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FAB CIDMS/MS analysis of partially methylated maltotrioses derived from methylated amylose: a study of the substituent distribution

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Abstract

Amylose was methylated with CH_3I in alkaline aqueous suspension, yielding methylated amylose (MeAl) with a degree of substitution of 1.44 ($s < 0.01$). Determination of the monomer composition showed that HO-6 and HO-2 were highly substituted in contrast to HO-3 (7:2:5.5, HO-2:HO-3:HO-6). By using partial acid hydrolysis, oligomers were prepared that varied both in degree of polymerisation and in methyl-content. Studies on the distribution of substituents in trimers showed large deviations from random distributions. By using CID tandem mass spectrometry, the substituent distribution in these trimers was determined in more detail. Various sets of trimers with equal amounts of methyl-groups but differing in substituted positions were quantified. From the monomer composition of MeAl, the probability of each trimer was calculated and compared to the outcome of the measured distributions. It was concluded that trimers with terminal tri- or non-substituted glucose monomers at the non-reducing end were formed preferentially during partial hydrolysis and that partial hydrolysis of MeAl yielded oligomers in a non-random way. This is the first study that describes the partial hydrolysis of MeAl in such detail.

Keywords: Methylated amylose; Starch; Mass spectrometry; Internal residue loss; Substituent distribution

1. Introduction

Starch is an important renewable raw material that is used in food, pharmaceuticals, paper and technical products. In higher plants starch, consisting of mixtures of amylose and amylopectin, is deposited in cold-water insoluble, partially crystalline granules [1]. Modifications of starches are carried out in order to provide starch products with properties that fulfil various demands. In most chemical modifications (i.e. derivatisations) of starch, hydroxyl groups are partially substituted [2]. The level of substitution in derivatised starch granules is expressed in the degree of substitution (DS) [3]. The distribution of the substituents determines the properties of the starch derivatives and is not expected to be random in view

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of the different levels of organisation within the starch granule [4,5].

In order to acquire knowledge about the relationship between structure and function, it is important to determine the distribution of substituents [6]. For this purpose, oligomers are prepared from the polymer of interest by using partial acid hydrolysis. A powerful method in the structure characterisation of such oligomers is mass spectrometry (MS) [3,7], in particular by using relatively soft ionisation techniques such as fast-atom bombardment (FAB) [8], electrospray (ES) [9] and matrix-assisted laser desorption/ionisation (MALDI) [10]. The structural information that can be obtained by MS improves significantly after derivatisation of oligosaccharides [11,12] and makes the determination of substituent distributions in partially methylated samples possible [3,13–15]. To this end, remaining free hydroxyl groups in partially methylated oligosaccharides are converted into OCD_3-groups. Permethylated oligosaccharides with the same degree of polymerisation (DP) but with different CD_3-content are chemically equivalent, but have different molecular masses. By using mass spectrometry, these oligomers have been quantified and results have been interpreted in terms of substituent distribution, in this study further referred to as primary distribution. Oligomers with the same DP and equal CD_3-content are mixtures of structures with equal molecular masses, but may differ in substituted monomers and positions. In primary distribution studies, these different oligomers are measured as a mixture only. Identification and quantification of each structure in a mixture adds new information about the distribution of substituents in methylated amylose. Here, it is shown that this so-called secondary substituent distribution within a trisaccharide can be determined using collision induced dissociation tandem mass spectrometry (CIDMS/MS) after quantification of well-defined fragments. For this purpose, partially methylated amylose (MeAl) is used as a model compound. In contrast to starch granules, amylose can be substituted conveniently over 50% [13]. Amylose is essentially a linear (1→4)-linked polymer of α-D-glucose, which can contain a few (1→6) branches [16]. Therefore, all oligosaccharides isolated from MeAl after partial hydrolysis are almost exclusively linear (1→4)-linked α-D-glucans.

