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Substitution patterns in methylated potato starch as revealed from the structure and composition of fragments in enzymatic digests

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ABSTRACT

The effect of the granule structure on the methylation of starch was investigated by comparing the substitution patterns of potato starch methylated in granular suspension and in solution to DS 0.3. Substitution patterns were analyzed by successive digestion with α-amylase and amyloglucosidase, fractionation of the resulting malto-oligosaccharide mixture by GPC on a preparative scale, and characterization of the fractions by GLC and MALDI–MS. The mass composition of fractions with intermediate and higher degree of polymerization was indicative of enhanced clustering of substituents in granular methyl starch. On the contrary, the composition of the smaller saccharides was governed by enzyme specificity, which was also reflected in strong deviations in their monomer composition. A sequencing study on selected 'pure' small saccharides confirmed and complemented previous conclusions on enzyme specificity.

1. Introduction

In nature, starch macromolecules are found within small semi-crystalline granules. To optimize and extend the application of starch, a small number of the hydroxyl groups of its monomer units is modified chemically.[1,2] The preferred process is modification of the intact granules in aqueous suspension or in the semi-solid state. Because of the organized structure of the granule, not all monomer units are believed to be equally accessible to reaction at those conditions. Current knowledge of the structure of the starch granule suggests that radially oriented amylopectin molecules, with a size in the order of 100 nm, traverse quite a number of successive amorphous and crystalline lamellae, which are tangentially oriented and have a spacing of ca. 10 nm.[3,4] This would give rise to a periodicity in substitution density along the amylopectin molecule: crystalline straight chain regions which are virtually devoid of substituents would alternate with amorphous branching regions which contain the majority of modifying groups. The position and orientation of amylose within the granule are still unknown, but there is evidence that amylose is located in the amorphous zones.[5–7]

A common approach to probe for non-uniformity of substitution patterns is based on the observation that the specificity of starch-degrading enzymes is altered by the presence of substituted monomer units. One or more glucosidic bonds adjacent to a modified glucose unit may not be available for hydrolysis by α-amylase or amyloglucosidase.[8,9] Consequently, a sequence of unsubstituted monomers of a certain minimum length is required for enzymatic hydrolysis to occur. Because the probability for longer unsubstituted sequences is higher for block-wise than for random substitution, a higher degree of digestibility is expected in the former case.

In a previous paper we have addressed this issue by studying the substituent distribution in malto-oligosaccharide fractions obtained by exhaustive digestion by α-amylase and amyloglucosidase of potato starches which had been methylated in solution and in granular suspension.[10] On the basis of distinct differences in malto-oligosaccharide composition and degree of substitution (DS = average number of substituents per monomer) between the two types of modified starch, we concluded that granular methyl starches are substituted in a more block-wise manner. Van der Burgt et al. demonstrated that the branched regions in the amylopectin fraction of methyl starch had a higher substitution density than the linear ones and that amylose was more highly substituted than amylopectin.[11] Similar conclusions were obtained for suspension-modified cationic starch,[12,13] and for hydroxypropyl starch.[14] This is in agreement with a higher accessibility of the amorphous regions, where the amylopectin branching zones and amylose are located.

Analytical techniques for studying substitution patterns include GLC–MS and non-fragmenting mass spectrometry like MALDI–MS. GLC yields average characteristics and probes regioselectivity, that

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is, the preferred site of reaction within a monomer unit, whilst MALDI–MS affords detailed molar mass and DS distributions of populations of modified oligosaccharides. These techniques are usually applied to unfractionated enzymatic digests.[13–15] More detailed information is obtained if the malto-oligosaccharides in the digests are separated prior to analysis, for example, by gel permeation chromatography (GPC).

