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Substitution patterns in methylated starch as studied by enzymic degradation

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Abstract

Potato starch was methylated in granular suspension and in solution to degrees of substitution (ds) up to 0.8. The distribution of methyl groups along the starch chains was probed by consecutive enzymic degradation with alpha-amylase and amyloglucosidase, followed by structural characterisation of isolated fragments. A minimum sequence length of two unsubstituted glucose residues is required for amylolysis to occur. At a given ds, larger amounts of glucose and more highly substituted material of high molecular weight were obtained from granular *O*-methylstarches than from starches methylated in solution. Although the differences between both classes of methylated starch were not very pronounced, this result is taken as evidence that granular *O*-methylstarches are substituted in a more blockwise manner.

1. Introduction

Apart from being a major constituent of foodstuffs, starch is important as a raw material for the manufacture of sweeteners, thickening and binding agents, and adhesives. For this purpose, starch is modified chemically by hydrolysis of glucosidic bonds or by partial substitution of the hydroxyl groups of individual glucose residues [1–3]. Almost all substitution reactions are carried out in heterogeneous systems, e.g., on intact starch granules to a very low degree of substitution (ds). The ds denotes the average number of substituents per glucose residue. The granular nature of starch in the reaction vessel enables high starch concentrations to be handled and ensures easy recovery of the product.

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Starch granules are semicrystalline. The crystalline lamellae are ordered tangentially with a spacing of ca. 10 nm [4–6]. The amylopectin molecules are arranged radially [7–9]. It is generally accepted now that the double-helical arrangements [10–12] of the short side-chains of amylopectin (dp ≃ 15) make up the framework of the crystalline domains [13]. The position and orientation of amylose within the granule are still unknown. However, there is evidence that amylose is located in the amorphous zones [13–15].

In view of the organised nature of the granules, it may be questioned whether or not substituents are introduced randomly in heterogeneous reactions. In a previous paper [16], topochemical effects of heterogeneous methylation were discussed at different structural levels and distance scales: granule surface, amylose and amylopectin, crystalline and amorphous zones, and HO-2,3,6 of the glucose residue. Acid degradation studies suggested that substitution takes place preferentially, if not exclusively, in the amorphous domains. As a consequence, a higher reactivity of the amylose fraction was observed. The same conclusion was reached by Hood and Mercier [17], based on enzymic degradation patterns. On the other hand, no topochemical effects were observed at length scales much larger or much smaller than the crystalline/amorphous domain size: surface effects were absent and no influence of the physical structure of the granule on the reactivity of different hydroxyl groups could be demonstrated.

In the present paper, we address the distribution of methyl groups along the chains of starch molecules. An individual amylopectin molecule (size ca. 100–500 nm) [18] traverses a large number of successive amorphous and crystalline layers, so one could expect a blockwise substitution pattern along the chains.

2. Experimental

Materials.—Potato starch was a gift from AVEBE b.a. (Veendam, The Netherlands).

General methods.—Samples were evaporated at reduced pressure at 40°C. Dry matter was calculated from the weight loss after heating (130°C, 2 h). Total carbohydrate, reducing sugars, and glucose were assayed with anthrone [19,20], the Nelson–Somogyi method [20], and the glucose oxidase method, respectively. Iodine-binding capacity (ibc) was measured by amperometric titration with potassium iodate in acidic medium [21].

The ds of methylated starches was calculated from methoxyl content by a modified Zeisel method [22]. Methoxyl groups were cleaved by treatment with boiling aq 57% HI and the resulting iodomethane was transferred into acidified standard $AgNO_3$ solution by a stream of N_2. Precipitated AgI was assayed by back titrating the standard solution with ammonium thiocyanate.

The monomer composition and the ds of column fractions recovered after enzymic degradation was estimated by GLC [23] after conversion into partially methylated glucitol acetates by hydrolysis with 2 M CF_3CO_2H [24], borohydride reduction, and acetylation, as described previously [16].

Table 1
Method of preparation, degree of substitution (ds), and iodine-binding capacity (ibc) of methylated starches

Method of preparation	Amounts (g)				Washing [a]	Ds	Ibc (%)
	Starch	Na_2SO_4	H_2O	Me_2SO_4			
Suspension	100	50	150	23	w	0.18	2.1
Suspension	100	50	150	53	w	0.29	0
Suspension	100	50	150	133	w/50%a	0.41	
Suspension	50 [b]	50	150	66	50%a	0.70	
Solution	25		600	13	80%a	0.12	3.4
Solution	25		600	40	80%a	0.27	2.2
Solution	25		600	66	90%a	0.37	1.5
Solution	12.5		250	100	90%a	0.76	0

[a] a = Aqueous acetone (% v/v); w = water.
[b] Starting from *O*-methylstarch ds 0.41.