2. Results and discussion

Preparation of methylated amylose (MeAl) and monomer composition.—Amylose was methylated in alkaline aqueous suspension using iodomethane [13], yielding methylated amylose (MeAl) with a DS of 1.44 ($s < 0.01$, $n = 5$). The monomer composition of MeAl as determined by monosaccharide analysis is given in Table 1 [14,17]. The HO-2 group has the highest degree of substitution in agreement with earlier studies on substituted starch granules [14,15,18]. The ratio of substitution is 7:2:5.5 at HO-2, HO-3 and HO-6. The relatively low substitution of the HO-3 group compared to the HO-6 group is in contrast to methylated starches, wherein the HO-3 and the HO-6 group were substituted to almost the same extent.

Table 1
Monomer composition of MeAl

Glc [a]	Glc2Me	Glc3Me	Glc6Me	Glc2,3Me_2	Glc2,6Me_2	Glc3,6Me_2	Glc2,3,6Me_3
14.2	21.9	3.0	10.8	6.4	33.6	2.2	7.9

[a] All monomers are characterised as trimethylsilylated methyl glucosides ($\alpha+\beta$) and are given in molar percentages. The ratio of substitution at HO-2, HO-3 and HO-6 is calculated according to Ref. [3]:

$X_2 = \text{Glc2Me} + \text{Glc2,3Me}_2 + \text{Glc2,6Me}_2 + \text{Glc2,3,6Me}_3$

$X_3 = \text{Glc3Me} + \text{Glc2,3Me}_2 + \text{Glc3,6Me}_2 + \text{Glc2,3,6Me}_3$

$X_6 = \text{Glc6Me} + \text{Glc2,6Me}_2 + \text{Glc3,6Me}_2 + \text{Glc2,3,6Me}_3$

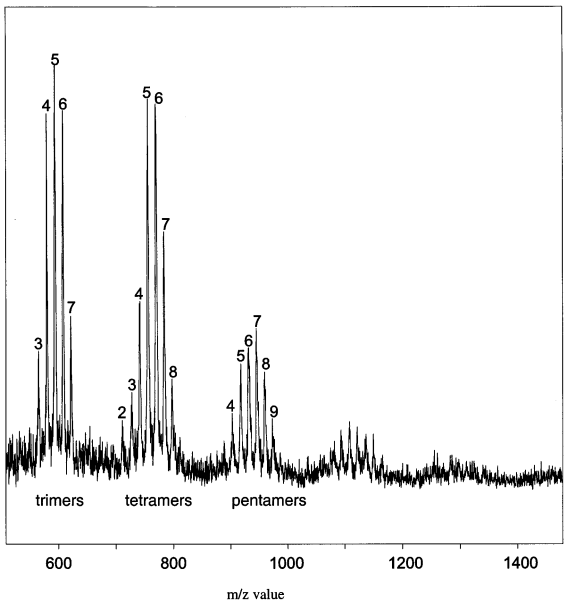
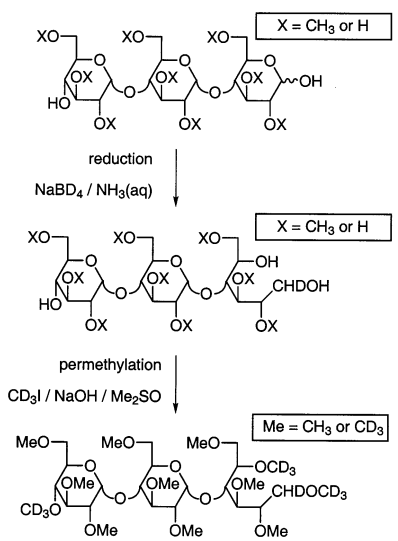


Fig. 1. MALDI mass spectrum of sodium-cationised trimers, tetramers and pentamers in the maltotriose fraction from MeAl before reduction and permethylation. Numbers on top of the peaks indicate the number of CH_3-groups present in the oligomer ($DS_{oligomer}$).



Scheme 1. Preparation of (reduced) per(deuterio)methylated maltotrioses.

Preparation of the reduced maltotriose fraction from MeAl.—MeAl was partially hydrolysed, and the maltotriose fraction was isolated by Bio-Gel P-2 gel permeation chromatography (GPC). Analysis of the maltotriose fraction by using MALDI mass spectrometry (Fig. 1) showed the presence of sodium-cationised pseudomolecular ions of mainly trimers, tetramers and pentamers, and of larger oligomers in minor amounts. Trimers in the maltotriose fraction could not be separated from the tetramers and pentamers by using GPC, because oligomers with identical DP overlap due to the presence of methyl substituents.