In this paper we present a GLC and MALDI–MS analysis of oligosaccharide fractions, obtained by preparative GPC, from enzymatic digests of starch methylated to DS 0.3 in granular suspension and in molecular solution. In order to gain more information on enzyme specificity, two fractions containing a single saccharide (disregarding isomers) were sequenced, applying a strategy based on methylation analysis. This revealed the location of the substituted and unsubstituted monomers within the saccharide. Part of this work has been reported previously in concise form.[16]

From a practical point of view, this work is of interest because it may offer guidelines in tailoring enzymatic digestibility of chemically modified food starches. Although methyl starch is not food grade, it may serve as a convenient model at least for starch ethers.

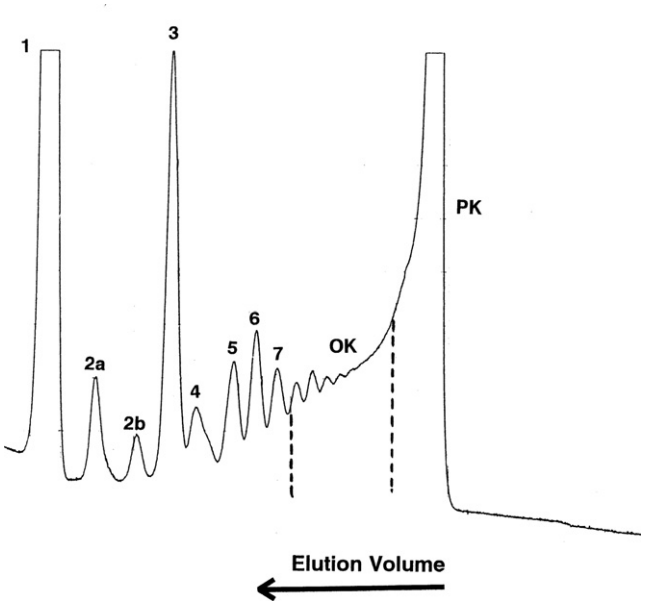

Figure 1. GPC elution profile on Biogel P2 of α-amylase/amyloglucosidase digest of granular methyl starch DS 0.31. Labels represent fraction numbers. OK and PK denote oligomer and polymer fractions, respectively. Reprinted from Ref. 16 with permission of Woodhead, UK.

2. Results and discussion

2.1. Preparation of oligosaccharide fractions

Potato starch was methylated to DS 0.31 both in aqueous granular suspension and in aqueous solution. After dissolution in water the methylated starches were digested exhaustively by α-amylase followed by amyloglucosidase. For the sake of a better defined enzyme specificity, purified α-amylase from *Bacillus amyloliquefaciens* and amyloglucosidase from *Aspergillus niger* were used instead of industrial enzymes. The dosage of the enzymes was adjusted so that the yield of glucose was maximal and insensitive to further variations in dosage. The resulting malto-oligosaccharide mixtures were fractionated by preparative GPC on Biogel P2 (Fig. 1). In comparison to our earlier work,[10] the GPC separation was improved by desalting the enzymatic digests and by a different column setup. Hereby the number of recovered fractions was increased from 5 to 10. Fractions were dried prior to their use in the GLC, MALDI–MS and sequencing studies.

2.2. GLC analysis of digests and oligosaccharide fractions

Average characteristics of the digests and oligosaccharide fractions are displayed in Table 1. Glucose contents of the digests (not shown) closely matched the amounts of fraction 1, which was found by GLC to have DS close to zero, indicating that no substituted monomer was released on digestion by the enzymes used.

We observed that DS of fractions 1–7 was not sensitive to reaction conditions (suspension or solution reaction). The release of a saccharide with given degree of polymerization (DP) and DS requires a monomer sequence within the saccharide that makes it resistant to further enzymatic attack (a) and the presence of unsubstituted sequences of sufficient length for cleavage at either side of the fragment (b). The insensitivity of DS of these fragments to reaction conditions together with requirement (a) suggest that DS of small saccharides is dictated by enzyme specificity: a DS of 0.35–0.48 appears to be a threshold for resistance to enzymatic attack. This threshold DS of the fragments ($\leqslant$0.5) together with requirement (b) indicate that these saccharides stem from sparsely to moderately substituted regions in the original starch. The oligomer and polymer fractions had DS equal to or higher than the threshold. Besides, DS became dependent on reaction conditions, being higher for the granular starch. These fragments stem from regions in the original starch that contain unsubstituted sequences too short to be hydrolyzed. After digestion, 56.8% of the methoxyl groups of the suspension-modified starch were detected in the

