matched those of 4-linked glucose (4-Glc) and 3-linked galactose (3-Gal) (Fig. 2b–c). Glucose is not observed in O-glycans [41]. In humans 4-linked glucose is most commonly observed in glycosphingolipids [42]. If present, glycosphingolipids could have remained bound to DBP during the purification process. We tested this hypothesis with the last remaining 15 μg of vitamin D affinity-purified DBP that we had available using the MALDI-MS method of Touboul et al. [43], but were unable to definitively identify the presence of glycosphingolipids. Contamination of the DBP protein with cellulose (e.g., from filters employed during the purification process) could also explain the presence of 4-linked glucose. Galactose linked at its 3-position corresponds to the PMAA anticipated from the linear sialylated core 1 O-glycan (NeuNAc-α(2-3)-Gal-β(1-3)-GalNac-Thr). A dibranched structure would have produced a terminal galactose residue, and there is no evidence for a PMAA representing terminal galactose, as it would be expected at 5.14 min [36] (Fig. 2a). Hence the linkage analysis evidence for the O-linked trisaccharide attached to the Gc1F allele product supports a linear glycan structure in which galactose is the middle residue and is connected to sialic acid via its 3-position hydroxyl group.

The linkage analysis procedure employed is not as sensitive to HexNAc residues as it is to hexoses [36] and we were unable to detect any HexNAc residues in the preparation of Gc1F allele product that was purified by vitamin D affinity chromatography. As such, a sample of DBP from pooled human plasma was obtained from Athens Research & Technology (Athens, GA) (Fig. 3). Linkage analysis of 500 μg of this protein preparation confirmed the findings observed in the vitamin D affinity-purified DPB preparation (Fig. 4a–c) and revealed that the single identifiable HexNAc residue was a 3-linked GalNAc residue (Fig. 4d–e).

3.2. Glycan structure of GcMAF

Having examined the linkage pattern and monosaccharide composition of the DBP O-glycan, our investigation turned to the analysis of active GcMAF. As a first step, the intact protein was analyzed by ESI-MS, which revealed the major glycan component as a Gal-GalNAc disaccharide and not the GalNAc monosaccharide as anticipated (Fig. 5). Linkage analysis was attempted with the