The degree of polymerisation (dp) and the degree of branching (db) of column fractions of enzyme-degraded methylated starches were determined by methylation analysis. Fractions containing ca. 10 mg of carbohydrate were evaporated to dryness, dissolved in Me_2SO (1 mL), and methylated with MeI (250 μL) in the presence of powdered NaOH (50 mg) during 15 min [25]. The mixture was extracted with $CHCl_3$ (4 × 3.5 mL). The organic layer was washed with water (4 × 25 mL) and evaporated. The permethylated oligosaccharides were converted into their partially methylated glucitol acetates and analysed by GLC as described above. The dp was calculated from the molar fractions of nonreducing end groups and branched monomer units in the oligosaccharide. Db is defined here as the molar percentage of branched glucose residues.

Methylation of potato starch.—For the preparation of granular *O*-methylstarches [26] (Table 1), potato starch was suspended in aq 25% (w/w) Na_2SO_4, and the pH was adjusted to 11. Following dropwise addition of dimethyl sulfate (1 h), the reaction was continued with stirring for 20 h (pH 11, room temperature). The pH was kept constant by addition of aq 0.75 M NaOH by means of an automatic titrator. The suspension was neutralised to pH 6.5, filtered, washed with water or aq acetone, then air-dried.

For homogeneous methylation (Table 1), starch was dissolved in distilled water in a pressure cooker (140°C, 15 min), and the reaction was carried out as described above, but in the absence of Na_2SO_4. After neutralisation, the mixture was poured into acetone (10 vol), and the methylated starch was filtered off, washed with aq acetone, then anhyd acetone, and air-dried.

Enzymic degradation.—Methylated starch (6 g) was dissolved in water in a pressure cooker (140°C, 15 min). Citrate buffer (pH 6; 0.1 M; 10 mL) and alpha-amylase from *Bacillus amyloliquefaciens* (BAN-L120, NOVO, 125 μL) were added and the total weight was adjusted to 110 g with aq $CaCl_2$ to a final $CaCl_2$ concentration of 0.02%. After incubating the sample (50°C, 24 h), alpha-amylase was inactivated by heating at 100°C (15 min). Subsequently, the pH was adjusted to

4.2 with aq citric acid (0.1 M). Amyloglucosidase from *Aspergillus niger* (NOVO, 125 μL) was added, and the total weight was made up to 125 g by addition of water. Following incubation (50°C, 24 h), the enzyme was inactivated (100°C, 15 min) and the sample was freeze-dried.

Analytical GPC.—Analytical GPC was performed on Biogel P2 (column length, 1 m; diameter, 16 mm; Pharmacia K16/100). Aqueous 5% carbohydrate (100 μL) was eluted with distilled water at 37°C at a rate of 7–8 mL/h. Fractions of ca. 2 mL were collected and weighed. Fractions corresponding to each peak in the chromatogram were pooled and the molecular weight distribution was calculated from their carbohydrate content.

Preparative GPC.—Preparative GPC was performed on Biogel P2 (column length, 1 m; diameter, 50 mm; Pharmacia K50/100). Aqueous 5% carbohydrate (10 mL) was eluted with distilled water at 37°C at a rate of 60 mL/h. Fractions (15 mL) were collected, combined as shown in Fig. 3, and stored at −18°C. In order to recover sufficient amounts of carbohydrate for analysis, each separation was repeated.

3. Results and discussion

Strategies for obtaining sequence information.—The sequence distribution of a copolymer can be defined as the chain length distribution of contiguous sequences of monomers of the same kind (in this case, substituted or unsubstituted glucose monomers). Partial substitution reactions are statistical in nature, so each molecule has its own sequence and only averages can be obtained, whatever method is used. In heteropolysaccharides, sequence information has been obtained conveniently from diad and triad frequencies as measured by NMR [27]. For substituted homopolysaccharides, this approach is not successful because of the large number of valence bonds between a substituent group and a neighbouring monosaccharide unit.

