Structural Studies on Methylated Starch Granules[*]

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Granules of potato starch and amylopectin potato starch were methylated in aqueous alkaline suspension. Here, an overview is given of all structural aspects that have been studied with respect to the determination of the location of the substituents. Methods, which are generally applicable, were developed for the separation of different areas of derivatised starch granules, followed by determination of the substitution level. For methylated starch granules it was found that methyl groups are predominantly present in branched regions and amorphous domains, and that amylose is higher substituted than amylopectin. No differences were found between substitution levels of granules of various sizes, nor radially across the granules.

1 Introduction

1.1 Starch derivatisation

Starch is the main energy reserve polysaccharide in plants. Next to cellulose, starch is the most abundant carbohydrate in the world. The raw material is available in sufficient amounts and in high purity. Its total annual world production is estimated to be between 25 and 45 million tons [1]. To fulfil the various demands for functionality in different starch products, industrially processed starch is modified enzymatically, physically or chemically [2]. For example, in the Netherlands about 70% of all native potato starch is modified [3]. In most chemical modifications of starch, usually referred to as chemical derivatisations, the granular form is maintained and hydroxyl groups are partially substituted, yielding starch ethers and esters, as well as anionic and cationic starches. Other types of chemical derivatisation are oxidation, cross-linking and grafting of starches. A broad range of modified starches are used in the food and non-food sectors with empirically tailored application profiles [4]. In contrast to petrochemicals, starch is a renewable, biodegradable and non-toxic raw material. In the long run, the restoration of an economy based on plant resources as existed before 1850 seems to be plausible due to the exhaustion of fossil fuels [5]. Currently, in search for (new) applications of (modified) polysaccharides, and starch in particular, research follows the strategy of "sustainable development". Here, the aim is to achieve a balance between growth of global population and economy, reduction of the burden on the ecosystem, and trends and demands of society in general.

1.2 Structural characterisation of starch derivatives

The **substitution level** of a chemically derivatised polysaccharide such as cellulose or starch is commonly used as a characteristic value in its structural definition. To determine the substitution level the amount of substituted monosaccharide residues is measured in comparison to the total number of monosaccharide residues. Usually, these measurements are carried out enzymatically, or by applying chromatographic methods on hydrolysed material, resulting in a percentage of non-substituted monosaccharide residues (%u) or the substitution index (SI = 100 − %u). However, these values do not correspond with the actual amount of substituents present in the polysaccharide derivative. Therefore, in a more accurate way of expressing the substitution level, amounts of mono-, di- and tri-substituted monosaccharide residues are measured separately, resulting in a number between 0 and a maximum of 3 (in the case of a linear polysaccharide). This number equals the average number of substituents per monosaccharide residue and is generally referred to as the degree of substitution (DS). The method applied in the determination of the DS depends on the nature of the polysaccharide and the substituent. Different spectroscopic techniques have been used [2] (^{1}H NMR [6, 7], ^{13}C NMR [8, 9], solid state NMR [10], IR [11] and UV [12]), as well as chromatography (GLC [13], high-performance anion-exchange chromatography, HPAEC [14]), elemental analysis (substituents containing N-, P-, S- or Si-atoms) and titrimetric methods [15]. Another way of expressing the substitution level is used for derivatives with substituents that contain substitution sites themselves. The so-called molar substitution (MS) is defined as the number of moles of substituent divided by the number of moles of monosaccharide residues, and varies between 0 and theoretically infinite. Sometimes, the MS is referred to as molar degree of substitution (MDS). In summary, the definitions for substitution levels of starch derivatives are:

$$\%u = \frac{\text{number of non-substituted glucose residues}}{\text{total number of glucose residues}} \times 100\%$$

$$DS = \frac{\text{number of mono-, di- and tri-substituted glucose residues}^{(*)}}{\text{total number of glucose residues}}$$

(*) Multiplied by one, two and three, respectively.

$$MS = \frac{\text{number of moles of substituents}}{\text{total number of moles of glucose residues}}$$

The most refined parameters DS_{pos} (positional degree of substitution) and MS_{pos} (positional molar substitution) correspond, in the case of cellulose- or starch derivatives, with the substitution levels of the hydroxyl groups at the 2-, 3- and 6-positions. The DS_{pos} ratio of a sample provides information on the relative reactivities of the different hydroxyl groups. The reactivities may vary during a derivatisation process, and therefore DS_{pos} ratios are unique for each sample. For cellulose derivatives, DS_{pos} values have been measured by using ^{13}C NMR [16-18] or were derived from GLC monomer composition data [19]. The values have been used frequently as input data for statistical models of substituent distribution that calculate the relative reaction constants of hydroxyl groups during derivatisation processes [20, 21]. For starch derivatives, DS_{pos} values have been determined after hydrolysis of the polysaccharide and analysis of the monosaccharide residues by using HPAEC and GLC [22-24].