The methyl-content in an oligomer is defined as $DS_{oligomer}$ and is an integer value [3]. As is clear from Fig. 1, for trimers the $DS_{oligomer}$ varies from 3 to 7, for tetramers from 2 to 8, and for pentamers from 4 to 9. All oligomers with identical DP but different methyl-content differ in chemical structure. Therefore, the intensities of their pseudomolecular ions are not suitable for quantification. This result demonstrates only, the type of oligomers that are present in the hydrolysate.

In order to prepare derivatives suitable for quantitative FABMS studies, the maltotriose fraction was reduced with $NaBD_4$, then permethylated with CD_3I (Scheme 1) [19]. The distribution studies are focused only on the trimers present in the maltotriose fraction, for reasons given later.

Primary substituent distribution in reduced trimers from MeAl.—The distribution of substituents in oligomer fragments represents the substitution pattern in the corresponding polymer backbone [3,13]. Quantification of oligomers in the reduced and per(deuterio)methylated maltotriose fraction with specific DP and varying $DS_{oligomer}$ provides insight into the deviation of substituent patterns from random distributions. Because such oligomers are chemically equivalent, they show the same behaviour in terms of ionisation efficiency. Therefore, quantification of all FAB mass spectrometric intensities in the pseudomolecular ion region ($[M + Na^+]$) of oligomers with the same DP is valid. The differences between observed intensities and statistically expected values are visualised in Fig. 2. In the statistical binomial distribution, the methyl substituents are distributed randomly over equal and independent glucose monomers [3].

The overall degree of substitution of the trimer mixture as determined by FABMS is7% higher than the substitution level of intact MeAl as measured by monosaccharide analy-

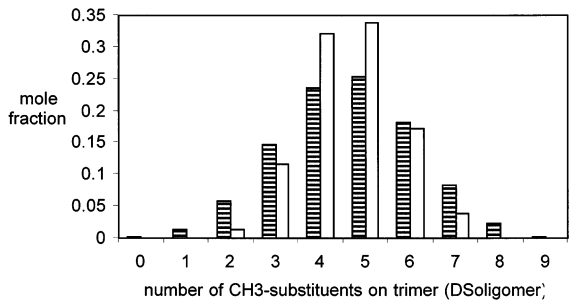


Fig. 2. Calculated (striped bars) and observed (white bars) primary substituent distribution in reduced and per(deuterio)methylated maltotrioses from MeAl.

sis. Therefore, the substitution pattern in MeAl is reasonably represented by these trimers [3,13]. It is clear from Fig. 2 that intensities of oligomers with $DS_{oligomer}$ 4 and 5 are higher than expected on the basis of a random distribution and all others are lower. These results clearly indicate that the primary substituent distribution in MeAl is heterogeneous. Previously, large deviations in the primary distribution of substituents were observed for amylose methylated under the same conditions [13].

The relatively low substitution of HO-3 in MeAl (Table 1) originates from the well known regioselectivity during the methylation process of (1→4)-linked α-D-glucans ([3,13]; references cited therein). The large substitution deviations from random distributions in the trimers prepared from MeAl (Fig. 2) are usually expressed in terms of heterogeneity [3,13]. Different areas within a polymer (or a granule in the case of starch) may differ in sensitivity towards partial hydrolysis. As a consequence, the oligomers that are studied might represent parts of the original polymer only [15]. For this reason, more information is needed considering the structure of these oligomers, which will give insight into the partial hydrolysis of the polymer.

Secondary substituent distribution in reduced trimers with specific $DS_{oligomer}$.—Trimers with identical $DS_{oligomer}$ can be mixtures of components, which may vary with respect to the substituted glucose monomers in the trimer and to the substituted positions in a glucose monomer (i.e. the 2-, 3- and/or 6-position). With nine available substitution sites in one trimer the number of different structures is given by

$$\frac{9!}{n!((9-n)!)}$$

where n equals the $DS_{oligomer}$ value of the trimer. The numbers of different structures are given in Table 2.

