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Synthesis and structural characterization of amino-functionalized polysaccharides

Research Article

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Abstract: A variety of carbohydrates, in particular polysaccharides can be subjected to chemical modification to obtain derivatives with amphiphilic properties, which enable biochemical or biological reactions at the polymer surface. In the present work, a polydisperse maltodextrin mixture of average molecular weight 3000 was coupled with 1,6-hexamethylenediamine (HMD) *via* reductive amination reaction. Resulting products were characterized by thermal analysis and positive nanoelectrospray quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Both thermal analysis and MS screening confirmed the formation of the HMD-polysaccharide coupling products. Moreover, HMD-linked polysaccharide chains containing 2 to 26 glucose building blocks were identified by nanoESI Q-TOF MS. MS/MS fragmentation using collision-induced dissociation (CID) at low ion acceleration energies provided strong evidence for HMD-maltodextrin linkage formation and the set of sequence ions diagnostic for the composition and structure of a HMD-linked chain containing 18 glucose residues.

Keywords: *Polysaccharides • Synthesis • Thermal analysis • Electrospray ionization • Quadrupole time-of-flight tandem mass spectrometry*

1. Introduction

Carbohydrates represent a particular class of biopolymers with high degree of structural complexity. They are polyhydroxylated aldehydic and ketonic compounds classified as monosaccharides, oligosaccharides and polysaccharides according to the size of the molecules, which is related to the number of monomeric units connected by glycosidic bonds. Carbohydrates are present as either oligosaccharides or glycoconjugates in which the oligosaccharide chain is covalently linked to an aglycon, frequently another biopolymer such as a protein and/or a lipid [1-3]. In nature, carbohydrates occur ubiquitously displayed on the surfaces of cell membranes or on the exposed regions of macromolecules and, as a result, they are suitable for storing biological signals in forms that are identifiable by other biological systems [4–6].

Polysaccharides form homopolymers and heteropolymers bearing a large number of monosaccharide units joined by glycosidic bonds. Various natural polysaccharides are readily available for chemical modification or as building blocks for the synthesis of hybrid materials [7-8]. In the past decade, chemical surface modification of nonpolar

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polymer matrices with hydrophilic carbohydrates has been pursued [9] in an effort to obtain block copolymers with amphiphilic properties, which enable biochemical or biological reactions at the polymer surface.

Block copolymers [10-11] are a special category of polymers that belong to a wider family known as soft materials. They can be considered as formed by two or more chemically homogeneous polymer blocks joined together by covalent bonds. This type of copolymers can be synthesized in many ways [12-14]; however, one of the most efficient methods is based on coupling of a functionalized hydrophobic block to a hydrophilic moiety. This procedure can be successfully applied for polyasaccharide modification at the reducing end, as described by us previously [15,16].

In the present study, a polydisperse maltodextrin mixture of average molecular weight 3000 was coupled to 1,6-hexamethylenediamine (HMD) at preparative scale. Attachment of diamine linker to the polysaccharide chain was achieved by reductive amination reaction [17]. Coupling products were characterized by thermal analysis and nanoelectrospray quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) in the positive ion mode.

2. Experimental Procedures

2.1 Materials

Methanol and formic acid (98%) were purchased from Merck (Darmstadt, Germany) and used without further purification. Distilled and deionized water from Milli-Q water system (Millipore, Bedford, MA, USA) was used for sample solution preparation. All sample solutions were dried in a SpeedVac Concentrator, SPD 111V-230 (Thermo Electron Corporation, Asheville, NC, USA) coupled to a vacuum pump, PC 2002 Vario with CVC 2000 Controller (Vaccubrand, Wertheim, Germany). Prior to chip-based MS analysis, sample solutions were centrifuged for 2 h in a SIGMA 2-16 model centrifuge (Sartorius GmbH, Göttingen, Germany).

NanoESI capillaries with external standard coating used in Q-TOF MS experiments were obtained from New Objective, Inc. (Woburn, MA, USA).