1.3 Current view on the structure of starch and starch granules

Theories about starch composition and structure have gone through many twists and turns. Although this search is far from concluded, the current view on the structure of starch is reasonably established. Starch is a polysaccharide consisting of glucose residues only, and as such it can be referred to as a homoglucan. However, complex hierarchical structures need to be considered in the starch granule [25, 26]. In addition, starch is a mixture of amylose and amylopectin that have very distinct structures. Amylose is an essentially linear $(1\rightarrow4)$-α-D-glucan that can contain few $(1\rightarrow6)$ branches. Its degree of polymerisation (DP) is controversial and seems to be dependent on the plant source and the growth stage. Amylopectin is a highly $(1\rightarrow6)$ branched $(1\rightarrow4)$-α-D-glucan with a molecular mass approximately 10^2–10^4 times higher than that of amylose. Starch granules consist of alternating amorphous and semi-crystalline growth rings (120–400 nm thick) [27]. The crystalline shells are made up of short side chains of amylopectin arranged in double helices that are oriented perpendicular to the shell [28, 29], and of amorphous lamellae. The degree of crystallinity is dependent on plant origin, amylose/amylopectin ratio and moisture content [30]. Generally, cereal starches exhibit A-type diffraction patterns, whereas tuber starches usually yield B-type patterns. The C-type crystallinity is more rare and is thought to be an intermediate form [31]. Recently, evidence was found for a model of the three-dimensional lamellar structure of starch granules [26], comprising intertwined amylopectin side chains that are assembled side-by-side in lamellae and organised in a superhelical fashion [32]. In addition, on the basis of electron and atomic force microscopic measurements it is proposed that these helices form spherical "blocklets" with diameters ranging from 20 to 500 nm [33]. However, this blocklet model still needs further experimental evidence. The structures of amylose and amylopectin have been analysed by using molecular modelling [34, 35]. Although this is a powerful tool in the study of three-dimensional features of macromolecules, a full spatial picture of the intact granular structure is still missing. Considering the increasing computational capacity in combination with developments in solid state NMR, mass spectrometry and microfocus X-ray diffraction techniques (from Synchrotron radiation), the unravelling of the spatial structure is within the realm of possibility in the decade(s) to come.

1.4 Relationship between molecular structure and functional properties of starch derivatives

The aim of the studies described in this review was to develop methods that are generally applicable in the structure elucidation of starch derivatives. Although derivatisations of starches have been carried out on an industrial scale for more than 50 years, the information on topochemical aspects is scarce. The nature, number, location and distribution of the substituents determine the properties of these starch derivatives. Detailed information on the location and distribution of substituents can contribute to the understanding of relationships between molecular structure and functional properties, thus opening ways to prepare "tailored derivatives" by selecting derivatisation procedures. For this purpose, methylated starch granules are chosen as model compounds and studied with regard to the following questions: how many substituents are present in the granules and how are they distributed?

2 Results and Discussion

2.1 Method development

In view of the different levels of organisation within the starch granule [25, 26], the distributions of substituents within glucose residues, in branched and linear regions, in crystalline and amorphous domains, and over amylose and amylopectin need to be determined. For this reason, methods for the separation of these parts of starch granules were developed [36], as is shown in the following paragraphs. These methods are applicable for structural analysis of derivatised starch granules in general.

2.2 Preparation of methylated potato and amylopectin potato starches and positional degrees of substitution

Methylated potato starches and methylated amylopectin potato starches, with molar substitutions (MS) varying between 0.040 and 0.300, were prepared by methylation of starch granules in an aqueous alkaline suspension using dimethyl sulphate. The determination of the MS values by using the Morgan-Zeisel assay has been reported previously, as well as GLC-measurements of the monomer compositions (DS_{pos}) by using methanolysis and trimethylsilylation [23]. In all methylated starches the HO-2 group has by far the highest DS_{pos} value and the HO-3 and the HO-6 group are substituted to almost the same extent (Fig. 1).
For all methylated starches investigated, the derivatisation was expressed against time from MS = 0 to the measured MS value of the specific sample by using the positional degrees
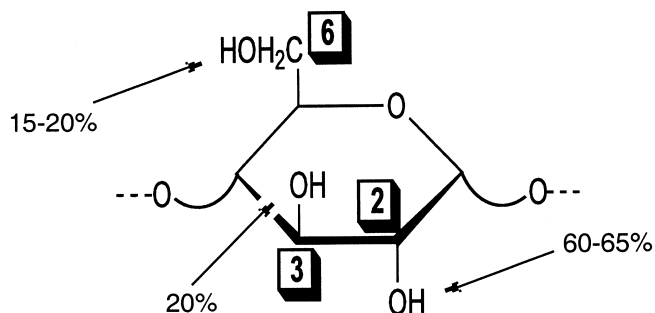