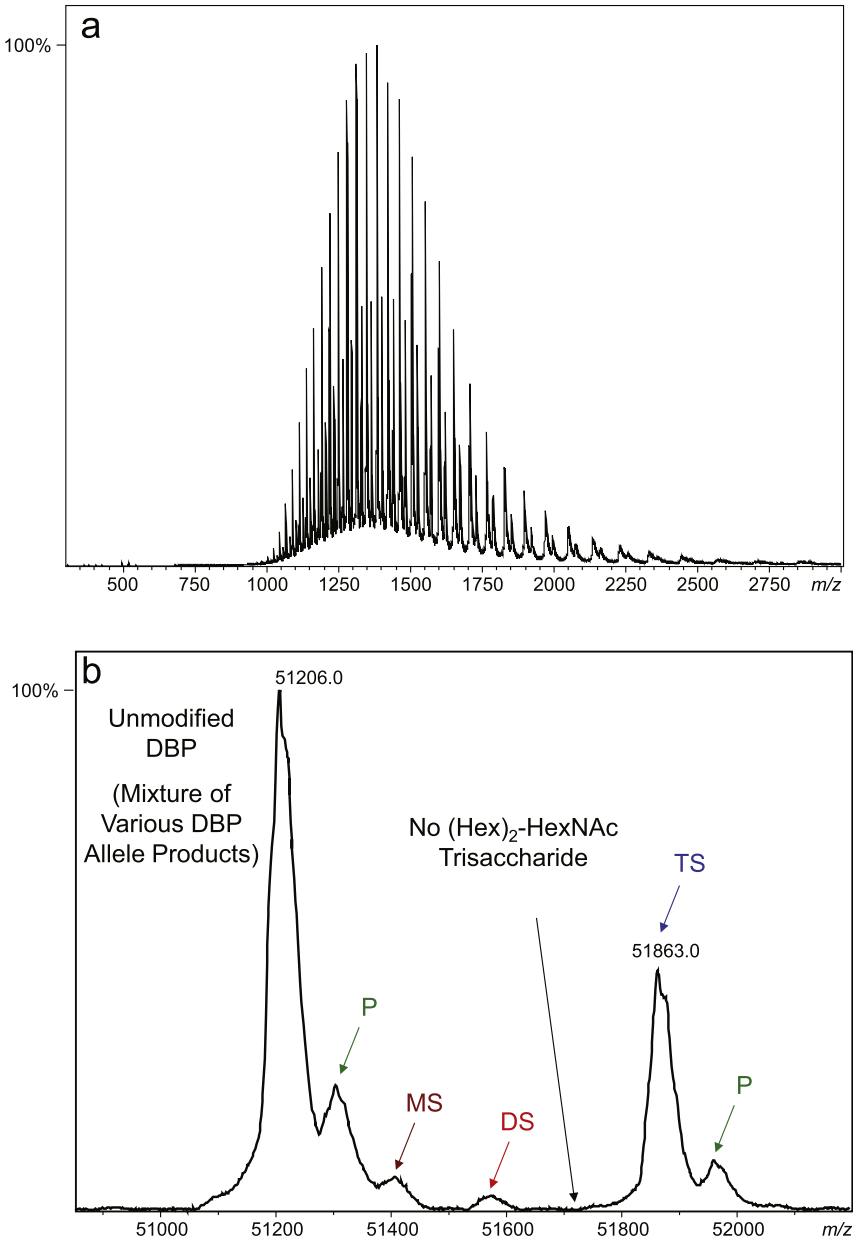


Fig. 3. Raw mass spectrum (a) and charge-deconvoluted ESI-mass spectrum (b) of DBP isolated from pooled human plasma. As expected, the asymmetrical peaks and mass values do not correspond exactly to those expected from individual DBP allele products [3] and reflect the pooled nature of the sample. Indeed, in addition to the Gc1F and Gc1S allele products, this preparation appears to contain DBP from a donor expressing a rare allele product that is heavier than the 1S protein but is also fully glycosylated [3]. Abbreviations for modified forms are supplied in Fig. 1. Calculated MH^+ masses for all forms are provided in Supplementary Material Table 1. These spectra indicate the purity of the protein preparation, that the protein preparation contains a variety of pooled DBP allele products (cf [3]), and the lack of evidence for a [dibranched], mannose-containing $(Hex)_2$-HexNAc-Thr trisaccharide.

limited quantity of material available, but only 4-linked glucose was detected along with a trace quantity of terminal galactose; no HexNAc residues were detected (data not shown).

3.3. Structural information revealed by glycosidase digestion

The discovery that the major glycan component of GcMAF is a Gal-GalNAc-Thr disaccharide and not the expected GalNAc-Thr monosaccharide prompted an investigation of what the various relevant glycosidic enzymes actually do to the DBP O-glycan. Using DBP from pooled human plasma (Fig. 3), over 30 serial glycosidic digestion experiments were conducted wherein the final intact protein was affinity-purified and detected directly by ESI-MS. The resulting data (Table 1) revealed a few interesting findings:

First, of the four β-galactosidase enzymes tested, only the enzyme from bovine testes was able to actually cleave the galactose residue (Table 1, Rows 3–6). Interestingly, putative GcMAF made using this particular enzyme (that actually cleaves the galactose residue) is reported to be no more active than negative controls [8,11,14]. Moreover, the sialic acid residue must be removed before β-galactosidase from bovine testes can cleave the galactose residue (Table 1, Rows 10–13). This supports a linear structure for the DBP trisaccharide and is in agreement with glycosidase digestion experiments previously reported by Ravnsborg et al. [8] as well as the linkage analysis results presented above. Notably, neither α-galactosidase nor α- or β-mannosidase are capable of cleaving the