By using FAB tandem mass spectrometry (MS/MS) with exclusive fragmentation of glycosidic linkages, the substitution level of each glucose monomer in a certain trimer in the maltotriose fraction can be determined, but not the positions of substitution within each

Table 2
Number of structures of partially methylated trimers with specific $DS_{oligomer}$

$DS_{oligomer}$	Total no. of possible structures	FAB CIDMS/MS observable sets of structures [a]	Total no. of observable sets of structures
0	1	{000} [b]	1
1	9	{**1**00 0**1**0 00**1**}	3
2	36	{**2**00 0**2**0 00**2**} {**11**0 **1**0**1** 0**11**}	6
3	84	{300 030 003} {**21**0 **2**0**1** **12**0 **1**0**2** 0**12** 0**21**} {**111**}	10
4	126	{**3**10 30**1** **13**0 **1**03 0**13** 03**1**} {**22**0 **2**0**2** 0**22**} {**112** **121** **211**}	12
5	126	{**3**20 30**2** **23**0 **2**03 0**32** 0**23**} {**113** **131** **311**} {**122** **212** **221**}	12
6	84	{330 303 033} {**213** **231** **123** **132** **321** **312**} {**222**}	10
7	36	{**331** **313** **133**} {**223** **232** **322**}	6
8	9	{**233** **323** **332**}	3
9	1	{333}	1

[a] Numbers indicate the number of CH_3-groups at each glucose monomer of the trimer. Sets with numbers indicated in bold contain glucose monomers with multiple possibilities of substitution sites.

[b] Groups of sets of structures are indicated between parentheses.

Table 3
Determined and statistically expected amounts of sets of structures for various $DS_{oligomer}$ values (in percentages)

$DS_{oligomer}$ 3 [a]		{300	030	003}	{**2**10	**2**01	**1**20	**1**02	0**1**2	0**2**1}	{**111**}	
cal [b]		1	1	1	12	12	12	12	12	12	25	
det [c]		2	3	2	19	3	15	17	0	10	29	
$DS_{oligomer}$ 4 [a]	{3**1**0	30**1**	**1**30	**1**03	0**1**3	03**1**}	{**22**0	**2**02	0**22**}	{**112**	**121**	**211**}
cal [b]	1.5	1.5	1.5	1.5	1.5	1.5	9.7	9.7	9.7	20.6	20.6	20.6
det [c]	0	2	0	2	2	4	15	5	4	18	24	24
$DS_{oligomer}$ 5 [a]	{3**2**0	30**2**	**2**30	**2**03	03**2**	0**2**3}	{**11**3	**1**3**1**	3**11**}	{**122**	**212**	**221**}
cal [b]	1.9	1.9	1.9	1.9	1.9	1.9	4	4	4	25.5	25.5	25.5
det [c]	0	8	0	0	12	0	18	0	11	0	0	51
$DS_{oligomer}$ 6 [a]		{330	303	033}	{**21**3	**2**3**1**	**12**3	**1**3**2**	3**21**	3**12**}	{**222**}	
cal [b]		0.6	0.6	0.6	8	8	8	8	8	8	50.4	
det [c]		2	2	2	4	8	7	2	16	15	42	

[a] Numbers indicate the number of CH_3-groups at each glucose monomer of the trimer. Sets with numbers indicated in bold contain glucose monomers with multiple possibilities of substitution sites.
[b] cal, calculated according to the monomer composition of MeAl.
[c] det, determined using FAB CIDMS/MS measurements.

glucose monomer. Therefore, oligomer structures containing glucose monomers, which have identical $DS_{oligomer}$ values but vary only in their substituted positions, are assembled into sets of structures that are observable using CIDMS/MS (Table 2). For example, (**1**33) is a set of three different trimers with one methyl-group at the 2-, 3- or 6-position of the terminal non-reducing glucose monomer, respectively, and all positions substituted at the other two glucose monomers. Sets of structures that contain identically substituted glucose monomers, but differing in their sequence are grouped in parentheses. From the monomer composition of MeAl (Table 1) the probability of each set of structures was calculated. It is evident that the probability of (03**1**), (**1**30), (**1**03), (3**1**0) and (301) is the same as that of (0**1**3). Thus obtained probabilities are summarised in Table 3.