Table 1
Amount, DS and substituent distribution of GPC fractions from α-amylase/amyloglucosidase digests of methyl starch DS 0.31

Fraction number[a]	Suspension reaction			Solution reaction		
	Amount (%)	DS[b]	HO-2:3:6[c]	Amount (%)	DS	HO-2:3:6
Total		0.36	76.0:14.4:9.6		0.31	75.5:14.4:10.1
1	36.1	0.04		28.9	0.02	
2a	2.0	0.01		0.9	0.02	
2b	0.7	0.47	19.8:3.0:77.1	1.1	0.48	15.8:2.9:81.3
3	6.9	0.35	89.0:5.6:5.4	7.9	0.34	83.7:10.6:5.7
4	1.8	0.35	59.4:9.9:30.7	3.0	0.34	47.8:32.6:19.6
5	2.1	0.48	74.8:11.8:13.4	3.3	0.49	68.5:16.1:15.4
6	2.5	0.44	79.3:11.0:9.7	4.0	0.42	76.4:15.2:8.4
7	2.0	0.48	75.2:13.3:11.5	3.6	0.45	73.0:16.8:10.2
Oligomer	12.2	0.55	75.3:12.8:11.9	23.9	0.49	76.1:14.1:9.8
Polymer	33.7	0.57	75.6:15.0:9.4	23.4	0.46	76.2:15.0:8.8

[a] See Figure 1.
[b] From GLC.
[c] Ratio of substituents on positions 2, 3 and 6 of the monomer.

polymer fraction. For the solution-modified starch this amounted to only 33.4%. This difference, together with the larger amount of liberated glucose, reflects the increased clustering tendency of substituents in granular methyl starch.

It is well documented that in etherification reactions at mild alkaline conditions, substitution at HO-2 is favoured.[7,17,18] Our results confirm this tendency for almost all the fractions, with notable exceptions for fractions 2b and 4. In fraction 2b, and to a much smaller extent in fraction 4, we observed an enhanced preference for position HO-6. On the other hand, the preference for HO-2 was even more pronounced in fraction 3. As we will show below, fractions 2b and 3 consist of maltose and maltotriose, respectively, bearing a single methoxyl group. These results confirm the earlier established fact that hydrolysis by starch-degrading enzymes is hampered more strongly by substitution at HO-2 than at HO-6.[10,19] A 6-*O*-methyl glucose monomer protects only one adjacent glycosidic linkage from scission, whilst a 2-*O*-methyl glucose unit blocks two adjacent linkages. More precisely, in the latter case one of these two linkages is cleaved at a much reduced rate. The near absence of 3-*O*-methyl glucose in fraction 2b and the deviating regioselectivity in fraction 4 will be discussed in Section 2.4.

2.3. MALDI–MS analysis of oligosaccharide fractions

Detailed information on the composition of the fractions was gained from MALDI–MS experiments. MALDI mass spectra of fractions 2–7 containing the small saccharides with DP up to six revealed the presence of at most two different oligosaccharides in each fraction, which is an indication of the quality of the GPC separation in this molar mass range (Table 2). Elution behaviour on Biogel P2 was affected by the presence of substituents: the elution volume at a specified DP shifted to lower values as the number of methoxyl groups increased. DS of each fraction was calculated from the mass and the relative abundance of molecular ions. The results were in good agreement with DS from GLC (compare Tables 1 and 2). Corresponding fractions from suspension- and solution-modified starch contained the same components (including isomers) and differed only in their relative amounts. This confirms the predominant influence of enzyme specificity on fraction composition in this molar mass range. Disregarding isomers, fractions 2a, 2b, 3 and 5 contained a single saccharide, which makes these fractions obvious candidates for the sequencing study discussed in Section 2.4. Our results demonstrate that fractions 2b and 3 indeed consist of a dimer and a trimer, respectively, bearing a single substituent. This corroborates our interpretation of the effects of substitution position on the availability of neighbouring glycosidic bonds to starch-degrading enzymes.