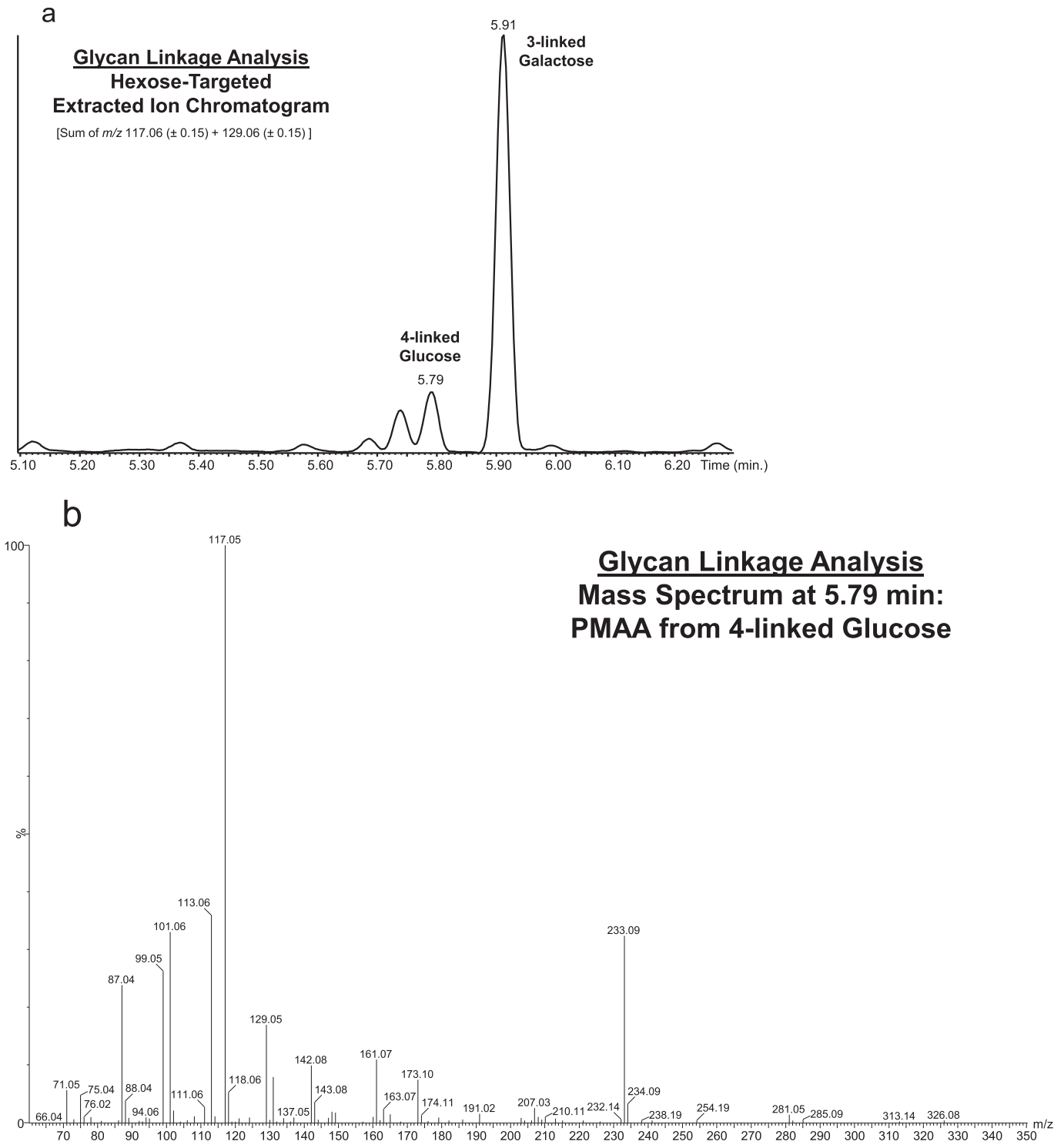