The approach adopted by us is based on the notion that the specificity of starch(and cellulose)-degrading enzymes is modified by the presence of substituted glucose residues. Glucosidic bonds adjacent to modified monomers are resistant or less amenable to amylolysis [28–34] or cellulolysis [35,36]. In fact, a minimum number of unmodified glucose residues adjacent to a substituted monomer is required for enzymic degradation. On this basis, a distinction between random and blockwise substitution can be made [37]. Randomly substituted polysaccharides contain more glucosidic bonds linked to one or two modified monomer units than blockwise-substituted polysaccharides and are therefore degraded to a lesser extent.

A general strategy for sequencing modified starches would involve the following steps: (*a*) the product is degraded with amylases, e.g., alpha-amylase followed by amyloglucosidase; (*b*) the molecular weight distribution is determined; (*c*) degradation products are isolated and characterised structurally; (*d*) the specificity of the enzymes used can then be established; (*e*) the actual product spectrum is

matched against models for various modes of substitution, e.g., random, regular, and blockwise. This approach is similar to that adopted by Valent et al. [38] for structurally well-defined polysaccharides and by McCleary et al. [39] for galactomannans.

Potato starch was methylated in solution (random substitution) and in granular suspension (heterogeneous substitution) up to ds 0.8 (Table 1). Methylated starches were degraded by consecutive treatment with alpha-amylase and amyloglucosidase. The molecular weight distribution was assessed by analytical gel permeation chromatography (GPC). The digest was fractionated according to molecular size by preparative GPC. The ds of each fraction was determined by GLC and the degree of polymerisation (dp) was confirmed by means of methylation analysis. In the final step of our analysis, product spectra of homogeneously and heterogeneously methylated starches were compared qualitatively.

Iodine-binding capacity and monomer composition.—Ibc values of *O*-methylstarches are shown in Table 1. The ibc of methylated starch prepared in granular suspension decreases much more rapidly with increasing ds than the ibc of starch ethers prepared in solution. As ibc reflects exclusively the properties of the amylose fraction, this result suggests a preferential substitution of amylose in granular starch, which corroborates the assumption that amylose is located within the amorphous regions of the starch granules [13–16].

The monomer composition of methylated starches was obtained by GLC of their acid hydrolysates after conversion into the corresponding partially methylated glucitol acetates (Table 2). The regioselectivity of the reaction (percentage of methyl groups located at HO-2,3,6) and the amounts of mono-, di-, and tri-substitution were calculated from the monomer composition (Figs. 1 and 2). The physical state of the starch in the reaction vessel (granular or dissolved) does not affect the regioselectivity and the monomer composition up to ds 0.8. This result is in agreement with our previous conclusion [16] that, in methylated starch of ds 0.15, topochemical effects could be demonstrated only at the level of crystalline/amorphous domains, but not at the level of the much smaller individual

Table 2
Monomer composition of methylated starches (in molar percentages)

Substituent position	Suspension reaction Ds				Solution reaction Ds			
	0.18	0.29	0.41	0.70	0.12	0.27	0.37	0.76
2,3,6-*O*			0.2	1.0			0.1	0.9
2,6-*O*	0.4	1.1	2.3	5.2	0.2	0.6	1.2	5.2
3,6-*O*		0.2	0.4	0.8		0.1	0.2	0.9
2,3-*O*		1.2	2.2	5.5	0.3	0.8	1.4	7.7
6-*O*	1.8	2.6	3.0	3.6	1.3	2.4	4.7	4.0
2-*O*	11.1	17.1	23.0	33.8	7.5	16.4	21.8	40.1
3-*O*	2.5	4.1	4.2	5.5	1.9	3.9	3.4	7.7
0	84.2	73.7	64.7	44.6	88.8	75.8	67.2	33.5

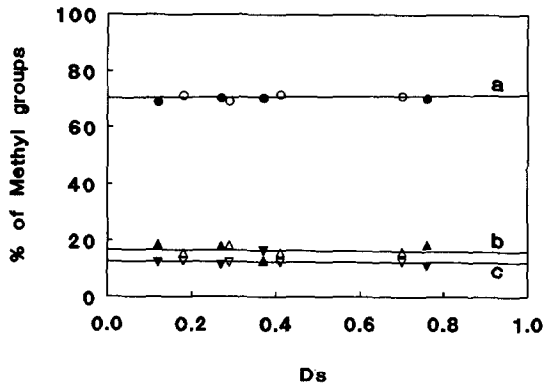

Fig. 1. Percentage of methyl groups located at HO-2 (a), HO-3 (b), and HO-6 (c) in starch methylated in suspension (open symbols) and in solution (filled symbols).