Maltodextrin and Paselli MD6 were obtained from AVEBE; 1,6-Diaminohexane (HMD), sodium cyanoborohydride, dimethylformamide (DMF) and acetic acid were purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and used without further purification. Dialysis membranes (MWCO=1000) were purchased from Spectrum Europe B.V. (Breda, The Netherlands) and the cationic exchange resin (Amberlite IR-120) was purchased from Sigma (Steinheim, Germany).

2.2 Samples

Preparation of HMD-linked maltodextrin. For the synthesis of HMD-linked maltodextrin, the procedure previously described by us was used [16]. Briefly, a 50 mL round–bottom flask equipped with teflon-coated magnetic stirrer and refrigerant was loaded with 1.44 g (12 mM) of HMD, 1.02 g (0.3 mM) of Dex3000, 0.35 mL of glacial AcOH and 10 mL of DMF. The mixture was stepwise heated for 3 hours up to 85°C. 0.6 g (8.3 mM) of sodium cyanoborohydride was added in small portions over 24 h. After cooling down the reaction mixture to room temperature, DMF was removed by vacuum distillation (temperature up to 45°C). Obtained product was dissolved in 50 mL water and the polysaccharide was precipitated with 200 mL of absolute ethanol. The precipitate was filtered and repeatedly washed with small portions of absolute ethanol and acetone. The obtained powder was dissolved in 50 mL of deionized water and passed through a strong cationic exchange column (2 cm i.d. × 30 cm length) packed with Amberlite IR-120. The unreacted polysaccharide (150 mg) was washed off with water and reused. The HMD linked polysaccharide was desorbed with 10% ammonia solution. The eluate was concentrated under vacuum pressure to 10 mL. This volume was dialyzed against water (dialysis membranes MW/CO=1000). The aqueous solution obtained after dialysis was concentrated by vacuum distillation to yield 910 mg of a light yellow powder of HMD-linked maltodextrin (Yield = 80% for maltodextrin).

2.3 Thermal analysis

Thermal decomposition was carried out using a TGA/SDTA 851-LF 1100 Mettler instrument. A 30 mg sample aliquot was placed in an alumina crucible of 150 µL. The experiments were conducted in static air atmosphere at a heating rate of 5°C min^{-1}, within 25 - 340°C temperature range. The air supplied by a compressor (4 - 5 bar) was passed over granular silica gel.

2.4 Mass Spectrometry

For MS analysis, the sample was dissolved to a concentration of about 5 pmol μL^{-1} (calculated for the average molecular weight) in MeOH/H_2O (1:1 v/v). In order to enhance the electrospray ionization process and taking into account the chemical structure of the modified maltodextrin, formic acid (98%) was added to the sample solution, in a proportion of 1/3 initial volume. MS was performed on a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Micromass/Waters Q-TOF Micro) with direct nanoelectrospray infusion in Micromass Z-spray

geometry. Q-TOF Micro mass spectrometer is interfaced to a PC running the MassLynx software to control the instrument, acquire and process the MS data. All mass spectra were recorded in the positive ion mode previously shown [15,16,18] to be advantageous for neutral oligosaccharide analysis. The ion source temperature was kept at 80^{0}C. For all experiments nitrogen at about 50 L h^{-1} flow rate was used as a desolvation gas.

MS/MS was performed by collision-induced dissociation (CID) at low energy using argon at a pressure of 15 psi as the collision gas. Collision energy was readjusted during an ongoing MS experiment to provide the full set of fragment ions fingerprint for the respective structure. The product ion spectrum was generated by combining across the total ion current (TIC) scans acquired at variable collision energies within 40-60 eV range. NanoESI Q-TOF MS system was programmed to record the signal at a scan speed of one scan per 2.1 s. MS/MS signal was acquired for about 15 min, which corresponds to 500 scans. Thus, the collision energy was varied with an average ramp of 1.5 eV min^{-1}. The LM and HM values on the Q-TOF MS for ion isolation were set for MS/MS experiments at 9 and 9 respectively. The m/z scale of the mass spectrum was calibrated by use of an external calibration standard G2421A electrospray ''tuning mix,'' from Agilent Technologies (Santa Rosa, CA, USA). The reference provided in the positive ion mode a spectrum with a fair ionic coverage of the m/z range scanned in both MS and CID MS/MS experiments. The mass accuracy obtained was within the normal range of a Q-TOF MS instrument.