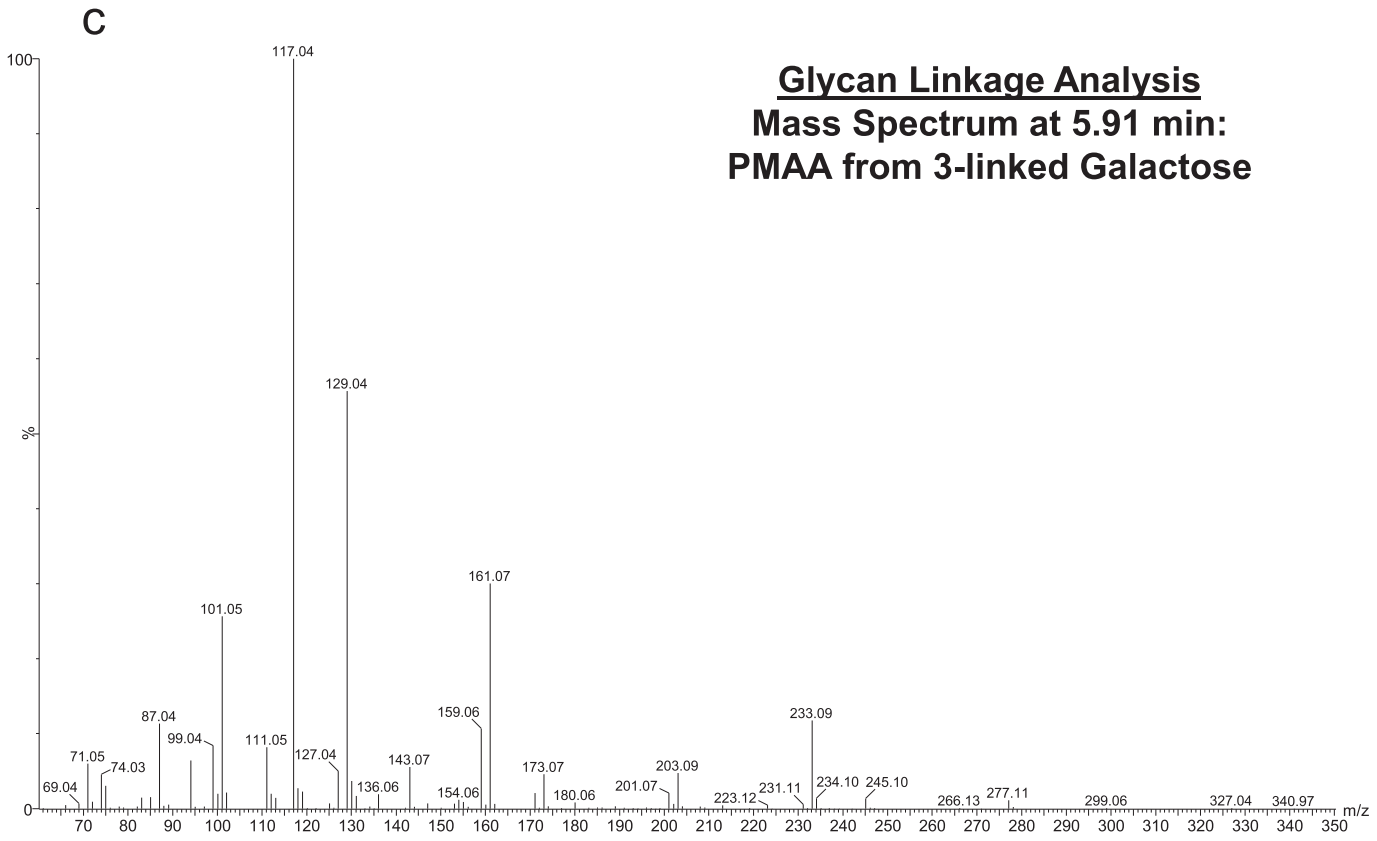