Figure 2 shows an expanded scale section of a MALDI mass spectrum of an oligomer fraction. It proves that this technique is capable in resolving all oligomers with different numbers of methoxyl groups within a family with the same DP. Compositional details of oligomer fractions are given in Table 3. The most significant observations are that DS of the most abundant species (DS at peak) is higher for the suspension reaction for oligomers with DP 7–10 (a) and that the range in *S*, the number of methoxyl groups per molecule, is broader for suspension-modified than for solution-modified starch, the lower *S* limit being about equal for both reactions (b). Accordingly, the upper *S* limit is higher for methylation in suspension. This, together with observation (a) is a consequence of substituent clustering. The lower *S* limit corresponds to DS 0.33–0.45 for all DP >6, irrespective of reaction conditions. This is in good agreement with the threshold DS for resistance to enzymatic attack, discussed in the previous section. It follows that also saccharides of intermediate size may originate from less densely substituted regions. Other phenomena such as the decrease in DS at peak with increasing DP at DP >11 and the narrower DP range in suspension-modified starch are explained by the elution behaviour of the GPC system.

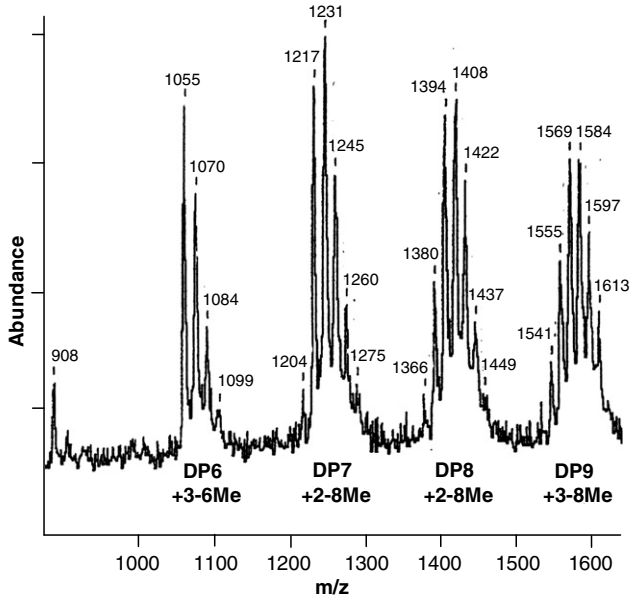

Figure 2. Expanded scale section of MALDI mass spectrum of oligomer fraction from enzymatically digested granular methyl starch. Labels denote the mass (m/z) of $[M-Na]^+$ molecular ions of methylated oligosaccharides.

Table 2
Composition of small saccharide fractions from α-amylase/amyloglucosidase digests of methyl starch

Fraction number[a]	Suspension reaction				Solution reaction			
	DP	*S*[b]	Amount (%)	DS[c]	DP	*S*	Amount (%)	DS
2a	2	0		0.00	2	0		0.00
2b	2	1		0.50	2	1		0.50
3	3	1		0.33	3	1		0.33
4	3	2	15	0.31	3	2	36	0.40
	4	1	85		4	1	64	
5	4	2		0.50	4	2		0.50
6	4	3	17	0.46	4	3	7	0.42
	5	2	83		5	2	93	
7	5	3	60	0.49	5	3	45	0.45
	6	2	40		6	2	55	

[a] See Figure 1.
[b] Number of methoxyl groups per molecule.
[c] DS of fraction calculated from mass spectrum.