The nomenclature of the fragment ions generally followed the rules established by Domon and Costello [19] considering glucose- hexamethylenediamine (GHD) moiety as an aglycon (Fig. 1).

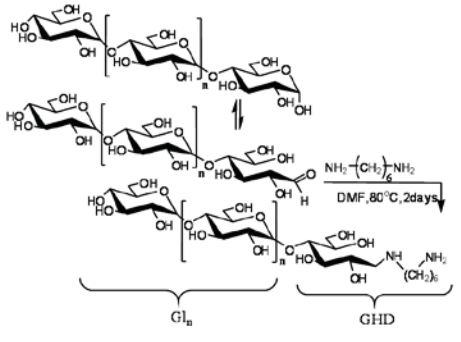

Figure 1. Synthesis of HMD-linked polysaccharides: reductive amination of maltodextrin with HMD and sodium cyanoborohydride as reducing agent

3. Results and discussion

The aim of this work was to develop a method for synthesis and purification that could be adopted specifically for production of maltodextrins with a modified reducing end. For this purpose, we employed reductive amination reaction in order to create a new C–N bond between a carbohydrate, which exists in solution either in a cyclic hemiacetal or in open-chain aldehyde form and a diamine residue (Fig. 1).

Previously, a series of fluorophores for enhancing UV detection such as 2-aminopyridine [20], p-aminobenzoic acid [21], 4-aminobenzonitrile [22], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [23], and 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) [24] were linked to oligosaccharides by reductive amination with a reaction yield varying from good to excellent. Here, we have modified the procedure of Williams *et al.* [25] usually used for short or branched oligosaccharides to correspond to long-chain polymeric carbohydrates. Large excesses of HMD were employed to achieve a high conversion of polysaccharides and avoid the formation of di-block polysaccharides as a result of double reductive amination on the same HMD molecule. HMD-maltodextrin compounds obtained were separated from uncoupled polysaccharides by anion exchange chromatography (AEC) and purified by dialysis against water. The result of the ninhydrin test of aqueous solution of purified HMD-linked polysaccharides was positive. Inspection by thin-layer chromatography (TLC) showed no presence of unreacted HMD. Modified maltodextrin (Gl$_n$GHD) was further subjected to thermal analysis. For comparison, separate thermal analyses of maltodextrin, HMD and their physical mixture were also performed. For these experiments the reacting maltodextrin and HMD components were introduced in the physical mixture at the molar ratio (1:1) they are present in the final product. The thermograms obtained are presented in Fig. 2 (a-d).

Inspection of Fig. 2 (a-d) reveals that the pattern of the three curves corresponding to differential thermal analysis (DTA), derivative thermal gravimetric analysis (DTG) and thermogravimetric analysis (TG) indicates significant differences between the physical mixture and HMD-maltodextrin reaction product.

DTG curve in Fig. 2d provides evidence for two endothermic and one exothermic (DTA curve) processes taking place within the 40 - 260 ^{o}C range. These three events are not observed in any of the Fig. 2a and 2c. For the reaction product, the massive mass loss by water elimination and oxidative processes starts at 190^{o}C (Fig. 2d) while in the case of maltodextrin (Fig. 2a) and the physical mixture (Fig. 2c) the mass loss