CIDMS/MS spectra of protonated reduced and perdeuteriomethylated maltotriose.—The fragmentation patterns of native and derivatised oligosaccharides using CIDMS/MS are well-defined [20]. The formation of B_i- and Y_i-fragment ions [21] is characteristic for the glycosidic bond fragmentation in protonated oligosaccharides. However, rearrangement processes occurring on FABMS/MS of native [22,23] and methylated [23] oligosaccharides have been described previously. In this rearrangement newly formed oligosaccharide ions result from so-called loss of an internal monosaccharide residue from larger precursors [24], yielding Y_i^*-fragment ions. As an example, the high-energy CID tandem mass spectrum of $[M + H]^+$ of reduced and perdeuteriomethylated maltotriose (m/z 712) is given in Fig. 3.

The ion at m/z 499 is the most abundant one, which can be assigned unambiguously to an 'internal residue loss' ion Y_2^*. Non-reduced perdeuteriomethylated maltotriose gives rise to the loss of two different internal residues (resulting in a Y_2^*- and a Y_1^*-fragment; unpublished results), however, with identical masses. These fragments have ambiguous assignments and are therefore not suitable for studies on the secondary substituent distribution. In Fig. 3, the ion at m/z 444 results from B_2 fragments only. Two other relatively abundant ions are Y_1 (m/z 269) and B_1-CD_3OH (m/z 196). Minor peaks are observed at m/z 482 (Y_2), m/z 429 ($Y_2^*-2CD_3OH$) and m/z 231 (B_1). The absence of peaks close to the assigned fragments makes a study possible on the secondary substituent distribution of the maltotriose fraction.

CIDMS/MS spectra of reduced trimers in the maltotriose fraction.—High-energy CID tandem mass spectra of $[M + H]^+$ of reduced and perdeuteriomethylated maltotriose with $DS_{oligomer}$ 3, 4, 5 and 6 (m/z values 703, 700, 697 and 694, respectively) are shown in Fig. 4.

The ions Y_2^*, Y_2, B_2, and Y_1 are chosen for determining the substituent distributions in the reduced and per(deuterio)methylated trimers. The combination of these fragments covers the substitution on all three glucose monomers of the trimers. The ions resulting from fragments that have lost CD_3OH could not be used due to the uncertainty about the CD_3-group that is lost from the monomer or oligomer (see Fig. 3). CID of trimers with $DS_{oligomer}$ 6 results in Y_2^*-ions at m/z 490, 487 and 484, containing three, four and five CH_3-groups, respectively. These three ions are chemically equivalent and are formed via identical fragmentation pathways. Therefore, the ratio of their intensities corresponds with the amounts of mono-, di- and tri-substituted ions, respectively. The same is true for the Y_2-ions at m/z 473, 470 and 467, containing three, four and five CH_3-groups, respectively, the B_2-ions at m/z 435, 432 and 429, containing three, four and five methyl-groups, respectively, and the Y_1-ions at m/z 266, 263 and 260, containing one, two and three methyl-groups, respectively. Within a deviation of less than 10%, the ratios of intensities are constant