Table 3
Composition of oligomer fractions from α-amylase/amyloglucosidase digests of methyl starch[a]

DP	Suspension reaction (DS_{GLC} 0.55)		Solution reaction (DS_{GLC} 0.49)	
	S range	DS at peak[b]	*S* range	DS at peak
5	4	0.80	4	0.80
6	3–6	0.50	3–5	0.50
7	3–6	0.57	3–5	0.50
8	3–7	0.62	3–5	0.50
9	3–8	0.61	4–5	0.56
10	4–8	0.65	4–6	0.55
11	5–9	0.64	5–7	0.64
12	5–7	0.58	5–9	0.59
13	7	0.54	5–8	0.62
14			6–9	0.57
18			8	0.44

[a] Results for DP 15–17 show a similar trend.
[b] DS of most abundant species.

Table 4
Composition of polymer fractions from α-amylase/amyloglucosidase digests of methyl starch

DP range	DS at peak, range[a]	
	Suspension reaction (DS_{GLC} 0.57)	Solution reaction (DS_{GLC} 0.46)
12–14	0.69–0.79	
15–20	0.69–0.76	0.52–0.60
21–27	0.71–0.76	0.52–0.57
28–34		0.50–0.57
35–41		0.47–0.51

[a] DS of most abundant species.

MALDI mass spectra of the polymer fractions are shown in Figure 3. The division between oligomer and polymer fractions was chosen arbitrarily at an elution volume, where the resolution of the GPC system was low (Fig. 1). Interestingly, the maximum DP encountered in the digest is higher for the solution-methylated than for the suspension-methylated starch. This suggests that the observed maximum DP 27 in granular methyl starch is related to the size of accessible regions in amylopectin. As DP increased it became difficult to completely resolve populations with specified DP and varying *S* by MALDI–MS. Hence, only DS at peak is reported in Table 4. The tendency already established for oligomers was observed: DS at peak was higher for the suspension reaction at a given DP. The lowest reported values of DS at peak exceed the threshold DS for resistance to enzymatic attack to a large extent for suspension-modified starch and to a modest extent for the solution-modified starch. This indicates that all these large oligomers have their origin in the more densely substituted regions. However, it has to be borne in mind that DS at peak will always be larger than minimum DS.

2.4. Substitution pattern and enzyme specificity

For a proper interpretation of the results it is essential that the effect of substitution on the specificity of the enzymes involved is known. Mischnick has addressed this issue for α-amylase/amyloglucosidase digests of methyl amyloses and arrived at the following conclusions.[20] The monomer unit at the reducing end of methylated oligosaccharides in the digest is usually unsubstituted (a) and amyloglucosidase is able to cleave the linkage directly adjacent to a 2-*O*- and 6-*O*-methylated glucose unit (b), but not the linkage adjacent to a monomer substituted at HO-3 (c). The observation that the dimer fraction 2b is enriched in 6-*O*-methylated glucose and depleted in 2-*O*-methylated glucose, whereas the reverse is true for the trimer fraction 3 (Tables 1 and 2), already suggests, together with Mischnick's conclusion (b), that the preferred position of methylation in these fractions is on the monomers at the non-reducing end.

This was checked by a sequencing study on fractions 3 and 5, which each contained a single oligosaccharide (disregarding isomers) (Table 2). Our approach was slightly different from that of Mischnick.[20] The oligosaccharide was reduced, per-deutero-methylated and converted into a mixture of partially methylated, partially deutero-methylated alditol acetates, which were analyzed by GLC–MS with chemical ionization (CI). In this way, mass differences were introduced between originally substituted and unsubstituted monomers, and also between internal monomers and monomers at the reducing and non-reducing terminals. Figure 4 displays the CI mass spectrum of 1,5-*O*-acetyl 2,3,4,6-*O*-methyl glucitol, corresponding to the monomer at the non-reducing end of the tetramer in fraction 5. Clearly the most abundant signals are the $[M-NH_4]^+$ and $[M-H]^+$ molecular ions with *m*/*z* 350 and 333, respectively, indicating predominant monosubstitution at that glucose unit. Weak signals at *m*/*z* 347 and 353 reveal that only very small amounts of this monomer bear 2 and 0 substituents, respectively. Table 5 summarizes the results, which show that the trimer has been substituted predominantly at its non-reducing monomer, whilst substitution in the tetramer has also occurred at the internal units.

