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RESEARCH ARTICLE

UP-HILIC-MS/MS to Determine the Action Pattern of *Penicillium sp*. Dextranase

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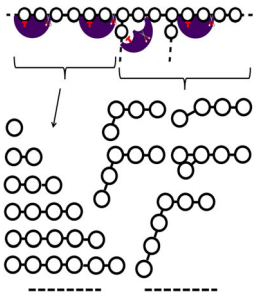

Abstract. Investigation of the action pattern of enzymes acting on carbohydrates is challenging, as both the substrate and the digestion products are complex mixtures. Dextran and its enzyme-derived oligosaccharides are widely used for many industrial applications. In this work, a new method relying on ultra-performance hydrophilic interaction liquid chromatography quadrupole time-of-flight tandem mass spectrometry (UP-HILIC- Q/TOF-MS/MS) was developed to analyze a complex mixture of dextran oligosaccharide products to determine the action pattern of dextranase. No derivatization of oligosaccharides was required and the impact of the α- and β-configurations of the native oligosaccharides on the chromatographic separation was eliminated. The 1→6, 1→3, 1→4 backbone linkages and the branch linkages of these oligosaccharides were all distinguished from diagnostic ions in their MS/MS spectra, including fragments corresponding to ^{0,2}A, ^{0,3}A, ^{0,4}A, B-H_2O, ^{2,5}A, and ^{3,5}A. The sequences of the oligosaccharide products were similarly established. Thus, the complex oligosaccharide mixtures in dextran digestion products were profiled and identified using this method. The more enzyme-resistant structures in dextran were established using much less sample, labor, time, and uncertainty than in previous studies. This method provides an efficient, sensitive, and straightforward way to monitor the entire process of digestion, establish the action pattern of the dextranase from *Penicillium sp*., and to support the proper industrial application of dextranase.

Keywords: HILIC, MS/MS, Dextran, Oligosaccharide, Sequence, Dextranase, Action pattern

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Introduction

Carbohydrate enzymes have been used widely to modify the physical/chemical properties of polysaccharides to meet different industrial needs [1–4]. They have also been used as tools to prepare oligosaccharide standards, and study the structures and sequences of complex carbohydrates [5–7]. Dextranase is a representative carbohydrate enzyme used to digest dextran for different purposes. Dextran is a complex, branched α-glucan exopolysaccharide usually produced by lactic acid bacteria [3, 8–10]. It is widely used as vessel volume expander, and iron carrier [11–14]. The average molecular weight (Mw) of a dextran typically decides the applications for which it is most useful [15, 16]. Dextrans of different Mw are often prepared through dextranase digestion [17, 18]. In

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addition, dextrans of different Mw and different dextran oligosaccharides often play important roles in carbohydrate analysis. They are usually used as standards and model molecules in developing methods, including size exclusion chromatography (SEC), whicih are useful in determining polysaccharide molecular weight [19, 20] and, following pre-column derivatization, for liquid chromatography- mass spectrometry (LC-MS) profiling the *N*- and *O*-glycans obtained from glycoproteins [21–23]. Dextranase is used as a tool to prepare these oligosaccharides and, thus, understanding the action pattern of dextranase is critically important.

The characterization of digestion products as a function of digestion time is the major way in which the action patterns of the carbohydrate enzymes are characterized. Two strategies were often used in carbohydrate enzyme action pattern studies. In the first approach, digested oligosaccharides can be purified in relatively large scale, and the structure or sequence of each purified oligosaccharide can be established using nuclear magnetic resonance (NMR) spectroscopy [24, 25]. In 2011, Maina et al. [24] established the action pattern of a dextranase using this strategy. In this study, oligosaccharides in the final product were separated by SEC and various two-dimensional NMR experiments were applied to elucidate the structures of these oligosaccharides. In a second approach, the digested oligosaccharides could be derivatized at their reducing ends with fluorescent reagents to improve separation and detection sensitivity, and to remove the confounding effect of α- and β-configurations for each oligosaccharide. Based on the retention times under different chromatographic conditions, these oligosaccharides could be identified and characterized [26].

However, both of these strategies exhibit clear disadvantages. Preparing an oligosaccharide with single structure from complex mixture is often difficult. For example, Maina et al. had to interpret NMR data on an oligosaccharide fraction composed of two oligosaccharide structures [24]. Since the NMR spectra of even pure carbohydrates are generally complicated, the analysis of an oligosaccharide mixture is even more challenging. Furthermore, the preparation of oligosaccharide fractions and the acquisition and interpretation of complicated NMR spectra is time-consuming and labor-intensive. Procedures involving carbohydrate derivatization can introduce potential impurities, result in the loss of structural information, and decrease the accuracy of analysis [27–29].

In the current study, an ultra-performance hydrophilic interaction liquid chromatography quadrapole time-of-flight tandem mass spectrometry (UP-HILIC- Q/TOF-MS/MS) method was developed to analyze digestion products produced from dextran through the action of dextranase from *Penicillium sp*. Optimized UP-HILIC afforded an effective separation of digested oligosaccharides based on both size and linkage positions. In addition, the presence of α- and β-configurations at the oligosaccharide's reducing end had no adverse impact on the quality of the separation and, hence, no pre-column derivatization was required. MS/MS was applied to sequence each oligosaccharide product, including those having the same degree of polymerization (dp) but different linkage positions. The action pattern of this enzyme was established by analyzing the separation and sequencing information obtained. This method was effective, sensitive, convenient, and straightforward for the analysis of dextranase action pattern.

Experimental

Materials

Dextran T-10 was provided by Hua Mao Corp. (Shanghai, China). It is a primarily 1-6 linked glucan (Supplementary Data). Dextranase from *Penicillium sp*. was purchased from Sigma-Aldrich (St. Louis, MO, USA). Maltotriose, maltotetrose, maltopentaose, and maltohexaose were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Acetonitrile and thin layer chromatography (TLC) plates (HPTLC silica gel 60, aluminium sheets 20 × 20 cm) were purchased from Merck (Darmstadt, Germany). High-purity water (resistivity ≥18.2 MΩ × cm, 25°C) was used throughout the study. All other chemicals and reagents were of HPLC grade.

