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The molecular structure of waxy maize starch nanocrystals

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Dedicated to Professor J.P. Kamerling on the occasion of his 65th birthday

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ABSTRACT

The insoluble residues obtained by submitting amylopectin-rich native starch granules from waxy maize to a mild acid hydrolysis consist of polydisperse platelet nanocrystals that have retained the allomorphic type of the parent granules. The present investigation is a detailed characterization of their molecular composition. Two major groups of dextrins were found in the nanocrystals and were isolated. Each group was then structurally characterized using β-amylase and debranching enzymes (isoamylase and pullulanase) in combination with anion-exchange chromatography. The chain lengths of the dextrins in both groups corresponded with the thickness of the crystalline lamellae in the starch granules. Only ~62 mol % of the group of smaller dextrins with an average degree of polymerization ($\overline{DP}$) 12.2 was linear, whereas the rest consisted of branched dextrins. The group of larger dextrins ($\overline{DP}$ 31.7) apparently only consisted of branched dextrins, several of which were multiply branched molecules. It was shown that many of the branch linkages were resistant to the action of the debranching enzymes. The distribution of branched molecules in the two populations of dextrins suggested that the nanocrystals possessed a regular and principally homogeneous molecular structure.

1. Introduction

Native starch is biosynthesized as granules with dimensions ranging from 1 to 100 μm.[1,2] The inner architecture of the granule is characterized by 'growth rings' that correspond to concentric semicrystalline thick shells with a thickness ranging from 100 to 400 nm. They are separated by amorphous regions. The crystalline shells consist of a regular alternation of amorphous and crystalline lamellae having a repeat distance of 9–10 nm clearly revealed by small-angle X-ray scattering.[3] Three-dimensional models describing the structural organization of the crystalline domains in starch granules have been established jointly using the data obtained from crystallographic studies of small oligosaccharides[4,5] as well as using diffraction and molecular modeling from recrystallized fibers[6] or single crystals grown in vitro.[7] Two allomorphs are known, namely A and B, corresponding, respectively, to a monoclinic and hexagonal packing of parallel-stranded left-handed double helices.[7,8] The structural models could be transposed to crystalline regions of native A-starch granules, such as waxy maize, which exhibit similar but much less resolved diffraction diagrams.

The principal component of the semicrystalline rings is amylopectin, a branched macromolecule that consists of short chains of α-D-(1→4)-linked glucosyl residues. The chains are interlinked through α-D-(1→6)-branches. The primary and secondary structures that describe the composition of unit chains[9,10] and the organization of these chains into clusters have been established.[11–13] Nevertheless, the intrinsic complexity of the secondary structure has not been fully translated into three-dimensional structures, and only models of fragments incorporating this information have been published.[14,15]

New morphological data on the individual crystalline lamellae of amylopectin-rich waxy maize starch granules were recently reported. By submitting native granules to a mild hydrochloric acid hydrolysis, insoluble residues were obtained.[16] They contained polydisperse platelet nanocrystals that retained the allomorphic type of the parent granules. These nanocrystals, which are not totally individualized, have a 5–7 nm thickness and characteristic geometrical features that were tentatively correlated to those of the monoclinic unit cell of A-starch.[16] The availability of such nanocrystals offers the unique opportunity to investigate their molecular composition, to characterize the type of chains that remain after acid hydrolysis of the parent amylopectin and to incorporate the three-dimensional features derived from the crystallographic investigation performed on crystals grown in vitro.[7] In a recent paper,[17] nanocrystals from waxy maize were prepared by

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treatment in mild sulfuric acid instead of hydrochloric acid, which is a well-known alternative described by Nägeli for the production of dextrins.[18] As a part of our attempt to fully explore the structure of the semi-crystalline growth rings in starch granules, the aim of the present investigation was to establish the molecular structure of these nanocrystals from waxy maize starch.

2. Experimental

2.1. Waxy maize starch nanocrystals

The details for the preparation of nanocrystals by H_2SO_4 hydrolysis of native waxy maize starch granules (Waxylis™, Roquette S.A., Lestrem, France) have been previously described.[17] The conditions of hydrolysis differed from those of the so-called 'lintnerization' procedure described by Robin et al.[19] and were defined as the result of the optimization of the treatment using a response surface methodology.[17] Native waxy maize starch granules (37 g) were mixed with 250 mL of 3.16 M H_2SO_4 during 5 days at 40 °C, with continuous stirring at 100 rpm. The suspension was then washed by successive centrifugations in distilled water until neutral and finally freeze-dried. The resulting nanocrystals will be referred to as WN in the following section.