monomers. Our previous study suggested that substitution occurs predominantly in the amorphous phase which grows in extent as the reaction proceeds. The relative reactivity of glucose hydroxyl groups in these amorphous domains is not different from that in solution. This is a requirement for obtaining meaningful sequence

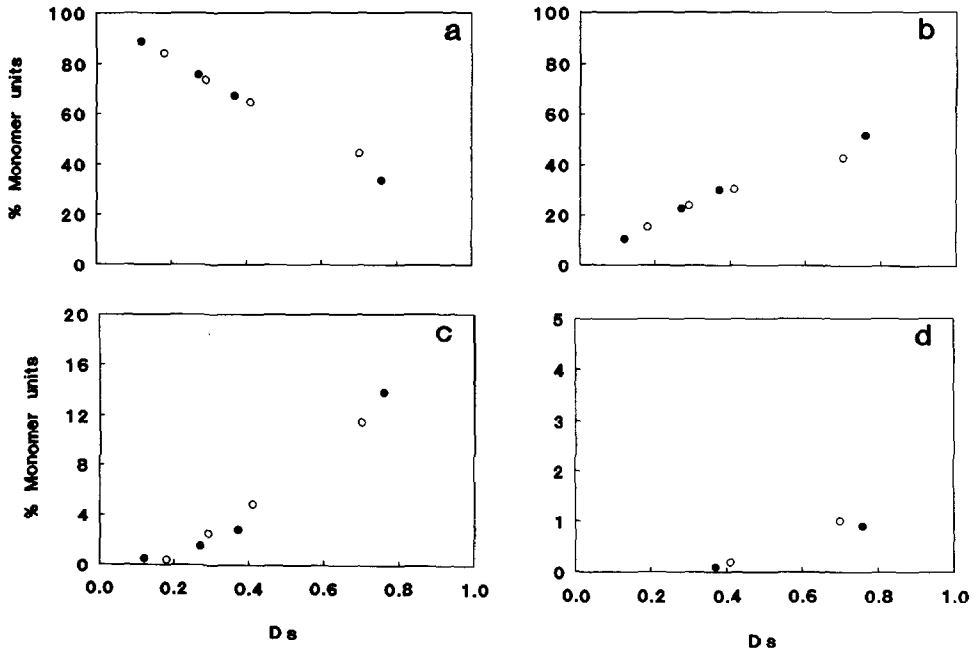

Fig. 2. Percentage of unsubstituted (a), mono- (b), di- (c), and tri-substituted (d) monomer units in starch methylated in suspension (○) and in solution (●).

information from enzymic degradation studies. Variations in monomer composition may well affect the specificity of the enzymes used, and hence lead to a different pattern of hydrolysis.

Molecular weight distribution of hydrolysates.—With the exception of Hood and Mercier [17], who used different enzymes to probe the location of substituent groups in modified starch, all investigations on the fine structure of starch derivatives were performed with alpha-amylase as the sole enzyme [28–34]. Braun et al. [29,40] used modified starches as model substrates for the examination of the action pattern of porcine-pancreatic amylase. We preferred consecutive digestion by alpha-amylase and amyloglucosidase. The main reason was that alpha-amylolysis generates a large number of degradation products, even when unmodified starch is used as a substrate [41,42]. In our approach, starch is degraded to a single product, glucose. All other constituents in the digest arise from the presence of substituents.

Methylated starches were degraded consecutively with commercial preparations of alpha-amylase and amyloglucosidase. In order to ensure exhaustive degradation, optimum conditions were established in preliminary experiments. The molecular weight distribution of the hydrolysates was determined by analytical GPC on Biogel P2. A representative chromatogram is shown in Fig. 3. The elution volumes of partially methylated malto-oligosaccharides are somewhat smaller than those of their unsubstituted counterparts. Therefore, the column was calibrated by checking the dp of some fractions by methylation analysis (see below). As buffer salts are eluted together with the high molecular weight material, fractions corresponding to each peak in the chromatogram were pooled and assayed for carbohydrate, rather than to rely on integrated peak areas. Molecular weight distributions from GPC (Table 3) were in good agreement with reducing power and glucose content

Table 3
Molecular weight distribution (in percentages) of hydrolysates of methylated starches obtained by consecutive action of alpha-amylase and amyloglucosidase