Enzymatic Digestion

Dextran T-10 (6 mg) was dissolved in 0.2 mL water and incubated with 0.15 U dextranase at 37°C. Aliquots were taken at 0, 1, 2, 3, 6, 9, and 12 h. Another 0.1 U of dextranase was added to the reaction and incubated for an additional 12 h for a total of 24 h. The digestions were stopped by boiling each aliquot for 20 min. The denatured enzyme was removed by centrifugation (15000 rpm) for 10 min. The digestion degree of each aliquot was monitored with TLC (for details see Supplementary Data) [30].

UP-HILIC

The UP-HILIC method was developed on an Agilent system equipped with an UHPLC (1290, dual pumps) and an ESI-Q/TOF-MS (6540; Agilent Technologies). Data were required with MassHunter 6.0 (Agilent Technologies). Chromatograms were obtained on an ACQUITY UPLC BEH Glycan column (2.1 × 150 mm, 1.7 µm; Waters, Milford, MA, USA) at 20°C. This column has a stable operating range from pH 2 to 11; caution is urged before substituting other silica-based columns. Mobile phase A was water:acetonitrile (5:95, v/v) solution, and mobile phase B was aqueous solution. Both mobile phases contained 10 mM ammonium acetate. A linear gradient with 85%–25% mobile phase B over 15 min was used at a flow rate of 0.2 mL/min. The pH of mobile phase A was considered as a critical factor to optimize in this work. Three different pH values were tested, including pH ~7.5 (without any adjustment after preparation), pH ~5.0 (adjusted with aqueous acetic acid), and pH ~10.0 (adjusted with aqueous ammonia).

MS and MS/MS

Nitrogen gas was used in the nebulizer at a pressure of 30 psi. The spray voltage was 3.5 kV and a flow of nitrogen gas of 8 L/min at 350 C assisted in the drying process. Fragment voltage was set to 120 V. A full MS scan between 100 and 2000*m/z* was performed. The collision-induced dissociation (CID) energy used in MS/MS to dissociate oligosaccharides was set as 30 V. All data were acquired in the negative mode [31, 32]. The resolution of MS and MS/MS is 40,000 Da; and the accuracy of MS and MS/MS are 1 ppm and 4 ppm.

Results and Discussion

TLC Analysis

The extent of digestion was monitored using TLC (Supplementary Figure S1). The oligosaccharides observed between the solvent front and origins were from dp 1 (monosaccharide) to about dp10 (decasaccharide). According to the TLC results, the polysaccharide was digested into large oligosaccharides within 1 h. These large oligosaccharides were digested to smaller oligosaccharides in 3 h, after which dp 2 and dp 3 became the major products. Interestingly, dp 3 started to be digested after 6 h and only a very light band, corresponding to dp 3, remained after a 9 h digestion, whereas dark bands, corresponding to dp 4 and dp 5, were observed in aliquot digested for 9 h and they became even darker in aliquots digested for 12 h and longer. These results suggest that some dp 4 and dp 5 domains are resistant to the digestion and accumulated in the final digestion product. A dark spot, corresponding to enzyme, was observed at the origin.

UP-HILIC Optimization

The chromatographic conditions were optimized by examining the final products (Figure 1). The mass-to-charge (*m/z*) of molecular ions for different oligosaccharides (*m/z* 179 for dp 1, *m/z* 341 for dp 2, *m/z* 503 for dp 3, *m/z* 665 for dp 4, *m/z* 827 for dp 5, *m/z* 989 for dp 6, *m/z* 1151 for dp 7, and *m/z* 1313 for dp 8) were extracted from the total ion chromatography (TIC). The extracted ion chromatography (EIC) was used throughout this study. Split peaks corresponding to mono-, di-, tetra-, and pentasaccharides were observed when mobile phase A was adjusted to pH ~5.0 (Figure 1a). It is believed that α- and ß-configurations contributed to the split peaks of these glycans. The peak splitting reduced as the peaks were eluted faster, when the mobile phase of pH ~7.5 was used (Figure 1b). The peak splitting could be eliminated at pH ~10.0, suggesting that the impact of α- and ß-configurations on chromatography could be eliminated in a basic environment (Figure 1c). Moreover, under basic conditions, all peaks eluted faster. Under these conditions, only one peak corresponding to a disaccharide was observed, implying that only one kind of disaccharide was dominant, isomaltose, in the digestion mixture. Multiple peaks corresponding to dp 4, observed in acidic and neutral conditions, collapsed to two peaks, suggesting that two different enzyme-resistant tetrasaccharides were present in the final product mixture. Two different enzyme-resistant pentasaccharides were also observed in the final product. In addition, additional oligosaccharides were observed using this method

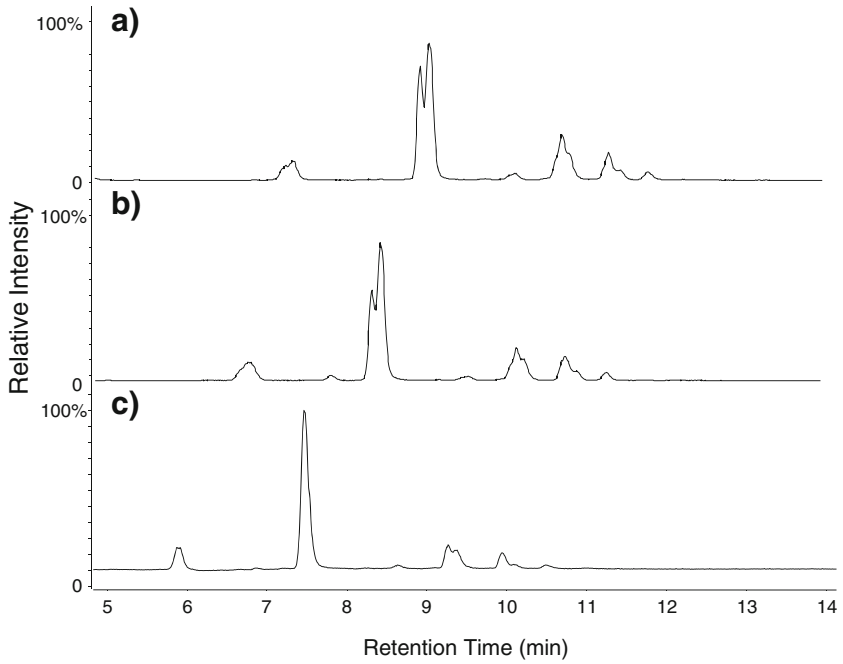

Figure 1. Optimization of chromatography with different pH values. **(a)** The EIC of final digestion product with pH ~5.0 mobile phase A; **(b)** the EIC of final digestion product with pH ~7.5 mobile phase A; and **(c)** the EIC of final digestion product with pH ~10.0 mobile phase A