Fig. 1. Positional degrees of substitution in methylated (amylopectin) potato starches. The percentages indicated are related to substituted glucose residues only. The amount of non-substituted glucoses varies with the molar substitution.
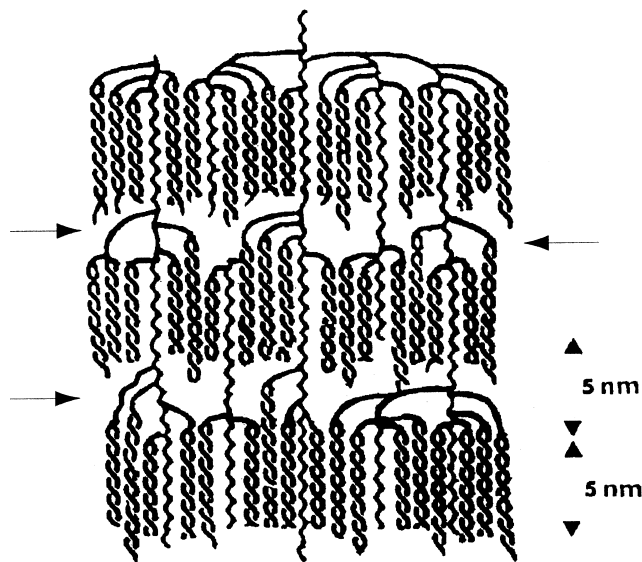
Fig. 2. Schematic model of a part of methylated amylopectin, proposed by *Imberty* and *Pérez* [34]. Total size of the amylopectin molecule is 100–500 nm. The arrows indicate the preferential positions of methyl substituents.

of substitution. In this way, a time model for the methylation process was obtained. It was shown that all samples are methylated in a similar fashion and only varied in their *extent* of methylation, which is expressed in the molar substitution. No differences were observed between monomer compositions of glucose residues from different areas in the starch granule.

2.3 Differences between branched and linear regions

After exhaustive digestion of the methylated starches with α-amylase, the highly branched fraction with a degree of polymerisation (DP) > 8 was separated from the linear oligomers by selective precipitation of the former in methanol [23]. The substitution levels of branched and linear regions were determined and it was found that methylation takes place preferably at the branched regions of amylopectin (Fig. 2).

The substitution patterns at the linear regions deviate significantly from a random substitution. Therefore, we hypothesise that most methyl substituents are present at glucose residues close to the non-reducing ends and the branching points.

2.4 Differences between crystalline and amorphous domains

Prolonged treatment of methylated starches with hydrochloric acid below the swelling temperature resulted in the release of D-glucose and small D-glucose oligomers from the amorphous domains. The granular structure was maintained during the treatment with acid, indicating that the crystalline lamellae were less affected by acid. The amorphous domains contained about two times more substituents per glucose unit than the remaining crystalline network.

2.5 Differences between amylose and amylopectin from methylated potato starches

Fractions containing either mainly amylose or mainly amylopectin were obtained after aqueous leaching of the methylated starch granules [37]. Amylopectin in these fractions was precipitated with concanavalin A to separate it from amylose. Amylose remained in solution and was enzymatically converted into D-glucose for quantification, thereby taking into account the decreased digestibility due to the presence of methyl substituents. It was found that the MS of amylose was 1.6 – 1.9 times higher than that of amylopectin in methylated potato starch granules. This is in agreement with the location of amylose in amorphous domains.

2.6 No differences between granules of varying sizes and no differences within one granule

Methylated starch granules were fractionated into three sieve fractions (< 45 mm, 45 – 60 mm, > 60 mm), which all had the same molar substitution. This is in agreement with earlier studies, where it was found that the substitution level of methylated starch granules was independent of granule size [38]. One middle size fraction from potato starch and one from amylopectin potato starch were treated with 4 M calcium chloride [39]. In this way, the outside layer of each starch granule gelatinised and diffused from the remaining granule that was isolated. This procedure was repeated four more times, yielding a series of "pealed" remaining methylated potato and amylopectin potato starch granules. As shown in Tab. 1, all remaining granules had the same molar substitution, indicating that the methyl substituents are distributed equally within each starch granule.

3 Conclusions

The results of the structural analysis on methylated starch granules corroborate the current view on the structure of starch. Crystalline domains are less accessible for chemical substitution than amorphous parts. These crystalline lamellae are built up from amylopectin side chains, of which the glucose residues close to the non-reducing ends and the branching points are substituted predominantly. Amylose is present in amorphous domains of the granule and is therefore highly substituted.

The development of methods that are generally applicable in the structure elucidation of starch derivatives has been successful. The distribution of substituents in methylated starch granules has been described considering different regions,

Tab. 1. MS values of partially gelatinised methylated starch granules.

Treatment with 4 M CaCl$_2$ [min]	Amount of remaining potato starch granules [mg granules/40 mL]	MS (GLC)	Treatment with 4 M CaCl$_2$ [min]	Amount of remaining amylopectin potato starch granules [mg granules/40 mL]	MS (GLC)
0	1055	0.114	0	1156.2	0.136
15	882.2	0.109	15	967.0	0.090
30	763.6	0.090	30	717.8	0.106
45	698.2	0.100	45	466.6	0.105
60	547.5	0.097	70	245.3	0.101
180	200.0	0.099	90	52.3	0.106

domains and molecules and granule sizes. These methods can now be used in the structural analysis of other kinds of starch derivatives. That would open the way to an understanding of the relationships between molecular structure and functional properties, like the viscosity or stability of a starch gel.

Acknowledgements

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