Dp	Suspension reaction Ds				Solution reaction Ds			
	0.18	0.29	0.41	0.70	0.12	0.27	0.37	0.76
Polymer [a]	8.9	27.4	47.4	68.5	2.5	17.2	33.0	93.6
Oligomer [b]	13.5	12.9	8.6	6.5	7.5	17.4	20.6	
7	1.1	2.2	1.5	1.2	1.0	3.6	1.9	
6	3.7	2.5	1.9	1.8	3.4	4.3	3.7	
5	2.5	1.9	1.4	1.0	2.1	3.2	3.6	4.5 [c]
4	1.8	1.9	1.4	1.0	2.3	3.3	2.4	
3	8.8	7.0	5.5	3.8	11.7	10.5	7.2	
2	1.5	1.5	1.0	0.7	2.9	2.0	1.8	
1	58.2	42.7	31.3	15.5	66.6	38.5	25.8	1.9

[a] Fraction 5 in Fig. 3.
[b] Fraction 4 in Fig. 3 with dp > 7.
[c] Dp 2 to oligomer.

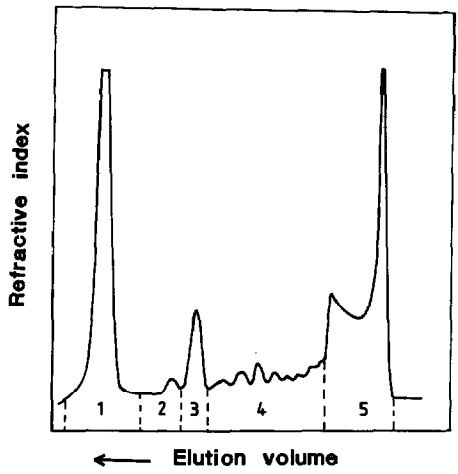

Fig. 3. Gel permeation chromatogram of a hydrolysate of granular *O*-methylstarch ds 0.29, obtained by consecutive treatment with alpha-amylase and amyloglucosidase, and classification of fractions used for structural studies: 1, monomer; 2, dimer; 3, trimer; 4, oligomer; 5, polymer and salt.

of hydrolysates, which were determined separately by colorimetric assays (not shown).

The most conspicuous features of the molecular weight distributions are the predominance of fractions with dp 1, dp 3, and polymer, and the comparatively small amount of dimer (Fig. 3, Table 3). In Fig. 4, we have plotted the amount of some fractions as a function of the ds of the parent methylated starches. Fractions are defined as indicated in Fig. 3. The most important result is that starches methylated in granular suspension afford significantly more monomer than starches methylated in solution. Following the arguments presented above, this result has to be taken as evidence that there exist longer sequences of contiguous unmodified glucose residues in granular *O*-methylstarch than in starch methylated in solution. Another interesting feature is the larger amount of trimer and oligomer liberated from *O*-methylstarches prepared in solution. On the other hand, the amount of polymer produced from both types of methylated starch seems not to be significantly different (Fig. 4).

Fine structure of degradation products.—In order to collect sufficient quantities of material for structural studies, large-scale separations were performed by preparative GPC on Biogel P2. From each separation, five main fractions were obtained, as shown in Fig. 3. Each separation was repeated in order to collect at least 10 mg of each fraction. GLC is a convenient method to determine the degree of methylation of saccharides if only small quantities of material are available. In addition, GLC analysis also affords the monomer composition of the degradation products. Some of the collected fractions were subjected to methylation analysis in order to establish the dp and the db. An advantage of the use of partially methylated starches as model substances in the study of substitution patterns is, of