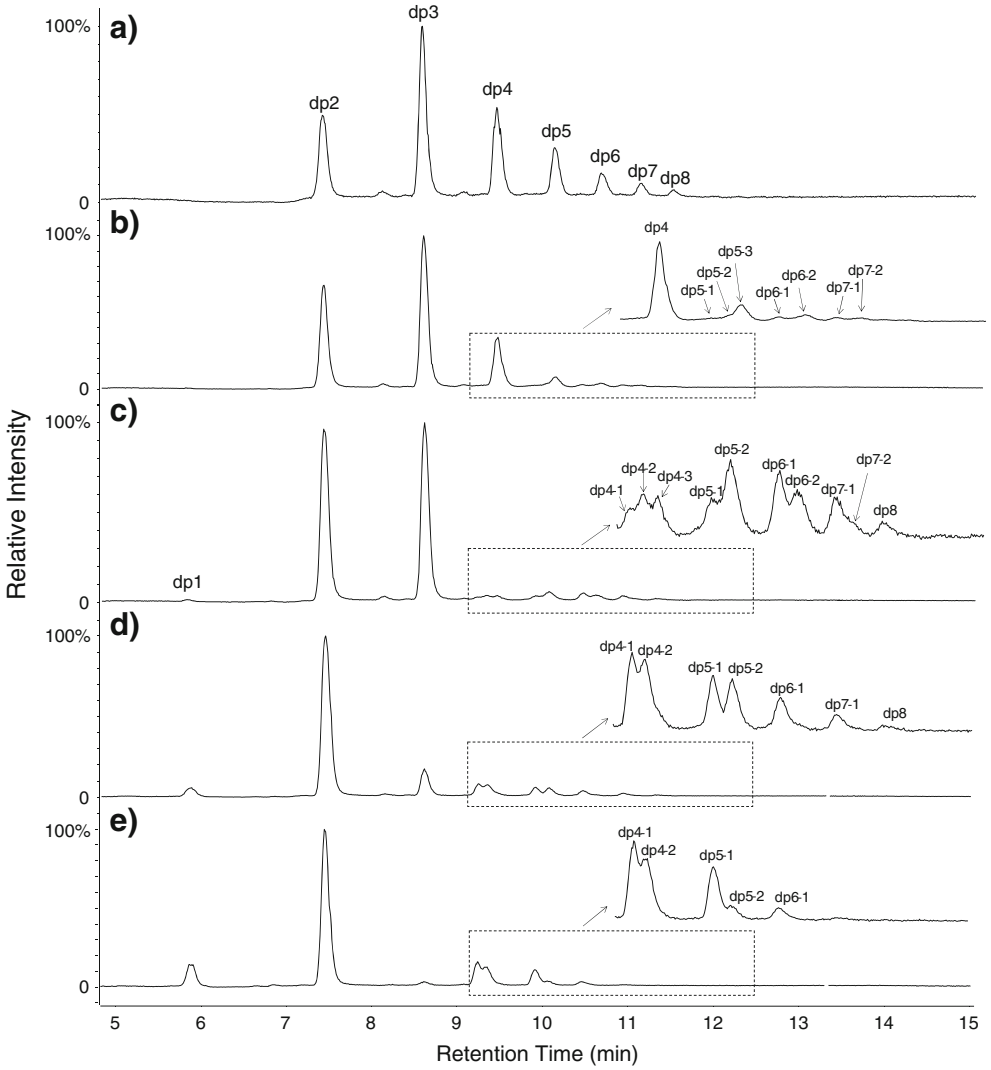

Figure 2. EICs of digestion products under different reaction time. **(a)** The EIC of 1 h digestion product; **(b)** the EIC of 3 h digestion product; **(c)** the EIC of 6 h digestion product; and **(d)** the EIC of 9 h digestion product; **(e)** the EIC of 12 h digestion product

in intermediate digestion mixtures (Figure 2). Thus, pH 10 was selected as the optimal pH of mobile phase A in this method. These results suggested that an efficient method to analyze different types of dextran oligosaccharides, without required derivatization, had been developed.

Analysis of the Aliquots with Developed UP-HILIC Method Followed by ESI-Q/TOF-MS

The developed method was applied to confirm the results of TLC and to provide additional sequencing information. Aliquots taken at 1, 3, 6, 9, and 12 h were analyzed using this method and their EIC are shown in Figure 2. Digestion for 1 h showed seven single peaks corresponding to dp 2 through dp 8 (Figure 2a). Three penta-, two hexa-, and two heptasaccharides were present in the 3 h digestion mixture (Figure 2b and its insert). Based on retention times, dp 5-3, dp 6-2, dp 7-2 were the penta-, hexa-, and heptasaccharides present in 1 h digestion product (Figure 2a). Monosaccharide, three tetrasaccharides, and only dp 5-1 and dp 5-2 were present in the 6 h digestion mixture (Figure 2c and its insert). Based on its retention time, dp 4-3 was identified as the tetrasaccharide present in 1 h digestion product (Figure 2a). The monosaccharide (dp 1) was present at higher levels after 9 h, whereas most of

Table 1. Assignments of Fragment Ions in MS/MS Spectra for Two Trisaccharides

	B_1	C_1	$^{0,4}A_2/^{2,4}A_2$	$^{0,3}A_2$	$^{2,5}A_2$	$^{0,2}A_2$	B_2	C_2	$^{0,4}A_3/^{2,4}A_3$	$^{0,3}A_3$	$^{2,5}A_3$	$^{0,2}A_3$	$[M-H]^-$
dp3-1	161	179	221^0	251	—	281	323	341	383^0	413	—	443	503
Maltotriose	161	179	221^2	—	263	281	—	341	383^2	—	425	—	503

trisaccharide (dp 3) had been digested. All dp 4-3, dp 6-2, and dp 7-2 (observed previously) were absent from the 9 h digestion product (Figure 2d and its insert). At 12 h, more monosaccharide was present whereas the trisaccharide was nearly absent. The ratio of dp 4-1 to dp 4-2 and dp 5-1 to dp5-2 increased and dp 7 and dp 8 were nearly absent after 12 h. The results observed in the EIC were consistent with observations from TLC (Supplementary Figure S1) but afforded more structural details.