By combining the results in Tables 1, 2 and 5 with those reported by Mischnick,[20] the preferred sites of scission by the successive action of α-amylase and amyloglucosidase on starch chains containing monomers methylated at different positions

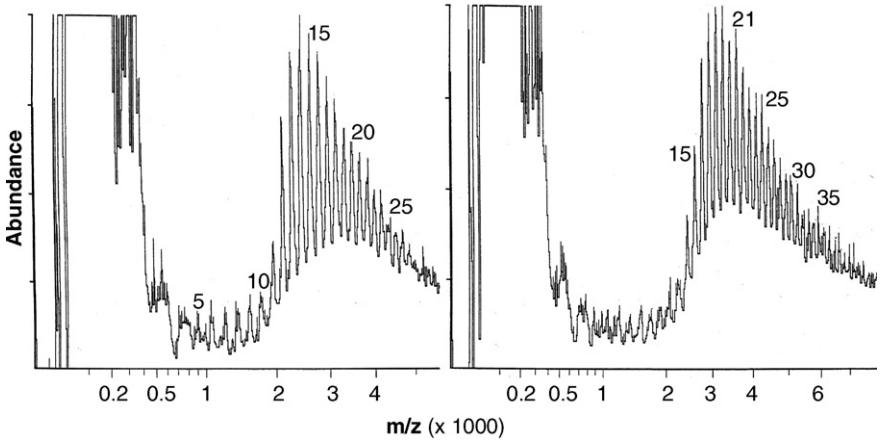

Figure 3. MALDI mass spectra of the polymer fractions of enzymatically digested starch methylated in suspension (left) and in solution (right). Numbers denote DP.

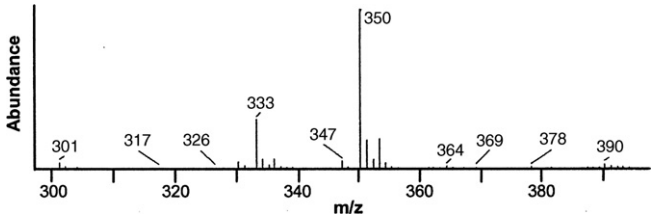

Figure 4. CI mass spectrum of 1,5-*O*-acetyl 2,3,4,6-*O*-methyl glucitol from DP 4 + 2 Me (=fraction 5), obtained from suspension-methylated potato starch.

Table 5
Mass (*m*/*z*) of most abundant $[M-NH_4]^+$ molecular ions in CI mass spectra of methylated alditol acetates from fractions 3 (DP 3 + 1 Me) and 5 (DP 4 + 2 Me)

Alditol acetate[a]	DP 3 + 1 Me		DP 4 + 2 Me	
	Unsubstituted	Substituted	Unsubstituted	Substituted
1,2,3,5,6-*O*-Me	328		328	
2,3,6-*O*-Me	378		378	375
2,3,4,6-*O*-Me		350		350

[a] Alditol acetates correspond to reducing, internal and non-reducing monomer, respectively (top to bottom).

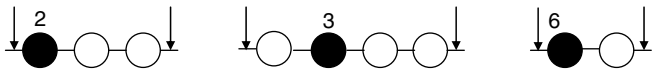

Figure 5. Sites of scission by amyloglucosidase (left arrows) and α-amylase (right arrows) in starch chains containing monomers methylated at positions 2, 3 or 6, subjected to successive digestion by α-amylase and amyloglucosidase.

were identified (Fig. 5). The predominance of 6-O-substitution in the dimer fraction 2b and its depletion in the trimer fraction 3 indicate that a glucose unit substituted at HO-6 protects one single linkage at its reducing side from scission by α-amylase. A glucose monomer substituted at HO-2 protects two adjacent linkages at its reducing side, as follows from the predominance of 2-O-substitution in fraction 3 and its depletion in fraction 2b.