Fig. 4. Glycan linkage analysis of the DBP isolated from pooled human plasma (Fig. 3). Hexose-targeted extracted ion chromatogram (a) and EI mass spectra (b–c) of the hexose PMAAs. As explained in the main text, 4-linked glucose (b) is not observed in O-linked glycans and is most likely a contaminant from glycosphingolipids or cellulose; 3-linked galactose (c) was the only other hexose identified and corresponds to the expected hexose residue from a linear DBP O-glycan. HexNAc-targeted extracted ion chromatogram and EI mass spectrum of the HexNAc PMAA are shown in (d–e), respectively: 3-linked GalNAc was the only clearly identifiable HexNAc (and there was no evidence of a HexNAc residue that had been linked at two different positions in the original glycan polymer—though this assay is readily capable of detecting such HexNAc-derived PMAAs [36]). Reference EI mass spectra of the PMAAs shown here are provided in Supplementary Material Fig. 1.



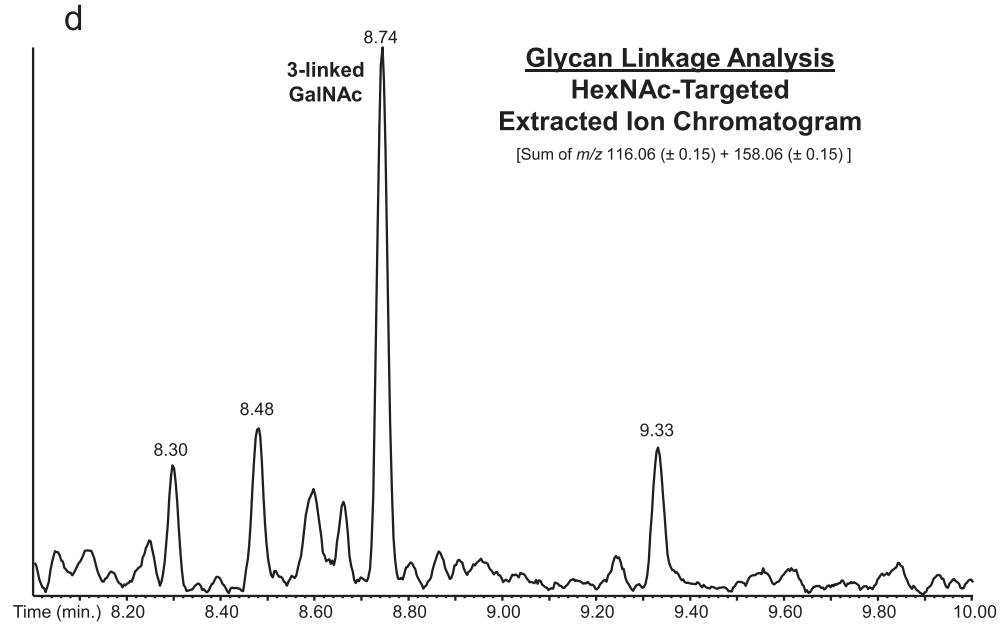


Fig. 4. (*continued*).