It is known that native dextran is mainly composed of 1→6 linked glucose [9, 20, 27]. Also the ^{1}H-NMR spectrum of dextran showed that it is primarily α1-6 linked glucan. (Supplementary Figure S2) Thus, the oligosaccharides observed at the beginning of dextranase treatment (1 h digestion product) can be assumed to contain 1→6 glycosylic bonds. Moreover, the 1→6 linkages present in the early products can be further confirmed by the observation that these oligosaccharides are completely absent in the final products.

Sequencing of Oligosaccharides in the Digestion Product with MS and MS/MS

The dp of each oligosaccharide was confirmed with MS analysis in negative mode. The sequence of each oligosaccharide in the digestion products was elucidated through the assignments of the fragment ions in their MS/MS spectra. The assignments of molecular ions and fragment ions utilize the nomenclature of Domon and Costello [33] and are summarized in Tables 1, 2, 3 and 4.

MS/MS was next applied to confirm that trisaccharide, observed at all digestion times, was 1→6 linked. In addition to the typical fragment ions from glycosylic bond cleavages, such as B_1 (*m/z* 161), C_1 (*m/z* 179), B_2 (*m/z* 323), and C_2 (*m/z* 341), cross ring cleavage ions, ^{0,2}A, ^{0,3}A, and ^{0,4}A, were observed corresponding to the second and third residues from the non-reducing end in the MS/MS spectrum of trisaccharide (Figure 3a). The observations of ^{0,2}A, ^{0,3}A, and ^{0,4}A all demonstrate the 1→6 linkages in these residues. In contrast, when maltotriose, a 1→4 linked glucose trisaccharide, was analyzed under the same conditions (Figure 3b), the cross-ring cleavage ion ^{0,3}A was not observed but diagnostic ions, $^{2,5}A_2$ (*m/z* 263) and $^{2,5}A_3$ (*m/z* 425), were exclusively present, corresponding to the second and third residues from the non-reducing end, confirming their 1→4 linkages. In addition, the ion peaks observed at *m/z* 221 and 383 were assigned as $^{2,4}A_2$ and $^{2,4}A_3$, respectively, for 1→4 linked residues. Thus, the trisaccharide (dp 3) from dextran could be unambiguously assigned as Glc 1-6 Glc 1-6 Glc.

The EIC for the tetrasaccharides were next examined (Figure 4). The dp 4-3, observed in the intermediate digestion products corresponded to the tetrasaccharide in the early 1 h digestion product and, thus, should be a 1→6 linked tetrasaccharide. In the MS/MS spectrum of dp 4-3 (Figure 4c), two series of cross-ring cleavage ions, ^{0,2}A, ^{0,3}A, and ^{0,4}A at the second and third residues from non-reducing end were observed, confirming their

Table 2. Assignments of Fragment Ions in MS/MS Spectra for Four Tetrasaccharides

	B_1	C_1	$^{0,4}A_2/^{2,4}A_2$	$^{0,3}A_2$	$^{2,5}A_2$	$^{0,2}A_2$	B_2	C_2	$^{0,4}A_3/^{2,4}A_3$	$^{0,3}A_3$	$^{2,5}A_3$	$^{0,2}A_3$	C_3	$^{0,4}A_4/^{2,4}A_4$	$^{2,5}A_4$	$[M-H]^-$
dp4-1	161	179	—	—	—	—	—	341	383^{0}	413	—	443	503	545^{0}	—	665
dp4-2	161	179	221^{0}	—	263	—	323	341	383^{0}	413	—	443	503	545^{0}	—	665
dp4-3	161	179	221^{0}	251	—	281	323	341	383^{0}	413	—	443	503	545^{0}	—	665
Maltotetrose	161	179	221^{2}	—	263	281	—	341	383^{2}	—	425	443	503	545^{2}	587	665

Table 3. Assignments of Fragment Ions in MS/MS Spectra for Four Pentasaccharides

	B_1	C_1	$^{0,4}A_2/^{2,4}A_2$	$^{0,3}A_2$	$^{2,5}A_2$	$^{0,2}A_2$	B_2-H_2O	B_2	C_2	$^{0,4}A_3/^{2,4}A_3$	$^{3,5}A_3$	$^{0,3}A_3$
dp5-1	161	179	221^0	251	—	281	305	323	341	—	—	—
dp5-2	161	179	221^2	—	—	—	—	—	—	—	401	—
dp5-3	161	179	221^0	251	—	281	—	—	341	383^0	—	413
Maltopentose	161	179	221^2	—	263	281	—	—	341	383^2	—	—

	$^{2,5}A_3$	$^{0,2}A_3$	B_3	C_3	$^{0,4}A_4/^{2,4}A_4$	$^{0,3}A_4$	$^{2,5}A_4$	$^{0,2}A_3$	C_4	$^{0,4}A_5/^{2,4}A_5$	$[M-H]^-$
dp5-1	—	—	—	503	545^0	575	—	605	665	707^0	827
dp5-2	—	—	485	503	545^0	—	—	605	665	707^0	827
dp5-3	—	443	—	503	545^0	575	—	605	665	707^0	827
Maltopentose	425	443	—	503	545^2	—	587	—	665	707^2	827

Table 4. Assignments of Fragment Ions in MS/MS Spectra for Three Hexasaccharides

	C_1	$^{0,4}A_2/^{2,4}A_2$	$^{0,3}A_2$	$^{2,5}A_2$	$^{0,2}A_2$	B_2	C_2	$^{0,4}A_3/^{2,4}A_3$	$^{0,3}A_3$	$^{2,5}A_3$	$^{0,2}A_3$	B_3-H_2O	B_3	C_3
dp6-1	179	221^0	251	—	281	323	341	383^0	413	—	443	467	485	503
dp6-2	179	221^0	251	—	281	—	341	383^0	413	—	443	—	—	503
Maltohexose	179	221^2	—	263	281	—	341	383^0	—	425	443	—	—	503