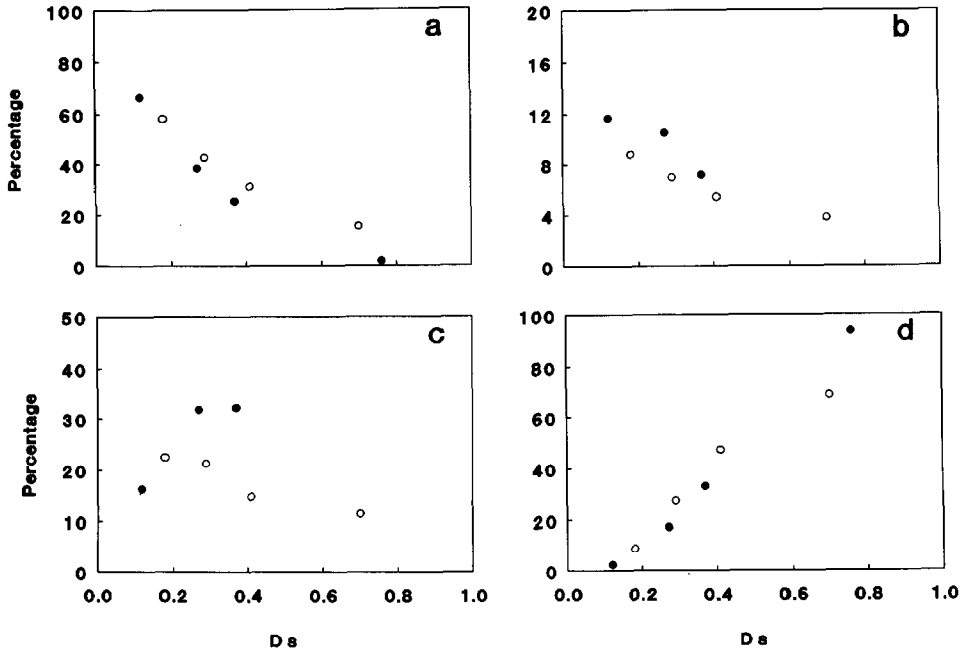

Fig. 4. Percentage of monomer (a), trimer (b), oligomer (c), and polymer (d) released by consecutive action of alpha-amylase and amyloglucosidase from starch methylated in suspension (○) and in solution (●).

course, that substituent groups already present do not interfere with the methylation analysis.

Ds values of fractions are presented in Table 4. The monomer fraction is essentially unsubstituted. This means that at least one of the glucosidic bonds adjacent to a modified glucose residue is not accessible to enzymic degradation. If, for the moment, we disregard the fraction with dp 2, which was obtained only in

Table 4
Degree of substitution of molecular weight fractions obtained from enzymically degraded methylated starches

Fraction	Suspension reaction Ds				Solution reaction Ds			
	0.18	0.29	0.41	0.70	0.12	0.27	0.37	0.76
Dp 1	n.d. [a]	0.01	0	0	0.03	0	0.05	0.04
Dp 2	0.37	0.42	0.38	0.42	0.44	0.44	0.54	
Dp 3	0.31	0.32	0.32	0.33	0.33	0.31	0.32	0.54 [b]
Oligomer	0.43	0.49	0.51	0.52	0.37	0.45	0.46	
Polymer	0.40	0.51	0.64	0.87	0.25	0.42	0.49	0.80

[a] Not determined.
[b] Dp 2 to oligomer.

Table 5
Dp and percentage of branched monomer units (db) of selected fractions obtained from enzymically degraded starches, methylated in solution

	Starch ds 0.27	0.27	0.27	0.27	0.27	0.37	0.76
	Fraction dp 1	dp 2	dp 3	Oligomer	Polymer	Polymer	Polymer
Dp	1.0	1.9	2.9	5.0	7.1	37	625
Db	0	1.5	1.2	2.9	7.5	7.1	4.7

minor amounts, the smallest substituted fragment encountered has dp 3. The ds of this fraction is 0.33, regardless of the ds and the preparation method of the parent *O*-methylstarch. So each liberated maltotriose bears exactly one substituent. The position of this methyl group is still unknown and cannot be deduced from the present study. As the majority of methyl groups are located at HO-2, it is very unlikely that this group is placed at the reducing glucose residue, where it is in close proximity to the glucosidic bond which was cleaved. For the present discussion, we assume that this methyl group is located on the central monomer. The fact that (apart from a minor amount of dimer) a trimer bearing one methyl group is the smallest substituted fragment suggests that at least two adjacent unmodified glucose residues are required for enzymic cleavage to occur. In other words, the trimer fragments in the digests originate from isolated substituted monomers in the original *O*-methylstarch separated from neighbouring *O*-methylglucose residues by at least two unmodified glucose residues.

The oligomer fraction comprises molecules with a dp ranging from 4 to 12. Methylation analysis established an average dp of 5 (Table 5), which seems somewhat low. The ds of these fractions increases slightly from 0.35 for lowly substituted starches to a plateau value of ca. 0.5. The oligomer fractions represent rather short sequences in the original *O*-methylstarch where, on average, one half of the monomer units bear a methyl group. As both the reduced and the nonreducing end of each oligosaccharide fragment are assumed to be unsubstituted, limited clustering of substituted monomer units may occur. Like the trimer fraction, the overall structure of the oligomer fraction does not depend on the reaction conditions employed.