2.2. Transmission electron microscopy (TEM)

A drop of dilute nanocrystal suspension was spread on a glow-discharged carbon-coated TEM grid. The preparation was negatively stained with 2% (w/v) uranyl acetate and was observed using a Philips CM200 microscope (FEI Company, Eindhoven, The Netherlands) operating at 80 kV. Images were recorded on Kodak S0163 film.

2.3. Enzymes

All enzymes were obtained from Megazyme (Wicklow, Ireland) and used without dilution. As given by the supplier, isoamylase from *Pseudomonas* (EC 3.2.1.68), pullulanase from *Klebsiella planticola* (EC 3.2.1.41) and barley β-amylase (EC 3.2.1.2) had specific activities of ca. 280 U/mg, ca. 42 U/mg, and ca. 1400 U/mg, respectively.

2.4. Fractionation on Bio-Gel

Freeze-dried starch nanocrystals (10 mg) were dissolved in 2 mL of water by gentle heating. The solution was then centrifuged at 1800 *g* for 20 min to remove trace amounts of solid matter. An aliquot of the supernatant (1.9 mL) was applied to a column (30 × 1.6 cm) of Bio-Gel P-4 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and eluted with water at 3.5 mL h^{-1}. Fractions of 1 mL were collected and analyzed for carbohydrates with the phenol–sulfuric acid reagent.[20] These fractions were pooled into four samples (I–IV) as shown in Figure 1. Finally, samples I–IV were freeze-dried.

2.5. Enzymatic analysis

Stock solutions of samples I and III in water (approx. 2 mg mL^{-1}) were prepared and divided into two parts that were treated with enzymes using the following procedure (Fig. 2). To the first part (0.45 mL) were added 50 μL of 0.01 M NaOAc buffer (pH 6.0), and 1 μL of β-amylase in order to produce β-limit dextrins, labeled β-LD. The second part (0.475 mL) was debranched by adding 25 μL of 0.01 M NaOAc buffer (pH 3.5), and 0.5 μL of isoamylase. After incubation the sample was diluted with 0.5 mL of water and 0.1 mL of 0.1 M NaOAc buffer (pH 5.5) and further treated with 1 μL of pullulanase. An aliquot (0.3 mL) of the debranched sample was diluted in 0.3 mL of 0.1 M NaOAc buffer (pH 6.5), and finally treated with 1 μL of β-amylase in order to give the β-LD of the debranched sample. All enzymatic incubations were performed overnight at room temperature. The reactions were stopped by heating in a boiling water bath (5 min).

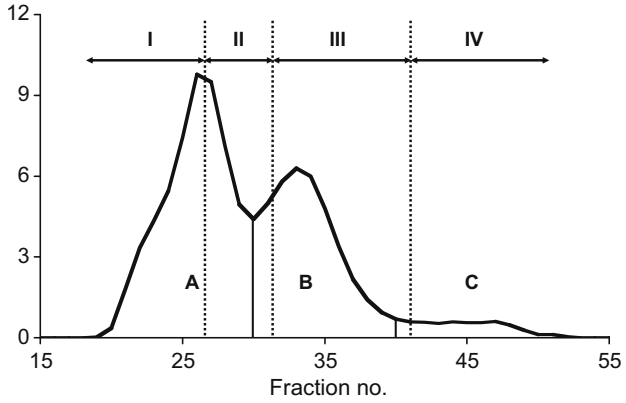

Figure 1. Bio-Gel P-4 fractionation of the solution of waxy maize starch nanocrystals into four samples (I–IV). Letters A, B and C describe the three populations of dextrins present in starch nanocrystals.

The principle of such a controlled enzymatic degradation procedure is exemplified in Figure 2. It shows how a double helical structure exhibiting α-(1→6) linkage between the parallel strands, can be progressively degraded into low molecular weight oligosaccharides.