	$^{0,4}A_4/^{2,4}A_4$	$^{0,3}A_4$	$^{2,5}A_4$	$^{0,2}A_3$	C_4	$^{0,4}A_5/^{2,4}A_5$	$^{0,3}A_5$	$^{2,5}A_5$	$^{0,2}A_5$	C_5	$^{0,4}A_6$	$^{2,5}A_6$	$[M-H]^-$
dp6-1	—	—	—	—	665	707^0	737	—	767	827	869	—	989
dp6-2	545^0	575	—	605	665	707^0	737	—	767	827	869	—	989
Maltohexose	545^2	—	587	605	665	707^0	—	749	—	827	869	911	989

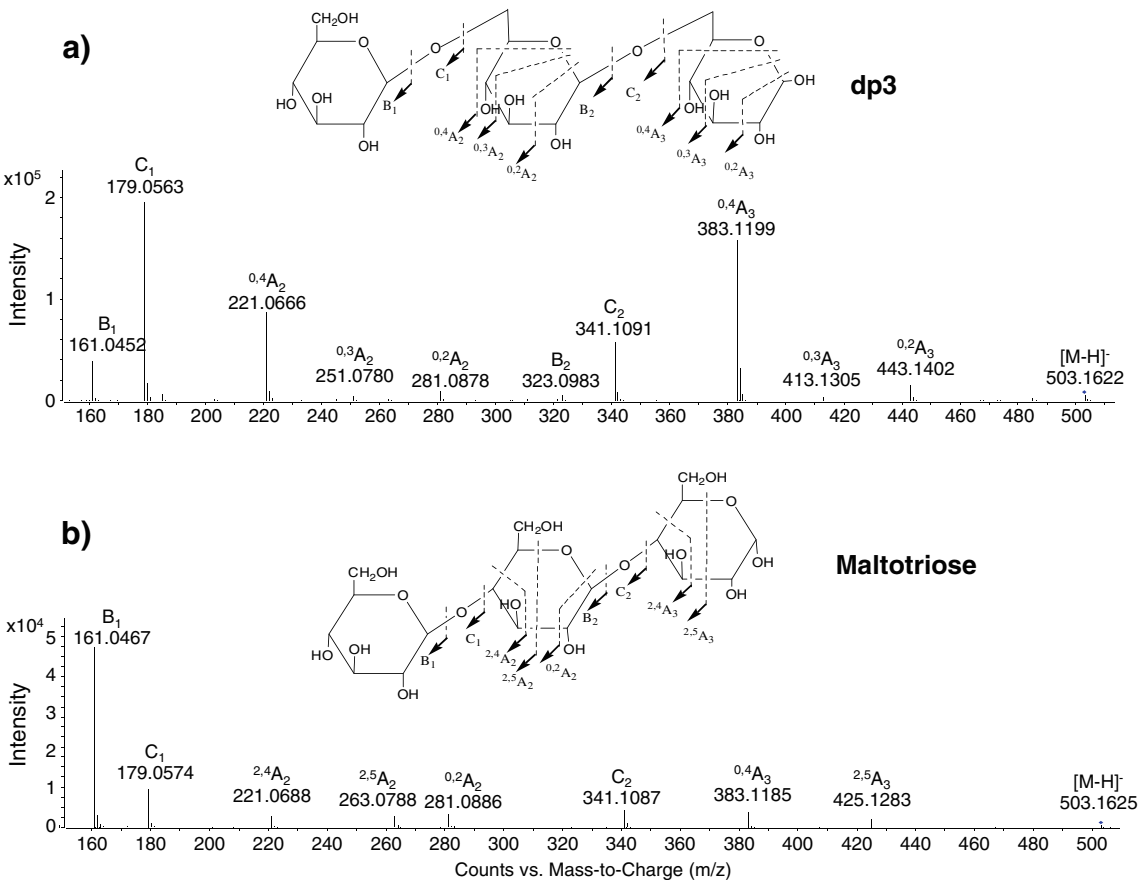

Figure 3. MS/MS spectra of trisaccharides. **(a)** Structure and MS/MS spectrum of dp3 from dextran; and **(b)** structure and MS/MS spectrum of maltotriose

1→6 linkages. The *m/z* 545 could be assigned as $^{0,4}A_4$, corresponding to 1→6 linkage at the reducing-end residue. In the MS/MS spectrum of dp 4-2, (Figure 4b) the presence of exclusive ion, $^{2,5}A_2$ (*m/z* 263), suggests the second residue from non-reducing end was 1→4 linked glucose. This was also confirmed by the observation of $^{2,5}A_2$ (*m/z* 263) in the MS/MS spectrum of 1→4 linked maltotetrose (Figure 4d). Since the $^{0,2}A_3$, $^{0,3}A_3$, and $^{0,4}A_3$ were also all observed, the third residue from non-reducing end was 1→6 linked glucose. The *m/z* 545 could be assigned from the $^{0,4}A_4$ fragmentation, corresponding to 1→6 linkage at the reducing-end residue. In the MS/MS spectrum of dp 4-1 (Figure 4a), the absence of significant cross-ring cleavage ion peaks at second residue from the non-reducing end suggests that it is neither 1→4 nor 1→6 linked glucose. Since 1→2 linked glucose is rarely reported in dextran [31], it was assumed that it was a 1→3 linked glucose. No significant cross-ring cleavage ions for 1→3 linked residues in negative mode have been previously reported [34–36]. The third residue from non-reducing end was confirmed as 1→6 linked by the presence of $^{0,2}A_3$, $^{0,3}A_3$, and $^{0,4}A_3$. The *m/z* 545 could be assigned as $^{0,4}A_4$, corresponding to 1→6 linkage at the reducing-end residue. Thus, three different tetrasaccharides (dp 4-1, dp 4-2, and dp 4-3) were confirmed as Glc 1-3 Glc 1-6 Glc 1-6 Glc, Glc 1-4 Glc 1-6 Glc 1-6 Glc, and Glc 1-6 Glc 1-6 Glc 1-6 Glc, respectively.