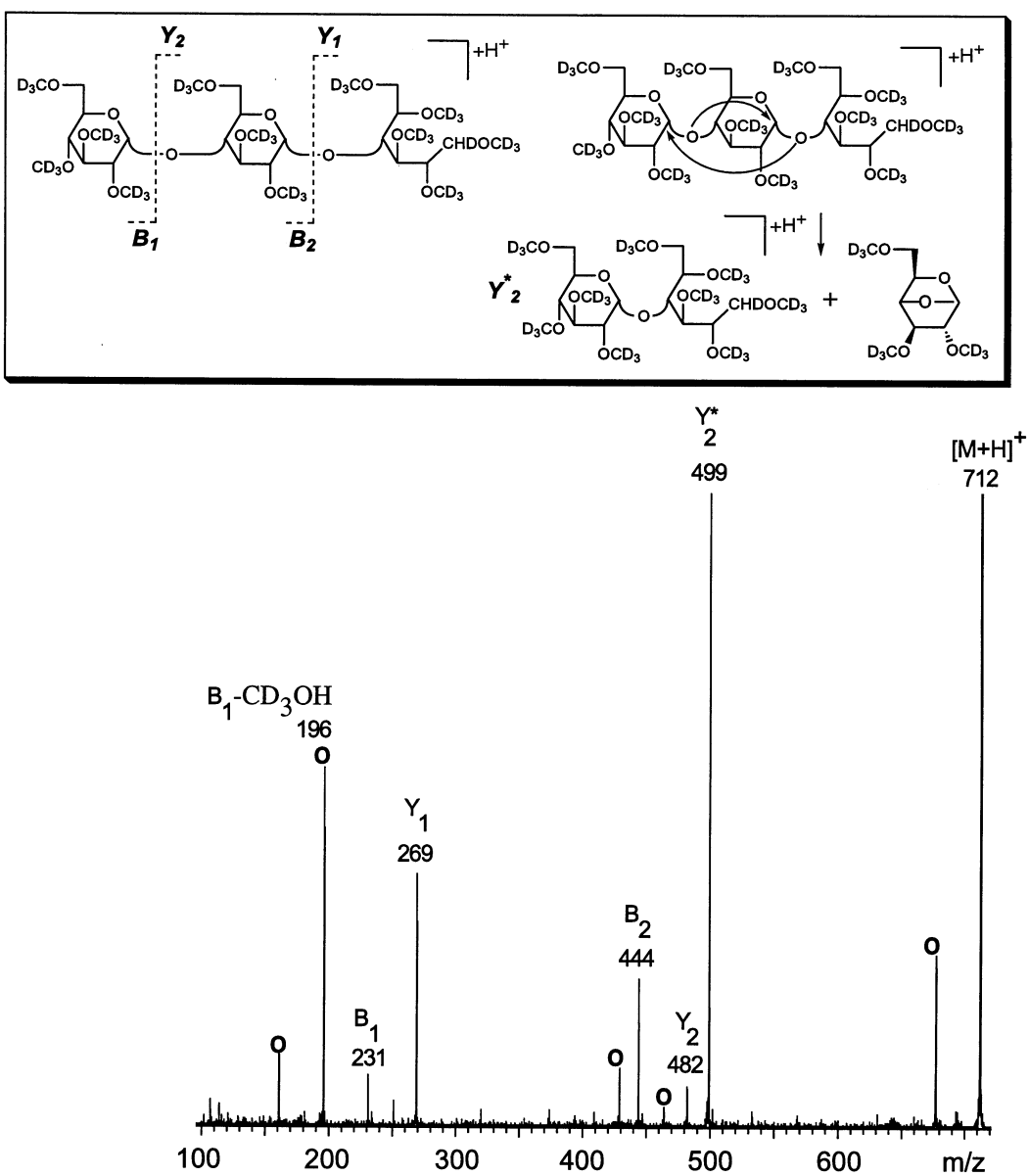


Fig. 3. CIDMS/MS fragmentation and spectrum of $[M + H^+]$ of reduced and perdeuteriomethylated maltotriose. The circles on top of the peaks indicate the loss of CD_3OH (an example is given at $m/z = 196$). The y-axis represents relative abundances.

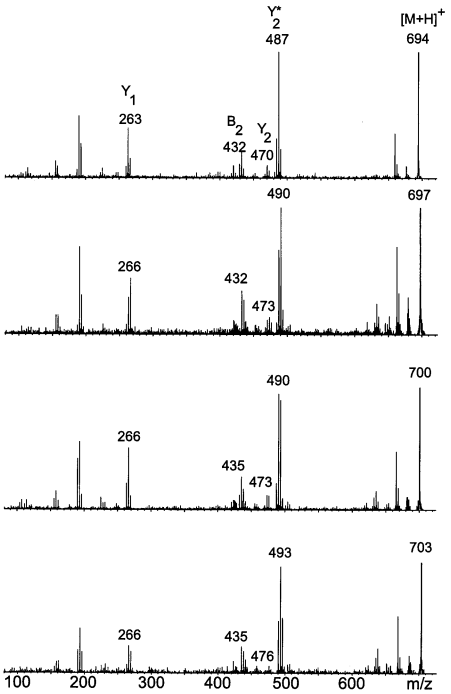


Fig. 4. CIDMS/MS spectra of $[M + H^+]$ of trimers ($DS_{oligomer}$ 6, 5, 4 and 3, respectively) from the maltotriose fraction. The satellite peaks of the fragments of interest, separated by three mass units, are clearly seen. *Y*-axes represent the relative abundances.

for each fragment during the first 3 min of CIDMS/MS. The measured intensities are given in Table 4.