2.6. Anion-exchange chromatography

The composition of the samples before and after enzymatic treatments was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD). The HPLC system was Dionex (Sunnyvale, CA, USA) series 4500i equipped with a BioLC gradient pump and a CarboPac PA-100 column (250 × 4 mm) in combination with a similar guard column. Filtered samples (25 μL) were eluted at 1 mL min^{-1} with 150 mM NaOH and a gradient of NaOAc as described earlier by Bertoft.[21] The PAD response was calibrated to quantitate the carbohydrate components as described by Koch et al.[22] It was assumed that branched dextrins of a certain degree of polymerization (DP) were eluted in front of linear dextrins of corresponding DP.[23,24] To be able to quantify the high amount of maltose obtained after β-amylolysis, the PAD signal was recorded at a less sensitive output range.[21]

The molar percentage of linear and branched dextrins in a given sample was calculated according to two independent methods from the chromatograms obtained before and after β-amylolysis. The molar percentage of linear chains was calculated as

$$\text{linear (mol \%)} = \frac{n_{\text{maltotriose,a}} \times 2}{n_{\text{total,b}}} \times 100 \quad (1)$$

where $n_{\text{maltotriose,a}}$ is the number of moles of maltotriose (DP 3) obtained after β-amylolysis and $n_{\text{total,b}}$ is the total number of moles of dextrins in the sample before β-amylolysis. The molar percentage of branched molecules was calculated as

$$\text{branched (mol \%)} = \frac{n_{\text{β-LD,a}}}{n_{\text{total,b}}} \times 100 \quad (2)$$

where $n_{\text{β-LD,a}}$ is the number of moles of β-limit dextrins (DP ⩾ 4) (abbreviated to as β-LD) obtained by β-amylolysis. Due to a large difference in molar amounts of maltotriose and β-LDs, the overall estimations of linear and branched dextrins were in the order of 100 ± 9%.