As with the assignments in MS/MS spectra of tetrasaccharides, dp 5-3 was confirmed as Glc 1-6 Glc 1-6 Glc 1-6 Glc 1-6 Glc by the presence of $^{0,2}A$, $^{0,3}A$, and $^{0,4}A$, corresponding to the second, third, and fourth residues from non-reducing end (Figure 5c). The *m/z* 707 could also be assigned as $^{0,4}A_5$, corresponding to 1→6 linkage at the reducing end residue. In the MS/MS spectrum of dp5-2 (Figure 5b), the absence of glycosidic cleavage ions, B_2 and C_2, suggested that there was a branch at the second residue from non-reducing end. The fragment ions from neutral oligosaccharides obtained in negative mode are all from non-reducing end direction [31], so the two-residue glycosidic fragment ions could be produced by single cleavage only if it is linear. In addition, the exclusive presence of $^{3,5}A_3$ at *m/z* 401 confirmed both 1→6 and 1→4 linkage occurred at the middle residue. The sequence of this domain at the non-reducing end was Glc 1-6 (Glc 1-4) Glc 1-. The presence of $^{0,2}A_4$, $^{0,3}A_4$, and $^{0,4}A_4$, although the fragment ion of $^{0,3}A_4$ was too small to be observed in Figure 5b, implies that the fourth residue from non-reducing end was a 1→6 linked glucose. The *m/z* 707 could also be assigned as $^{0,4}A_5$,

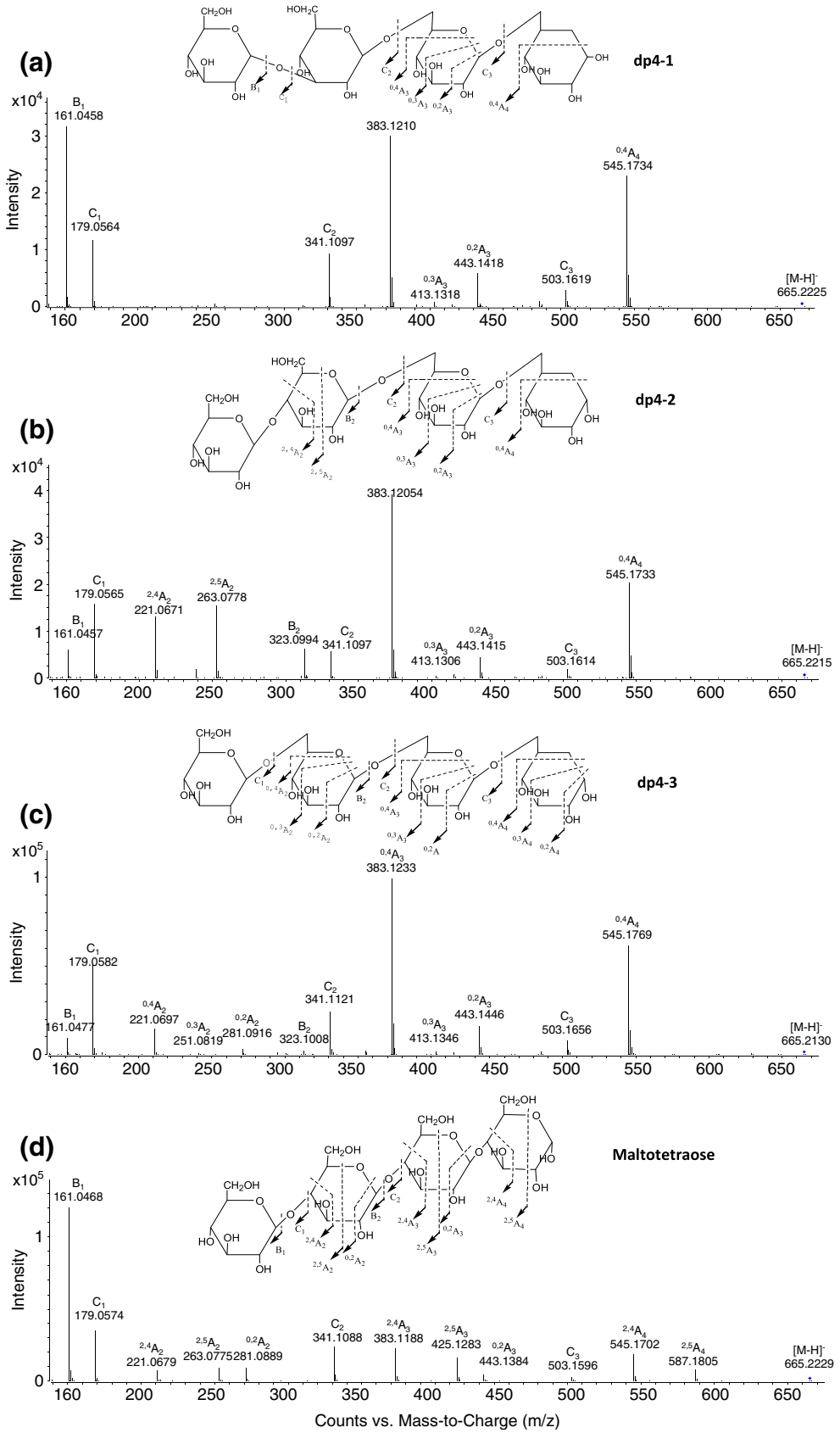

Figure 4. MS/MS spectra of tetrasaccharides. **(a)** Structure and MS/MS spectrum of dp4-1 from dextran; **(b)** structure and MS/MS spectrum of dp4-2 from dextran; **(c)** structure and MS/MS spectrum of dp4-3 from dextran; and **(d)** structure and MS/MS spectrum of maltotetrose