For the interpretation of thus obtained data, the correlation between the peak intensities in the CIDMS/MS spectra and the sets of structures for a certain $DS_{oligomer}$ was established in detail. Information on the substitution of the non-reducing glucose monomer in the trimers is present in the fragments Y_2^* and B_2. The fragments Y_2 and B_2 contain information on the substitution of the internal glucose monomer in the trimers, whereas substitution of the reduced glucose monomer can be derived from the fragments Y_1, Y_2 and Y_2^*. To tackle the relations between peak intensities and substitution of the monomers, the Solver-tool in Microsoft Excel was applied. Using this approach, a unique solution was determined for each set of structures from measured peak intensities. This was done with the absolute intensities as well as the weighted intensities (taking into account the ratio between the fragments Y_2^*, Y_2, B_2, and Y_1 in Fig. 3). Both methods gave almost identical results. Three assumptions were made during

Table 4
Measured relative intensities of relevant peaks in spectra from Fig. 4 (arbitrary units)

Fragment	Y_2^*							Y_2						B_2						Y_1					
No. of CH_3-groups	0	1	2	3	4	5	6	0	1	2	3	4	5	6	0	1	2	3	4	5	6	0	1	2	3	4	5	6
$DS_{oligomer}$ 3	3.8	29	57	28					1	3	0.8					7	12	14				11	14	4	1			
$DS_{oligomer}$ 4		6	56	60	12					7	7.3	2				4	11	17		7.8		7	32	14	0.5			
$DS_{oligomer}$ 5			10	63	35	5			5	7.5	6	2					5	14	19	3		2	23	15	4			
$DS_{oligomer}$ 6				15	66	20	3.6			4	6.8	2						5	14	7			10	26	6			

the Solver-optimisation: (i) each peak is composed of structures that contribute proportionally, (ii) peak intensities correlate with the composition of the mixture of structures, and (iii) the ratio between the fragments Y_2^*, Y_2, B_2, and Y_1 is constant. Combining the data obtained from the four different ions Y_2^*, Y_2, B_2, and Y_1 derived from the trimers with $DS_{oligomer}$ 3, 4, 5 and 6, all sets of structures (Table 2) were quantified (in the case of $DS_{oligomer}$ 3, the abundance of Y_2-ions was insufficient for quantification). The results are summarised in Table 3. By comparing calculated and determined data from Table 3, it can be seen that tri-substitution and non-substitution at the terminal glucose monomer at the non-reducing end occur more than expected on the basis of a random partial hydrolysis. Apparently, cleavage of a glycosidic linkage in MeAl is influenced by methyl substituents. In addition, substitution at the neighbouring glucose monomer has an effect on the hydrolysis at the potential terminal glucose monomer.

3. Conclusions

The substituent distribution in methylated amylose (MeAl) with a degree of substitution of 1.44 ($s < 0.01$) was studied. HO-6 and HO-2 were highly substituted, whereas HO-3 contained few methyl substituents (7:2:5.5 = HO-2:HO-3:HO-6). Oligomers were prepared with various DP and various methyl-content ($DS_{oligomer}$) by using partial acid hydrolysis. Studies on the primary distribution of substituents in trimers [3] showed a large deviation from a random distribution, as was found previously for methylated amylose [13]. In this study, secondary substituent distribution in oligomers for each $DS_{oligomer}$ was determined using CID tandem mass spectrometry. Various sets of trimers (derived from MeAl) with identical $DS_{oligomer}$ were quantified and the results were compared with the distribution expected on the basis of the monomer composition. Trimers with terminal tri- or non-substituted glucose monomers at the non-reducing end were preferentially formed during partial hydrolysis of MeAl. This indicates that MeAl hydrolyses in a non-random way in 0.2 M HCl. Reduced and permethylated oligomers with $DP > 3$ are not suitable for such studies by using FAB CIDMS/MS, because they consist of at least two glucose monomers with identical masses. However, by using new ion traps or time-of-flight (TOF) in combination with (nano-) electrospray ionisation (ESI), and by selecting other parent ions, secondary distribution studies on larger oligomers will soon be feasible.