In contrast to fractions with low dp, the ds of the polymer fractions increases strongly with the ds of the parent methylated starch (Fig. 5). At a specified ds value, *O*-methylstarch prepared in granular suspension yields a high molecular weight fraction that is more highly substituted than the polymer fraction obtained from starch methylated homogeneously. In our view, this important result has to be taken as evidence that, in granular *O*-methylstarches, substituted monomer units have a greater tendency to occur in clusters. The occurrence of polymer fragments with a ds much lower than 0.5 seems at first sight puzzling. It could be demonstrated that these fractions are enriched in branching points, which are likely to inhibit alpha-amylolysis equally well as substituent groups (Table 5).

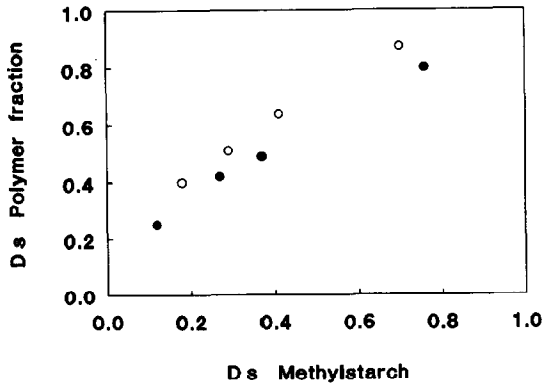

Fig. 5. Degree of substitution of polymer fraction released by consecutive action of alpha-amylase and amyloglucosidase from starch methylated in suspension (○) and in solution (●).

The presence of small amounts of dimer in the digests is intriguing. This fraction has an average ds somewhat below 0.5. The distribution of methyl groups among HO-2,3,6 in the parent methylated starches and in the fractions of dp 2, dp 3, oligomer, and polymer is presented in Table 6. Compared to the parent *O*-methylstarch, the dimer and the trimer fractions are enriched with methyl groups at HO-6 and HO-2, respectively. This suggests that glucosidic bonds adjacent to 6-*O*-methylglucose residues are cleaved more easily than bonds adjacent to glucose residues methylated at secondary positions. This is consistent with the observation that starch hydroxyethylated preferentially at HO-6 is hydrolysed more extensively by alpha-amylase than *O*-(2-hydroxyethyl)starch substituted predominantly at HO-2 [43]. On the other hand, substitution at HO-2 still prevails in the dimer fraction. Hence, it is probable that glucosidic bonds adjacent to 2-*O*-methylglucose are eventually cleaved, but at a much reduced rate.

Enzyme specificity and sequence information.—The specificity of starch-degrading enzymes has been studied in some detail [29–34,44–46]. Alpha-amylase is an

Table 6
Percentage of methyl groups located at HO-2,3,6 in fractions obtained from enzymically degraded *O*-methylstarches

Fraction dp	Solution reaction Ds				Suspension reaction Ds			
	0.18	0.29	0.41	0.70	0.12	0.27	0.37	0.76
2	56: 3:41	59: 2:38	61: 4:35	69: 4:27	54: 3:43	51: 2:47	49: 2:49	
3	84:11: 5	83:10: 7	86: 9: 5	90: 6: 4	80:14: 6	80:14: 6	78:16: 6	70:17:13 [a]
Oligomer	74:15:11	75:13:12	75:13:12	79: 8:13	71:19:10	72:16:11	71:17:12	
Polymer	77:14: 9	76:14:10	71:17:11	73:15:12	78:10:12	75:14:11	74:16:10	72:17:11
Starch	71:16:13	70:18:12	72:16:12	71:16:13	69:19:12	70:18:12	71:13:16	71:18:11

[a] Dp 2 to oligomer.

endo-enzyme that cleaves α-(1 → 4) linkages at random. Action on terminal linkages varies with the source. Amyloglucosidase is an exo-enzyme that breaks successive α-(1 → 4) bonds starting from the nonreducing end; α-(1 → 6) linkages at branching points are also cleaved by the latter enzyme. The active site of porcine-pancreas alpha-amylase is reported to have five "subsites", i.e., five glucose residues of a malto-oligosaccharide chain are bound to the active centre in the course of hydrolysis [34]. The glucosidic bond between subsites 2 and 3 is cleaved if substitution at each of these positions does not occur. Substitution of glucose residues at subsites 1, 4, and probably 5 is allowed in the case of starch hydroxyethylated at HO-2 [34], which is the preferred position for substitution [47]. This is in accordance with our observation that a minimum sequence length of two contiguous unsubstituted glucose residues is required for alpha-amylolysis to occur, although the enzyme specificity depends generally on the type of substitution and the source of alpha-amylase [29]. So alpha-amylase from *Bacillus subtilis*, which is closely related to the enzyme from *Bacillus amyloliquefaciens* used in the present work, is known to contain 9–10 subsites in its active centre [34].