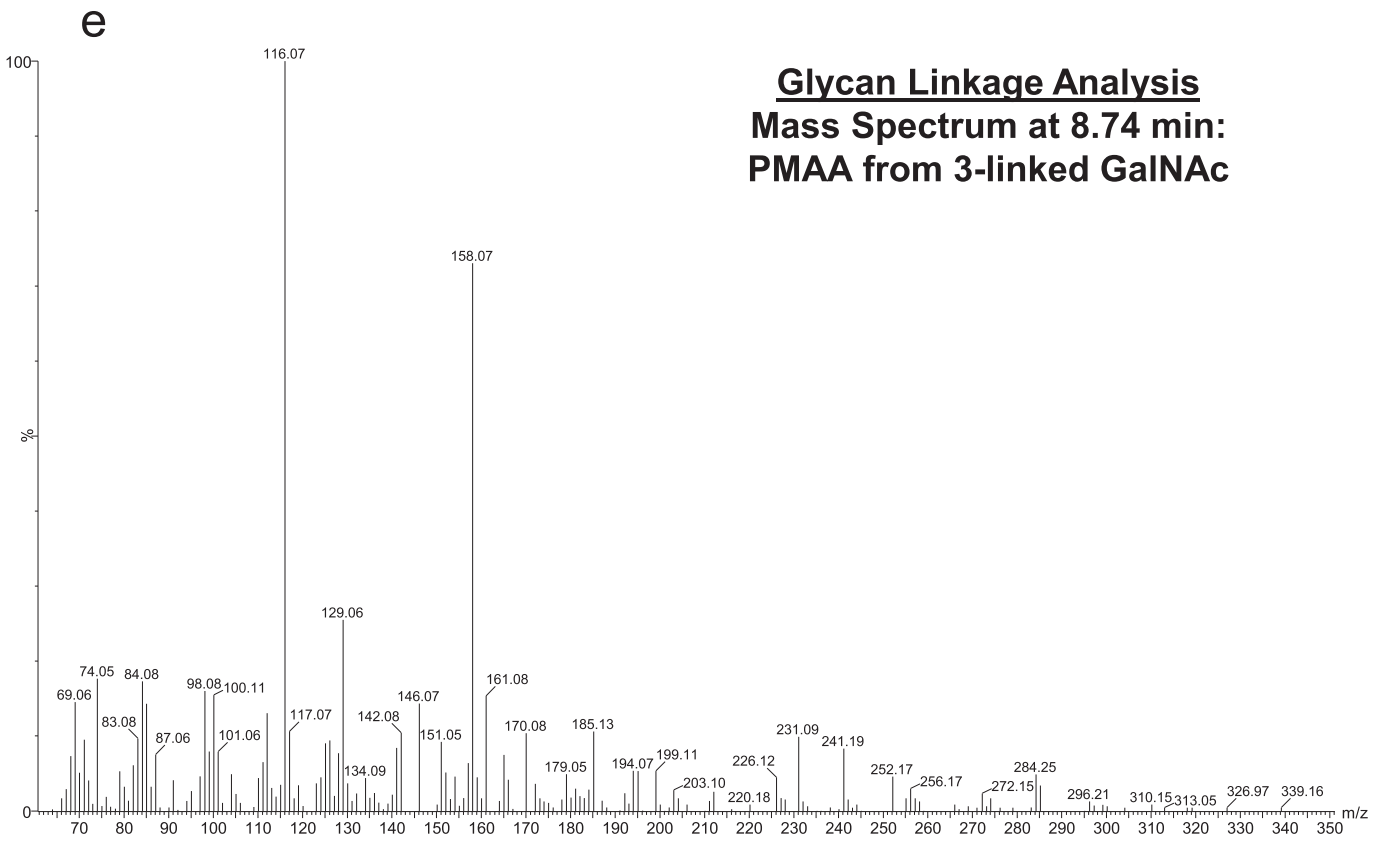


Fig. 4. (*continued*).

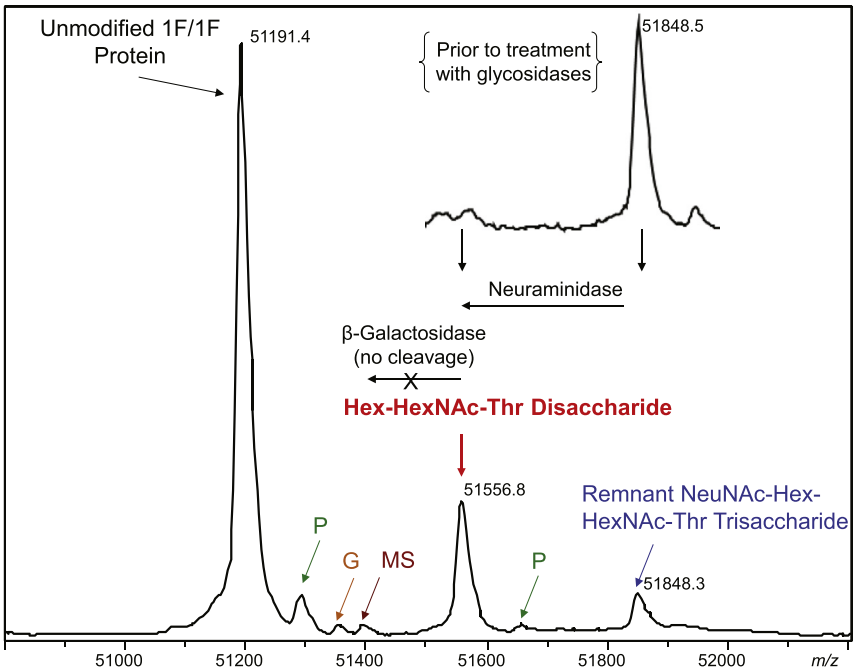


Fig. 5. Charge deconvoluted ESI mass spectrum of active GcMAF made from vitamin D affinity-purified DBP (Fig. 1) obtained from a plasma donor homozygous for the 1F allele product. Neuraminidase cleaved most of the NeuNAc, but β-galactosidase from *E. coli* did not cleave the galactose residue, even though the enzyme is required to make active GcMAF. No other changes occurred relative to a spectrum of the material prior to treatment with glycosidases (inset). Calculated MH^+ masses are provided in Supplementary Material Table 1.

galactose residue (Table 1, Rows 7–9).

Second, α-*N*-Acetylgalactosamindase (nagalase) lacks endoglycosidic function and is only able to cleave the DBP O-glycan once it has been trimmed down to a GalNAc-Thr monosaccharide (Table 1, Rows 25–26 and 19 & 24). This precludes the possibility of this enzyme removing the O-glycan trisaccharide from cancer-patient DBP *in vivo* as has long been claimed [12,17,25–27], but is in agreement with our previously published data on the glycosylation status of DBP in cancer patients [5,6]. Interestingly, however, regardless of whether it was applied to either the trisaccharide or disaccharide, nagalase did appear to impede the ability of O-glycanase to cleave the Gal-GalNac-Thr disaccharide (Table 1, Rows 16, 22–23) —a phenomenon that appeared to be partially counteracted by *E. coli* β-galactosidase (Table 1, Rows 32–35; additional description provided below), but was not due to inhibition of the O-glycanase enzyme. Lack of enzyme inhibition was verified by a lack of difference in the initial rate of absorbance of 4-nitrophenol (at 405 nm) released by O-glycanase acting upon 4-nitrophenyl-galacto-*N*-bioside in the presence or absence of nagalase.