4. Materials and methods

Preparation of methylated amylose.—Amylose (15.6 g; Sigma, from corn) was methylated (41.0 mL CH_3I) in alkaline aqueous suspension (37.0 g KOH in 100 mL 2 M KCl) for 72 h at 20 °C [13,25]. MeAl was isolated after filtration from the suspension and repeated washing with water.

Partial acid hydrolysis of MeAl and isolation of the maltotriose fraction.—Methylated amylose (40 mg) was suspended in 0.2 M HCl (10 mL) and partially hydrolysed for 1.5 h at 85 °C (TLC-control). After neutralisation with NaOH and cooling, the mixture was concentrated to dryness. The residue was fractionated on a column (40 cm × 30 mm) of Bio-Gel P-2 (Bio-Rad) using water as eluent, and the maltotriose fraction was isolated, lyophilised, and analysed by MALDI–TOF mass spectrometry. The spectrum in Fig. 1 was recorded on a Voyager-DE (PerSeptive Biosystems) instrument, using 2,5-dihydroxybenzoic acid as the matrix (linear mode, acceleration voltage of 24.0 kV).

Reduction of the maltotriose fraction.—A solution of the lyophilised maltotriose fraction (1 mL) in 0.5 M NH_4OH containing $NaBD_4$ (10 mg/mL) was kept for 1 h at rt. Then, the solution was neutralised with glacial HOAc and concd in vacuo to dryness. Residual borate was removed by co-evaporation with 9:1 MeOH–HOAc (4 ×), then MeOH (3 ×).

Analytical procedures.—Monosaccharide analysis was carried out by subjecting partially methylated MeAl to methanolysis (methanolic 1 M HCl, 18 h, 85 °C). The resulting mixture of methyl glucoside derivatives was trime-

thylsilylated (1:1:5 hexamethyldisilazane–trimethylchlorosilane–pyridine), identified by GLC–MS [16], and quantified by GLC using empirical molar response factors [14]. GLC analyses were performed on a WCOT CP-SIL 5CB fused-silica capillary column (25 m × 0.32 mm) using a temperature program of 110–230 °C at 4 °C/min. GLC–MS of (O-methylated) glucose derivatives, measured as trimethylsilylated methyl glucosides, was carried out on an MD800/8060 system (Fisons instruments; electron energy, 70 eV), equipped with a DB-1 fused-silica capillary column (30 m × 0.32 mm, J&W Scientific), using a temperature program of 110–230 °C at 4 °C/min. The reduced maltotriose fraction was permethylated with CD_3I as described previously (Me_2SO–NaOH) [18], and analysed by FABMS, performed on a JEOL JMS SX/SX 102A four-sector mass spectrometer, operated at 10 kV accelerating voltage, and equipped with a JEOL MSFAB 10 D FAB gun operated at a 10 mA emission current, producing a beam of 6 keV xenon atoms. The per(deuterio)methylated oligosaccharide sample was measured over a mass range of m/z 10–1200 in a matrix of *m*-nitrobenzyl alcohol saturated with NaI, using the standard resolution of 1500. CID tandem mass spectra were obtained using the collision cell in the third field-free region of the mass spectrometer, with air as collision gas at sufficient pressure to reduce the intensity of the selected ion beam by approximately 50%. As the collision cell was kept at ground potential, the collision energy in the MS/MS experiments was 10 keV. CID spectra of the per(deuterio)methylated trisaccharide sample were measured over a mass range of m/z 10–800 in a matrix of 4:1 *m*-nitrobenzyl alcohol–glycerol by selecting their corresponding $[M+H]^+$ pseudo-molecular ion.

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