On the other hand, amyloglucosidase is reported to liberate unmodified glucose linked to the nonreducing end of a modified glucose residue [44–46]. In that case, however, substantial amounts of substituted dimer should be expected, which is contrary to our observations. Again, influence of enzyme source and type of substitution may be involved.

Based on the afore-mentioned considerations, the following picture emerges regarding the specificity of the enzymes used by us. From the presence of a trimer as the smallest substituted fragment in the digests, it follows that a sequence of at least two contiguous unsubstituted glucose residues is required for alpha-amylolysis. Hence, glucose can be liberated only from a sequence of at least three unsubstituted monomer residues. This conclusion can be extended to the more general statement that a sequence of n contiguous unsubstituted glucose residues yields $n - 2$ molecules of glucose. In order to completely establish the enzyme specificity, the structure of the degradation products must be elucidated in more detail.

Finally, the sequence information that is extracted from enzymic degradation patterns is summarised. On exhaustive amylolysis with alpha-amylase and amyloglucosidase, granular *O*-methylstarches yield a polymer fraction with a ds that is significantly higher than for starches methylated homogeneously. At the same time, more trimer and oligomer are released from *O*-methylstarches prepared in solution. These fractions, with ds values of 0.33 and 0.4–0.5, respectively, originate from sequences with isolated *O*-methylglucose residues (trimer) and from sequences with approximately equal amounts of unsubstituted and substituted glucose residues (oligomer). In the latter fraction, extensive clustering of substituted monomers is not likely to occur. At a specified ds, more glucose is liberated from starch methylated in suspension, which is a consequence of a smaller number of glucosidic bonds linked to one or two modified monomer units. All these observations point to a more blockwise substitution pattern in granular *O*-methylstarches as compared to the presumed random substitution in solution.

Table 7
Average sequence length (n) of unsubstituted monomers in methylated starches

	Suspension reaction Ds				Solution reaction Ds			
	0.18	0.29	0.41	0.70	0.12	0.27	0.37	0.76
G_t	84.2	73.7	64.7	44.6	88.8	75.8	67.2	33.5
G_1	58.2	42.7	31.3	15.5	66.6	38.5	25.8	1.9
n	6.5	4.8	3.9	3.1	8.0	4.1	3.2	2.1

With the afore-mentioned assumption that a sequence of n unsubstituted monomers yields $n-2$ glucose molecules, an average unsubstituted sequence length n can be calculated as

$$n = 2G_t/(G_t - G_1) \quad (1)$$

where G_t and G_1 denote the quantities of unsubstituted monomer present in the parent starch ether and of glucose liberated by enzymic action, respectively. Values of G_t and G_1 are adopted from Tables 2 and 4. Results are shown in Table 7 and Fig. 6. As expected, the average sequence length of unsubstituted monomers at a specified ds is somewhat larger for granular *O*-methylstarch. The differences between homogeneous and heterogeneous methylation are not very pronounced, probably because the crystallinity of starch is moderate [48–50]. The calculation gives approximate values only because the amount of liberated glucose depends on the length distribution of unsubstituted sequences. Tentative calculations show that increasing the width of the distribution tends to underestimate n (if, at least, $n > 3$). For a more rigorous treatment, more details of the sequence distributions must become available. Nevertheless, our results imply that a rough estimate can be made simply by measuring total glucose after complete hydrolysis with acid, and glucose liberated by consecutive action of alpha-amylase and amyloglucosidase. A

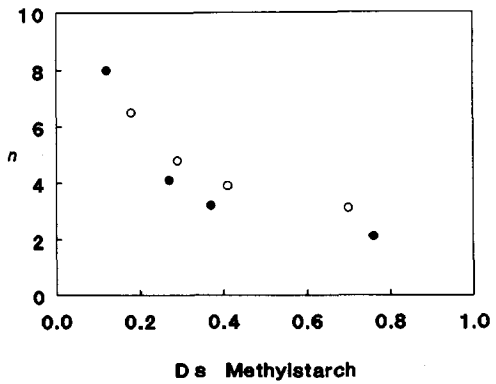

Fig. 6. Approximate average sequence length (n) of unsubstituted monomer units in starch methylated in suspension (○) and in solution (●).

similar approach to the substitution pattern of *O*-carboxymethylcellulose was published recently by an independent group of workers [37]. For application to other starch derivatives, Eq. *1* must be adjusted to possible alterations in enzyme specificity.

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