Much of the data in Tables 1 and 2 can be explained on the basis of this result. The depletion of substitution at HO-3 in fractions 2b and 3 and its enrichment in fraction 4 (the latter observed only in solution-methylated starch, Table 1) support Mischnick's observation (c). This enrichment is reflected by the presence in fraction 4 of a tetrasaccharide bearing a single methoxyl (Table 2). The enhanced substitution of HO-6 in fraction 4 is due to a trisaccharide with two methoxyls (Tables 1 and 2).

The methods and results presented here can assist in the design of modified food starches with tailored digestibility. If our results are also valid for food grade starch derivatives and for the starch-degrading enzymes in the human intestinal tract, we may expect that reaction conditions which favour uniformity of substitution (e.g., use of pregelatinized starch in the reaction) and promote substitution at HO-3 and HO-2 lead to enhanced resistance against starch-degrading enzymes at a given DS.

3. Experimental

3.1. General methods

Dry matter content of modified starches was calculated from the weight loss after heating (130 °C, 2 h). Total carbohydrate and glucose were assayed with anthrone[21] and the glucose oxidase method, respectively. DS of methylated starches was determined from the methoxyl content as measured by a modified Zeisel method.[10,22]

The monomer composition and DS of methyl starches and GPC fractions recovered after enzymatic digestion were estimated by GLC, after conversion into partially methylated glucitol acetates by hydrolysis with 2 M CF_3CO_2H (0.1 mL/mg, 125 °C, 1 h),[23] removal of acid by repeated evaporation with ethanol (60 °C, N_2) as an adjuvant, sodium borohydride reduction and acetylation, according to Jansson et al.[24] Sample size was 1 mg. GLC with flame ionization detection was performed on CP-Sil 5CB (25 m × 0.53 mm) with temperature programme: 150 °C (1 min), to 200 °C (2 min), to 250 °C (10 min), to 290 °C (1.6 min), 290 °C (1 min). Monomer compositions were calculated from GLC peak areas which were corrected by accounting for the effective carbon number of the components.[25] Improved procedures for reduction and acetylation were adopted in the course of the research and are described in Section 3.5.

3.2. Methylation of potato starch

Potato starch was a gift from AVEBE. It was methylated with Me_2SO_4 in granular suspension (0.426 g Me_2SO_4/g air-dry starch) and in solution (1.384 g Me_2SO_4/g air-dry starch) to DS 0.31 at a constant pH 11 as described previously.[10] Aqueous 0.75 M NaOH was added by controlled dosage to ensure constant pH. Medium for suspension reactions was aqueous 25% (w/w) Na_2SO_4, which served to inhibit starch granule swelling. In order to prevent a too strong decrease of the salt concentration by NaOH addition, NaOH solutions were prepared in aqueous 10% Na_2SO_4. Granular methylated starches were filtered, and washed with water or aqueous acetone. No Na_2SO_4 was used in solution reactions where, if necessary, some water was added to maintain an acceptable viscosity level. Solution reaction products were precipitated in excess acetone and washed repeatedly with aqueous 80% (v/v) acetone.