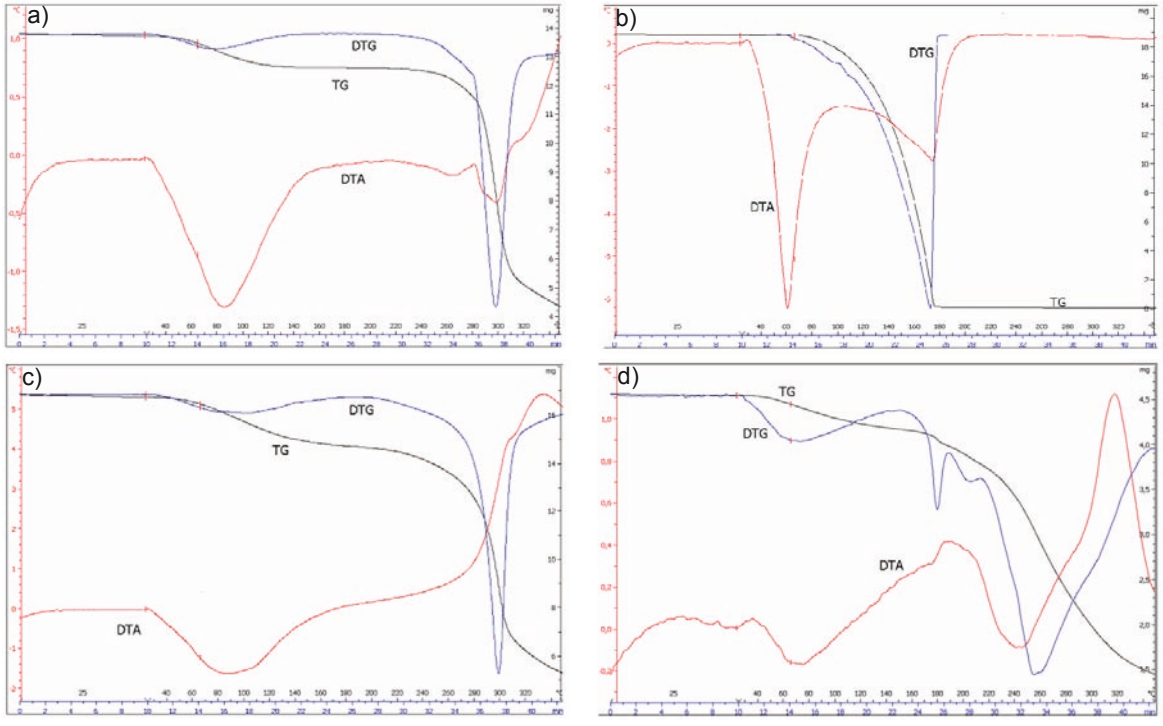

Figure 2. Thermal curves (DTA, DTG, TG) of a) maltodextrin; b) hexamethylene diamine (HMD); c) physical mixture of maltodextrin and HMD (1:1 molar ratio) and d) HMD-modified maltodextrin. Al crucible, static air atmosphere, 5°C min^{-1} heating rate