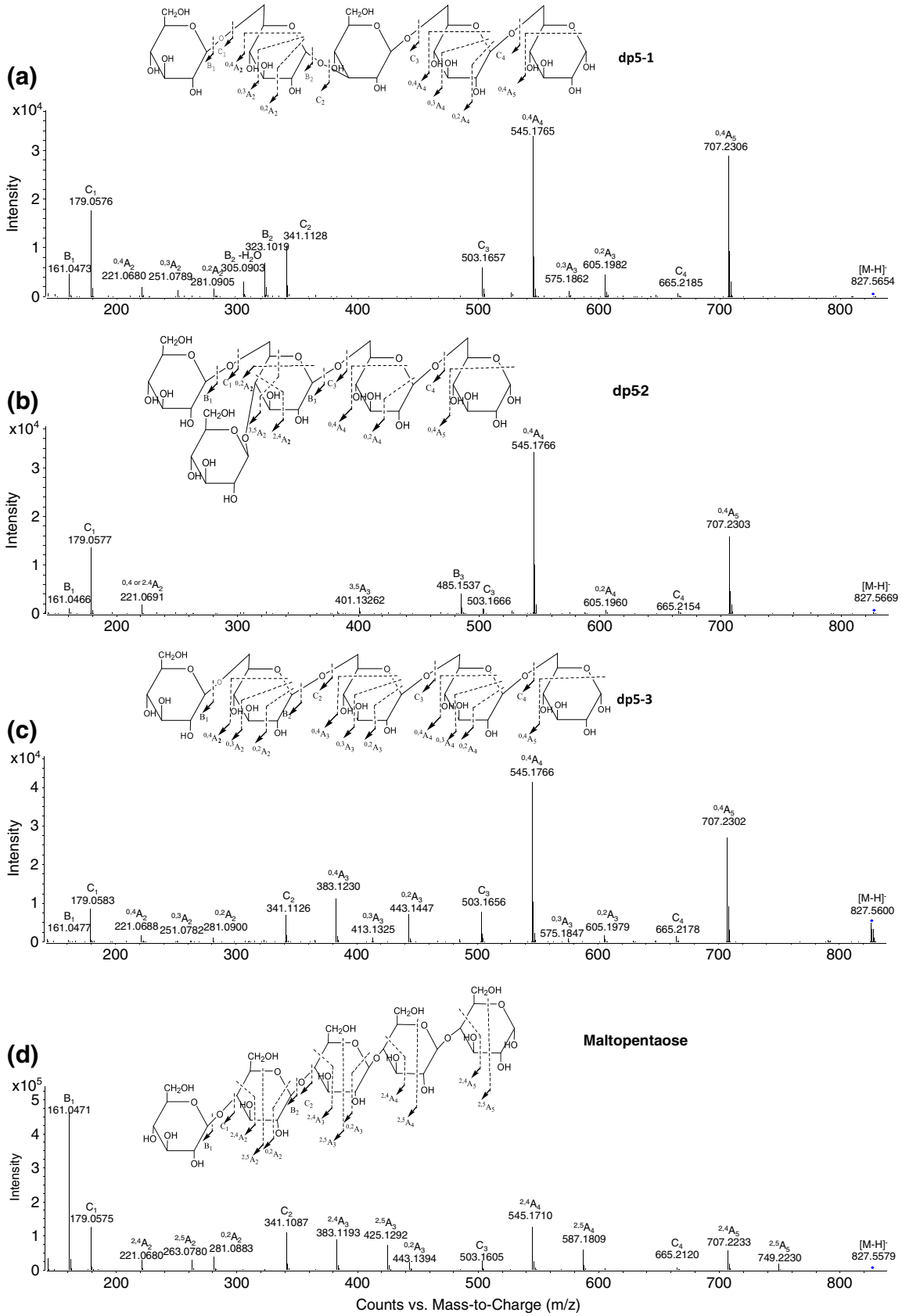

Figure 5. MS/MS spectra of pentasaccharides. **(a)** Structure and MS/MS spectrum of dp5-1 from dextran; **(b)** structure and MS/MS spectrum of dp5-2 from dextran; **(c)** structure and MS/MS spectrum of dp5-3 from dextran; and **(d)** structure and MS/MS spectrum of maltopentose

corresponding to 1→6 linkage at the reducing end residue (Figure 5b). In the MS/MS spectrum of dp 5-1, the absence of significant cross-ring ion peaks at the third residue from the non-reducing end suggests a 1→3 linkage at this residue (Figure 5a). In addition, a corresponding glycosidic cleavage ion, B_2-H_2O, present at *m/z* 305, which was not observed at 1→4 (maltopentose, Figure 5d) or 1→6 (dp5-3, Figure 5c) linked the middle residues. The B-H_2O ions could be used to diagnose 1→3 linkage in middle residues of the oligoglucan. The presence of two series of ^{0,2}A, ^{0,3}A, and ^{0,4}A corresponding to the second and fourth residues from non-reducing end in the MS/MS spectrum of dp 5-1 confirmed the 1→6 linkages at these two residues. The *m/z* 707 could also be assigned as $^{0,4}A_5$, corresponding to 1→6 linkage at the reducing end residue. Thus, the three different pentasaccharides dp5-1, 5-2 oligoglucan and 5-3, were assigned as Glc 1-6 Glc 1-3 Glc 1-6 Glc 1-6 Glc, Glc 1-6 (Glc 1-4) Glc 1-6 Glc 1-6 Glc, and Glc 1-6 Glc 1-6 Glc 1-6 Glc 1-6 Glc, respectively.

For the hexasaccharides, the presence of ^{0,2}A, ^{0,3}A, and ^{0,4}A in the MS/MS spectrum of dp 6-2 confirmed their 1→6 linkages throughout its sequence (Figure 6b). The absence of cross-ring cleavage ions at the third residues in the MS/MS spectrum of dp 6-1 suggested that it is 1→3 linked residue at this position (Figure 6c). Moreover, the exclusive presence of B_3-H_2O at *m/z* 467 confirmed the 1→3 linkage at this position. All other positions were confirmed as 1→6 linkages by the presence of the corresponding ^{0,2}A, ^{0,3}A, and ^{0,4}A fragments. Thus, two hexasaccharides, dp 6-1 and dp 6-2 were confirmed as Glc 1-6 Glc 1-6 Glc 1-3 Glc 1-6 Glc 1-6 Glc and Glc 1-6 Glc 1-6 Glc 1-6 Glc 1-6 Glc 1-6 Glc, respectively.