3.3. Enzymatic digestion

Methylated starch (5 g dry base) in tap water (92 g) was cooked with stirring and subsequently treated in a pressure cooker (140 °C, 15 min). The solution was incubated (20 °C, 1 h) with a solution (1 mL, 10 mg/mL) of α-amylase (*Bacillus sp.*, Sigma A 6380, from *Bacillus amyloliquefaciens*, 1800 units/mg). After inactivation (100 °C, 15 min), a solution in 1 M sodium acetate buffer, pH 4.6 (2 mL, 25 mg/mL), of amyloglucosidase (*Aspergillus niger*, Sigma A 7420, 36 units/mg) was added. After incubation (50 °C, 20 h), the enzyme was inactivated (100 °C, 15 min), and the digest was centrifuged (10,000 rpm, 20 min) and filtrated (0.45 μm). Part of the filtrate was desalted successively with a strong acidic cation exchange resin (Dowex AG 50W-X8, H-form) and a weakly basic anion exchange resin (Dowex AG 3-X4A, OH-form). Solutions were stored (−18 °C) until use.

3.4. Preparative GPC

GPC was performed on Biogel P2 (columns 2 × 1 m, diameter 50 mm, Pharmacia K50/100). Enzymatically degraded methyl starch solution (5% dry matter, 20 mL) was eluted with water (120 mL/h, 37 °C) and collected in portions (20 mL). Portions corresponding with the bulk of the peaks enumerated in Figure 1 were pooled into separate fractions. Aliquots of each fraction, corresponding to 5 mg carbohydrate, were freeze-dried. Intermediate material between peaks was used only for establishing mass balances.

3.5. MALDI–MS and sequencing study

A Finnegan MAT Vision 2000 time-of-flight mass spectrometer equipped with a UV nitrogen laser (337 nm) was used for

MALDI–MS of freeze-dried GPC fractions. Pulse duration was 3 ns. 2,5-Dihydroxy benzoic acid (10 mg/mL in water) was used as the matrix. Matrix and analyte solutions were mixed in a ratio 2:1. Analyte concentration in the sample solution was 100 μg/mL.

For the sequencing study presented in Section 2.4, fractions 3 and 5 (2 mg) were reduced (3 h, with occasional agitation) in aqueous $NaBH_4$ (400 μL, 5 mg/mL). Boron compounds were removed by adding 0.5 mL Dowex AG 50W-X8 (H-form), filtration (0.2 μm), washing with water (0.5 mL), evaporation (50 °C, N_2) and repeated evaporation after addition of methanol (5 × 0.5 mL).[24] The dried residue (P_2O_5, 1 h, 60 °C, reduced pressure) was dissolved in dry DMSO (0.2 mL) and methylated (15 min) with CD_3I (50 μL) in the presence of powdered NaOH (10 mg).[26] Water (1 mL) and CH_2Cl_2 (1 mL) were added and the organic layer was washed with water (3 × 5 mL, with centrifugation) and evaporated (40 °C, N_2). The residue was hydrolyzed (100 °C, 16 h) with H_2SO_4 (0.13 M, 3 mL). The hydrolysate was neutralized with $BaCO_3$ and filtered (0.2 μm). The filter residue was washed with water (2 × 1 mL). Filtrate and washings were combined and freeze-dried. Reduction with $NaBD_4$ was performed as described above (no removal of boron compounds) and the solution was freeze-dried. The sample was acetylated by suspending it in glacial HOAc (80 μL), adding ethyl acetate (400 μL), acetic anhydride (1.2 mL) and perchloric acid (70%, 40 μL) with intermittent mixing, and allowing the reaction to complete (5 min).[27] The mixture was cooled on ice, and water (4 mL, caution) and 1-methyl imidazole (80 μL) were added. After 5 min, the partially methylated alditol acetates were extracted into CH_2Cl_2 (1 mL) with centrifugation and an aliquot of the extract (0.5 mL) was filtered (0.2 μm, PTFE). The filter was washed with CH_2Cl_2 (0.3 mL). Washing fluid and filtrate were combined.

GLC–MS with chemical ionization was performed on a HP Ultra 2 column (25 m × 0.2 mm), with the temperature programme: 50 °C (1 min), to 200 °C (15 min), to 250 °C (10 min), to 290 °C (1.6 min). Ammonia in methane was used as the chemical ionization gas.

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