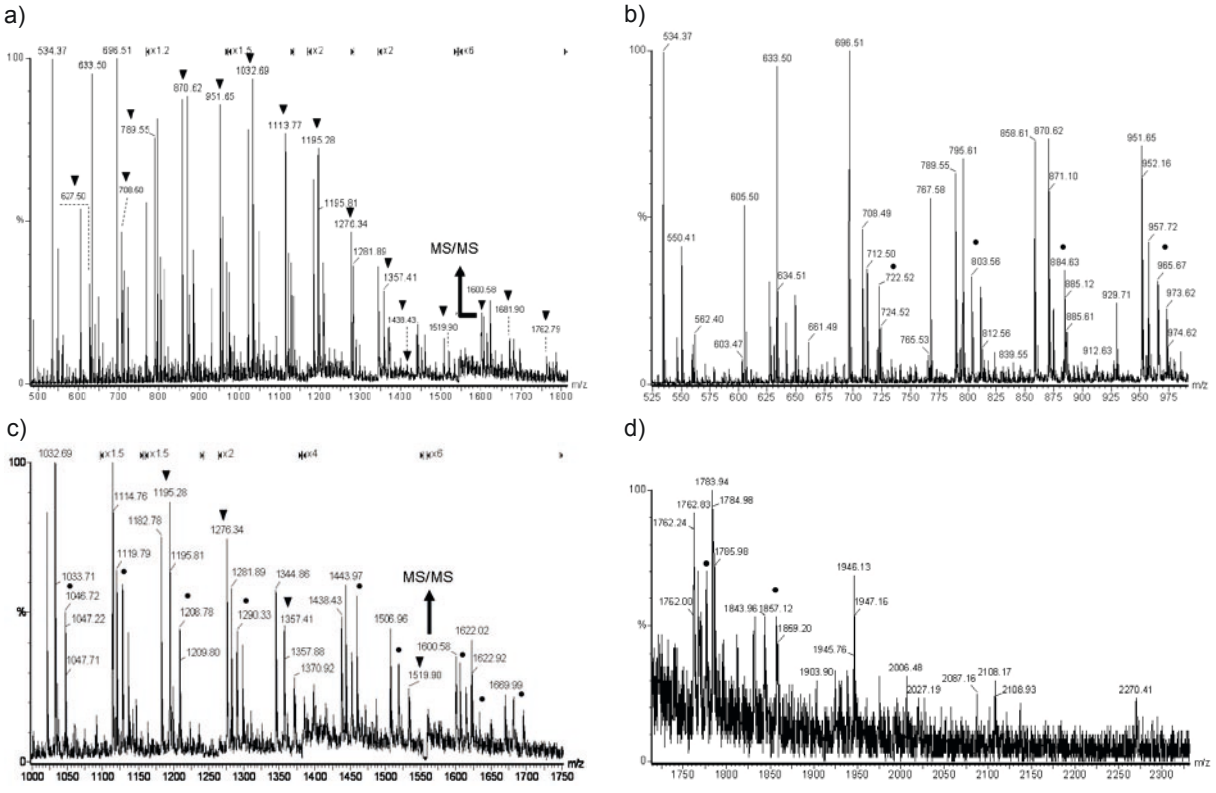

Figure 3. Positive nanoESI Q-TOF MS of HMD-modified maltodextrin mixture. a) m/z (500 - 1800); b) m/z (525 - 975); c) m/z (1000 - 1750); d) m/z (1725 - 2300). Capillary voltage: 1.2 kV. Cone voltage: 30 - 60 V. Acquisition: 850 scans. ▼-Gl_nGHD series; ●- products resulting from a side reaction of Glc_nGHD N-formylation

process is observed only around 250°C. This feature is attributable on one side to the fact that as compared to pure maltodextrin the packing of the functionalized polymer is looser and, on the other side, to the higher sensitivity of the aliphatic amine residue to oxidative thermal degradation.

To confirm HMD-maltodextrin linkage formation and identify obtained coupling products (+) nano ESI Q-TOF mass spectrometry was performed. 10 µL of sample solution in MeOH/H_2O/HCOOH were loaded into the coated nanoESI capillary. To generate optimal ionization of each mixture component the electrospray capillary voltage was set to 1.2 kV while the cone potential was varied within 30-60 V range. The signal was acquired for about 30 min, thus about 850 scans were accumulated. This is equivalent to approximately 26.66 pmols of material used in the MS screening experiment. The spectrum summed over the 850 scans acquired under variable cone voltage is depicted in Fig. 3a. To ensure the visibility of all components detected, Fig. 3 b-d present detailed views obtained by zooming out the areas within the m/z range: 525 - 975 (Fig. 3b); 1000 - 1750 (Fig. 3c); 1725 - 2300 (Fig. 3d). The assignment of major components detected by nanoESI QTOF MS screening is presented in Table 1.

Fig. 3 a-d reveals that the spectrum is dominated by ions belonging to the major series of modified maltodextrin (Gl_nGHD). All ions labeled in Fig. 3 a-d with their afferent m/z values and assigned in Table 1 to their structure are doubly charged Gl_nGHD molecules without adducts. The spectrum indicates a mass dispersion centered on the molecular weight range 1580 – 3680 Da and a degree of polymerization (DP) ranging from 2 to 26.

The m/z interval within this series is 81, equivalent to one-half the mass of the glucose repeat unit. Interestingly, as visible in Fig. 3, the major Gl_nGHD series (marked by ▼) is accompanied by three additional ion groups, all connected to the major series and covering the same m/z range as the Gl_nGHD. The first two groups form an envelope of doubly charged ions shifted by either m/z 14 (28 Da) or 28 (56 Da) that are attributable to compounds resulting from a side reaction (N-formylation) of Glc_nGHD product with dimethylformamide (DMF) during the synthesis (side reaction, Fig. 4).