The exact commercial β-galactosidase enzyme(s) employed to make GcMAF have rarely, if ever, been named in the literature. But given the commercial sources listed and the large quantities of enzyme used, it seems that the *E. coli* enzyme is the most likely source—unless otherwise specified—see, for example, Nagasawa et al. [11] and Ohkura et al. [14]. Moreover, the T/Tn antigen-based carcinoma vaccine, developed by Springer before the advent of GcMAF [44–49], was created from the sialylated T-antigen (a.k.a. core 1 O-glycan) using a combination of neuraminidase and *E. coli* β-galactosidase [44,50]. Given this information and the fact that *E. coli* β-galactosidase does not cleave the galactose residue from DBP (Table 1), we attempted to determine what the glycosidase might be doing to the core 1 O-glycan to "activate" it:

Since *E. coli* β-galactosidase is well known for its ability to convert lactose (Gal-β(1-4)-Glc) to allolactose (Gal-β(1-6)-Glc) [51–53] (which is the natural inducer of the lac operon), we examined the possibility that *E. coli* β-galactosidase might invert Gal-β(1-3)-GalNAc linkage anomercity from β-to α- (Table 1, Rows 14–15) or in some other way modify the ability of downstream enzymes to cleave galactose, GalNAc, and/or the Gal-GalNAc disaccharide (Table 1, Rows 15, 17–18, 20–21, 31–35). At most *E. coli* β-galactosidase appeared to have a modest ability to enhance cleavage of the DBP O-glycan by O-glycanase that had been exposed to nagalase (Table 1, Rows 22–23, 32–35), but no clear answers with regard to the precise activity of *E. coli* β-galactosidase on the DBP O-glycan were obtained.

3.4. What does "activating" β-galactosidase do?

Based on numerous literature reports from independent sources describing the activation of DBP with neuraminidase and β-galactosidase to generate GcMAF, β-galactosidase must do something to DBP to convert it to GcMAF. We have shown here that "activating" β-galactosidase does not cleave the galactose residue. So what could possibly explain the requirement of β-galactosidase for conversion of DBP to GcMAF? We pose two hypotheses:

One possibility is that activating β-galactosidase isomerizes the anomeric configuration or linkage pattern between galactose and GalNAc. As mentioned, we did not detect anomeric inversion in our glycosidase studies (Table 1, Rows 14–15). But as described above, linkage rearrangement of lactose (Gal-β(1-4)-Glc) to allolactose (Gal-β(1-6)-Glc) by *E. coli* β-galactosidase is a well-established phenomenon [51–53]. Perhaps an analogous process occurs on the DBP O-glycan—though to our knowledge such a reaction upon an existing glycan is without precedent.

A second possibility is that activating β-galactosidase and/or neuraminidase transfer immunoreactive lipids, glycolipid(s), and/or lipopolysaccharides to DBP (or at least the final resulting GcMAF preparation)—imparting its ability to activate macrophages. Evidence (presented above) of 4-linked glucose associated with DBP supports the hypothesis that glycolipid(s) may be associated with DBP—even after extensive purification of the protein. DBP is capable of binding large quantities of fatty acids [54] and is well known to associate with cell membranes *in vivo* [55–57]; moreover, there have been recent reports that its association with oleic acid dramatically increases its potency as a macrophage activating [58].

4. Conclusions

The primary O-glycan of GcMAF derived from the 1F allele product is a Gal-β(1-3)-GalNAc-Thr disaccharide and not a GalNAc-Thr monosaccharide as has long been believed based on inference from activity studies. This disaccharide is derived from the common sialylated core 1 O-glycan—a linear trisaccharide in which the sialic acid residue is attached to galactose at its 3-position. Indeed, based on glycosidase digestion studies followed by analysis of the intact protein by ESI-MS as well as glycan linkage analysis data presented here, both the Gc1F and Gc1S trisaccharides are linear, sialylated core 1 O-glycans (i.e., NeuNAc-(2-3)-Gal-(1-3)-GalNAc-Thr) and neither contains mannose. Moreover, α-*N*-Acetylgalactosamindase (nagalase) lacks endoglycosidase activity and is only capable of removing the DBP O-glycan GalNAc residue once the glycan has been trimmed down to just the single reducing-end GalNAc residue—a result that corroborates previous findings demonstrating that DBP is not deglycosylated in cancer patients.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2016.08.006.

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