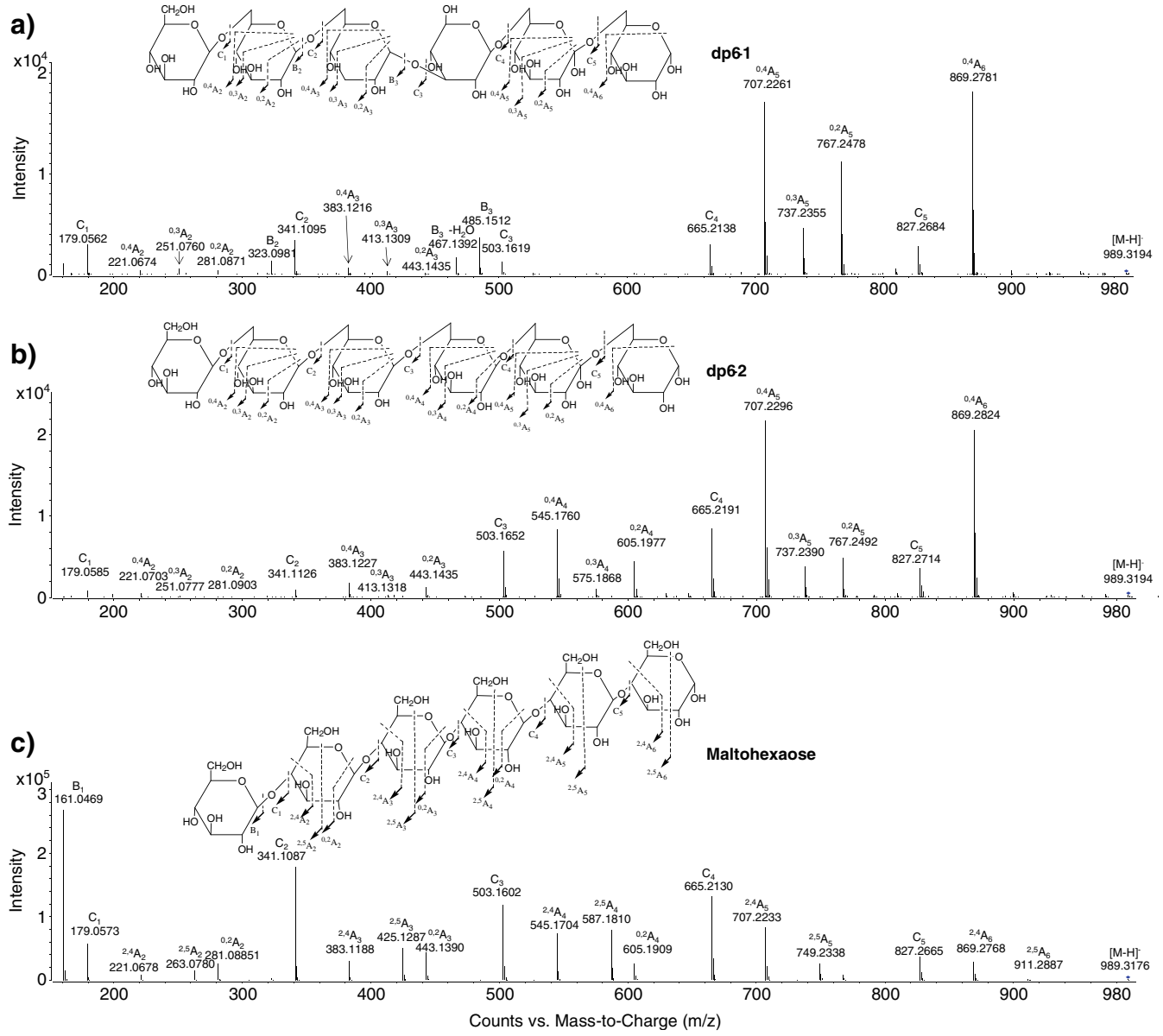

Figure 6. MS/MS spectra of hexasaccharides. **(a)** Structure and MS/MS spectrum of dp6-1 from dextran; **(b)** structure and MS/MS spectrum of dp6-2 from dextran; and **(c)** structure and MS/MS spectrum of maltohexose

Conclusions

In this work, a new method with UP-HILIC-Q/TOF-MS was developed to analyze neutral oligosaccharides having different linkage patterns. No derivatization of these oligosaccharides was needed and the impact of α- and β-configurations of native sugar on chromatography was eliminated by conducting the separation under basic conditions. The complex sugar mixtures in dextran digestion products were profiled and analyzed using this method, providing an efficient, sensitive, and straightforward way to monitor dextran digestion and to support the proper application of dextranase.

Moreover, the complex dextran oligosaccharides from digested products were all sequenced unambiguously using MS/MS analysis. In addition to glycosidic cleavage ions, cross-ring cleavage ions were often diagnostic of linkage position. The 1→6 linked glucose is the major component of dextran, which always appears as ^{0,2}A, ^{0,3}A, and ^{0,4}A fragments in the MS/MS spectrum. Glycosidic linkage to position 6 of the sugar does not significantly impact cross-ring cleavages in CID, whereas glycosidic linkages to position 3 stabilize the bonds 2 and 3 on the sugar ring, which are the most active bonds to be dissociated. Thus, the absence of A type ions usually corresponds to the presence of a 1→3 linkage. In addition, a new diagnostic ion, "B-H_2O", can be used to distinguish 1→3 from 1→4 and 1→6 linkages in the middle of oligosaccharides and is first reported in this work. An exclusive ion, ^{2,5}A, was observed in every 1→4 linkage as a diagnostic ion, which was confirmed by experiments on the 1→4 linked malto-oligosaccharides. The absence of the glycosidic cleavage ions at the branch site residues and presence of specific cross-ring cleavage ions, such as ^{3,5}A, implied that there could be branches on the backbone. According to the observations of these diagnostic ions in the MS/MS spectra, the sequences of one trisaccharide, three tetra- and pentasaccharides, and two hexasaccharides were unambiguously confirmed. The same or even better work could be done compared with previous work using sample preparation and NMR analysis strategies with much less sample, labor, time, and uncertainty using this method [24].

Based on the identification of oligosaccharides in the intermediate and final products, the action pattern of dextranase from *Penicillium sp*. can be summarized: (1) the 1→6 linkage is the optimal cleavage point for this enzyme; (2) the glucose and isomaltose are the final digestion products from dextran; and (3) isomaltotriose is the minimum digestion domain, except when an additional residue is attached at the non-reducing end with linkage types other than 1→6. This action pattern implies that the binding of enzyme to dextran requires a disaccharide domain or longer, and only one with 1→6 linkage, and the cleavage occurs at the glycosylic bond linked to the reducing end of the disaccharide domain. Branches at the non-reducing end of disaccharide domain can block the binding of enzyme to dextran resulting in the absence of cleavage.

Thus, an efficient UP-HILIC-Q/TOF-MS/MS method was developed, providing the effective separation and abundant sequencing information for the analysis of neutral oligosaccharides containing different linkage types. Dextran-derived oligosaccharides were profiled and analyzed using this method. The action pattern of dextranase from *Penicillium sp*. was clearly established based on the results presented.

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