Most likely, this process arises by addition of a formyl group at the terminal amino sequence, which induces the effective mass increase of 28 Da (14 m/z shift, marked by ● in Fig. 3 b-d). Molecular composition derived from the accurate mass measurement is consistent with the theoretical composition of the N-formyl side products.

The third accompanying series consists of a few low intensity singly charged ions corresponding to di-N-formyl derivative (Fig. 4, Table 1).

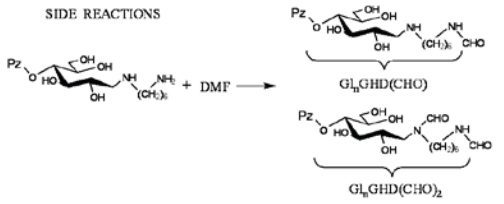

Figure 4. Side reactions in the synthesis of Gl_nGHD by reductive amination

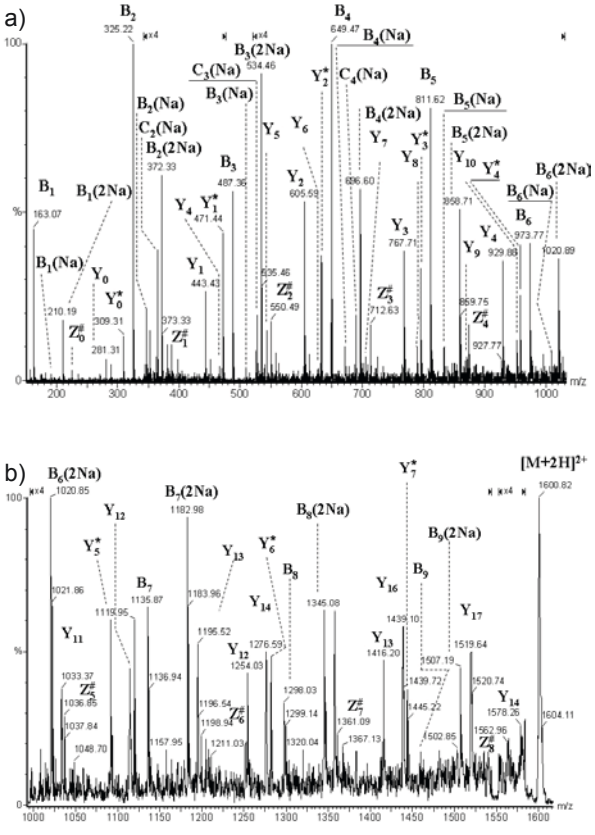

Figure 5. Positive nanoESI Q-TOF MS/MS of the doubly charged ion at m/z 1600.82 detected in HMD-modified maltodextrin sample. a) m/z (150 - 1020); b) m/z (1000 - 1600). Capillary voltage: 1.2 kV. Cone voltage: 50 V. CID at variable collision energy (E_{lab}) within 40-70 eV. Argon pressure (P_{lab}): 10-15 p.s.i. ($Z^{\#}$=Z-2H_2O; Y*=Y+2Na-H_2O). Assignment of fragment ions is according to the nomenclature introduced by Domon and Costello [19]

In order to provide hard evidence for the linkage formation between the HMD linker and the polysaccharide chains, the doubly protonated ion at m/z 1600.82 assigned, according to the mass calculation, to Gl_{18}GHD was submitted to detailed structural investigation by fragmentation in CID MS/MS experiment. The product ion spectrum obtained after 15 min. which corresponds to about 430 scans signal accumulation in the variable collision energy regime developed by us previously [26-30] is depicted in Fig. 5 a, b.

Table 1. Assignment of the major pseudomolecular ions detected in HMD-modified maltodextrin sample

Peak nr.	m/z	charge	Proposed structure	Peak nr.	m/z	charge	Proposed structure
1	534.37	+1	Gl_3(2Na)	52	1182.78	+1	Gl_7(2Na)
2	550.41	+1	Gl_2GHD -3H_2O	53	1195.28	+2	Gl_{13} GHD
3	560.42	+2	Gl_5GHD(CHO)	54	1198.78	+2	Gl_{15}-2H_2O
4	574.43	+2	Gl_5GHD$(CHO)_2$	55	1208.78	+2	Gl_{13} GHD(CHO)
5	605.50	+1	Gl_2 GHD	56	1217.63	+2	Gl_9 GHD(2Na)
6	627.44	+2	Gl_6 GHD	57	1222.81	+2	Gl_{13}GHD$(CHO)_2$
7	631.49	+2	Gl_8-$_2H_2O$	58	1253.78	+1	Gl_6 GHD
8	633.50	+1	Gl_2 GHD(CHO)	59	1276.34	+2	Gl_{14} GHD
9	641.46	+2	Gl_6 GHD (CHO)	60	1279.83	+2	Gl_{16}-2H_2O
10	650.01	+2	Gl_6 GHD(2Na)	61	1281.89	+1	Gl_6 GHD(CHO)
11	655.46	+2	Gl_6GHD(CHO)$_2$	62	1290.33	+2	Gl_{14} GHD(CHO)
12	696.51	+1	Gl_4($_2$Na)	63	1297.82	+1	Gl_8
13	708.49	+2	Gl_7 GHD	64	1302.79	+2	Gl_{14}GHD$(CHO)_2$
14	712.50	+2	Gl_9-2H_2O	65	1344.86	+1	Gl_8(2Na)
15	722.52	+2	Gl_7 GHD(CHO)	66	1357.88	+2	Gl_{15} GHD
16	730.04	2+	Gl_7 GHD(2Na)	67	1360.92	+2	Gl_{17}-2H_2O
17	736.51	+2	Gl_7GHD(CHO)$_2$	68	1370.92	+2	Gl_{15} GHD(CHO)
18	767.58	+1	Gl_3 GHD	69	1384.94	+2	Gl_{15}GHD$(CHO)_2$
19	789.55	+2	Gl_8 GHD	70	1415.41	+1	Gl_7 GHD
20	793.06	+2	Gl_{10}-2H_2O	71	1438.43	+2	Gl_{16} GHD
21	795.61	+1	Gl_3HMD(CHO)	72	1441.37	+2	Gl_{18}-2H_2O
22	803.56	+2	Gl_8 GHD(CHO)	73	1443.97	+1	Gl_7 GHD(CHO)
23	812.09	+2	Gl_7GHD(2Na)	74	1451.93	+2	Gl_{16} GHD(CHO)
24	817.56	+2	Gl_8GHD$(CHO)_2$	75	1459.84	+1	Gl_9
25	858.61	+1	Gl_5(2Na)	76	1465.87	+2	Gl_{16}GHD$(CHO)_2$
26	870.62	+2	Gl_9 GHD	77	1506.96	+1	Gl_9(2Na)
27	874.61	+2	Gl_{11}-2H_2O	78	1519.00	+2	Gl_{17} GHD
28	884.63	+2	Gl_9 GHD(CHO)	79	1533.01	+2	Gl_{17} GHD(CHO)
29	892.59	+2	Gl_7 GHD(2Na)	80	1600.58	+2	Gl_{18} GHD
30	898.61	+2	Gl_9GHD$(CHO)_2$	81	1605.34	+1	Gl_8 GHD(CHO)
31	912.24	+3	Gl_{15} GHD(Na)	82	1614.02	+2	Gl_{18} GHD(CHO)
32	929.71	+1	Gl_4 GHD	83	1622.02	+1	Gl_{10}
33	951.65	+2	Gl_{10}HMD	84	1669.99	+1	Gl_{10}(2Na)
34	955.68	+2	Gl_{12}-2H_2O	85	1684.78	+2	Gl_{21}-2H_2O
35	957.72	+1	Gl_4 GHD(CHO)	86	1695.23	+2	Gl_{19} GHD(CHO)
36	965.67	+2	Gl_{10} GHD(CHO)	87	1739.11	+1	Gl_9 GHD
37	973.62	+2	Gl_8 GHD(2Na)	88	1762.83	+2	Gl_{20} GHD
38	979.69	+2	Gl_{10}GHD$(CHO)_2$	89	1767.98	+1	Gl_9 GHD(CHO)
39	1020.70	+1	Gl_6(2Na)	90	1775.50	+2	Gl_{20} GHD(CHO)
40	1032.69	+2	Gl_{11} GHD	91	1783.94	+1	Gl_{11}
41	1036.70	+2	Gl_{13}-2H_2O	92	1830.00	+1	Gl_{11}(2Na)
42	1046.72	+2	Gl_{11} GHD(CHO)	93	1843.96	+2	Gl_{21} GHD
43	1054.20	+2	Gl_9 GHD(2Na)	94	1846.19	+1	Gl_{10} GHD -3H_2O
44	1060.70	+2	Gl_{11}GHD$(CHO)_2$	95	1857.12	+2	Gl_{21} GHD(CHO)
45	1091.78	+1	Gl_5 GHD	96	1929.19	+1	Gl_{10} GHD(CHO)
46	1114.76	+2	Gl_{12} GHD	97	1937.78	+2	Gl_{22} GHD(CHO)
47	1117.23	+2	Gl_{14}-2H_2O	98	1946.13	+1	Gl_{12}
48	1119.79	+1	Gl_5 GHD(CHO)	99	2004.16	+2	Gl_{23} GHD
49	1127.76	+2	Gl_{12} GHD(CHO)	100	2085.01	+2	Gl_{24} GHD
50	1135.71	+1	Gl_7	101	2108.17	+2	Glc_{26}
51	1141.75	+2	Gl_{12}GHD$(CHO)_2$	102	2269.96	+2	Gl_{26} GHD(2Na)

Inspection of spectrum in Fig. 5 indicates that optimized sequencing conditions yielded a high sequence coverage documented in particular by the complete set of Y-type ions detected as either high intensity mono-protonated (Y_0 to Y_{14}), monocharged disodiated monodehydrated (Y_0* to Y_7*) or doubly-protonated (Y_4 to Y_{17}) signals. The spectrum exhibits also peaks corresponding to B-type of fragment ions such as the monoprotonated and monoprotonated disodiated B_1-B_9 series, three monosodiated C-type of fragment ions (C_2, C_3, C_4) and monocharged doubly dehydrated Z-type ions of low intensity ($Z_0^{\#}$ to $Z_8^{\#}$). Fragmentation pathway obtained by CID MS/MS and the generated sequence ions corroborate Gl_{18}GHD structure of the precursor ion and implicitly HMD-polysaccharide linkage.

4. Conclusions

We developed and have presented here a reliable protocol for obtaining modified linear maltodextrins with an amino linker attached at the reducing end followed by thermal analysis at preparative scale and ESI Q-TOF MS/MS characterization of the coupling products. DTA, DTG and TG data substantiated the linkage formation between the amino group and the polysaccharide. Subsequently, elucidation of HMD-polysaccharide mixture composition and individual component structure was accomplished by positive ion mode nanoESI Q-TOF MS and tandem MS by CID at low energies. Accurate molecular weight measurement achieved by nanoESI MS screening indicated the formation of amino-derivatized maltodextrin species exhibiting degrees of polymerization ranging from 2 to 26. In the last stage of MS analysis, optimized sequencing conditions induced an efficient fragmentation of $[M+2H]^{2+}$ ion corresponding to Gl_{18}GHD component. In the fragmentation spectrum we were able to identify the set of sequence ions, which are diagnostic for the structure of a linear HMD-linked chain containing 18 Glc residues and support the concept of Gl-HMD bond formation.

This study has particular significance due to the foreseen applications of the reaction products and method developed. HMD-linked polysaccharides can be used for production of di-block copolymers and for functionalization of peptides and proteins in order to increase their degree of solubilization. Moreover, polysaccharides modified at their reducing end by a diamine linker exhibit higher ionization efficiency in positive ion electrospray. For this reason, the method described here can also be regarded as a novel derivatization procedure for long carbohydrate chains, which do not possess readily ionizable groups and can not be investigated by electrospray mass spectrometry in their native form. Although this protocol is very efficient particularly when the reaction is performed in an organic solvent, further studies to develop a method using pure water will be undertaken.

Acknowledgements

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