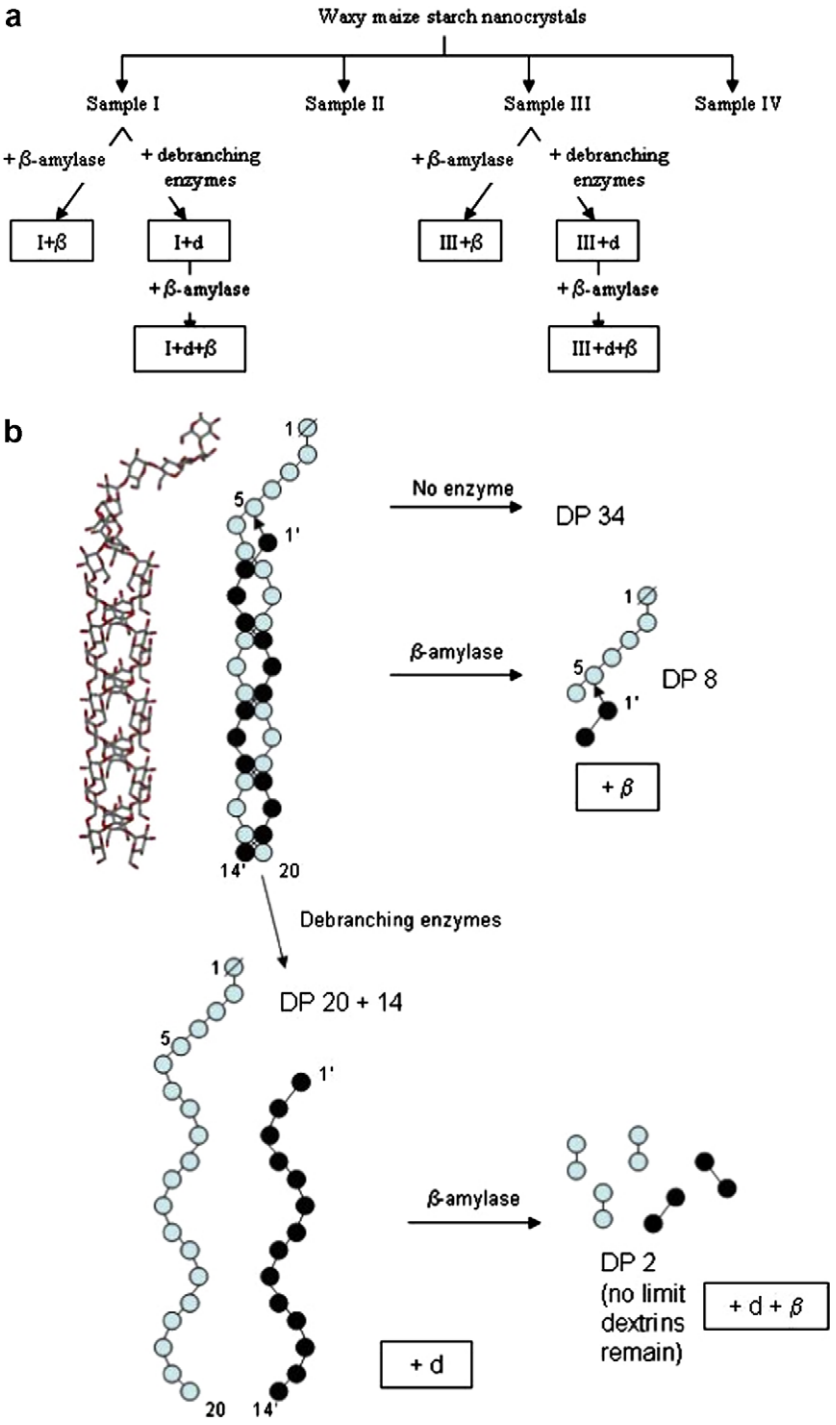

Figure 2. (a) Scheme of the enzymatic analysis. (b) An example of the analysis of a single branched dextrin of DP 34 and the products obtained after treatment with β-amylase, debranching enzymes, and with the successive treatment with debranching enzymes and β-amylase.

The average apparent number of chains per molecule (NC) was calculated as the ratio between the average DP ($\overline{DP}$) before and after debranching.

3. Results

3.1. TEM observation of waxy maize starch nanocrystals

After hydrolyzing waxy maize starch granules for 2 days with 3.16 M H_2SO_4 at 40 °C, micrometer-sized aggregates are observed, constituted by fragments of granules exhibiting a clear lamellar structure (Fig. 3a). The 5–7 nm-thick white lamellae likely correspond to an edge-on view of the platelets formed by crystallization of the amylopectin side chains.[3,16] After 5 days of hydrolysis, as more and more α-(1→6) branching points are cut by the acid, the lamellae are more disorganized, and some of them can be observed lying flat on the carbon film (Fig. 3b).[17] The aggregates are similar to those observed by Putaux et al.[15] after a 40-days hydrolysis of waxy maize starch granules with 2.2 N HCl at 36 °C. They appear to be made of parallelepiped units with average length and width of 20–40 nm and 15–30 nm, respectively, and exhibiting 60–65° acute angles.

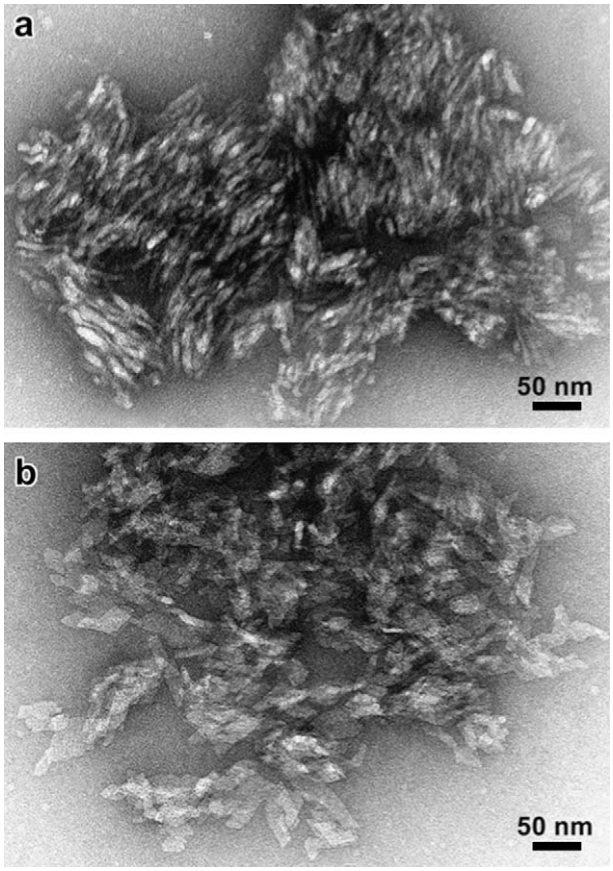

Figure 3. TEM images of negatively stained preparations from waxy maize starch granules treated with H_2SO_4 at 40 °C, under continuous stirring, during: (a) 2 days; (b) 5 days.

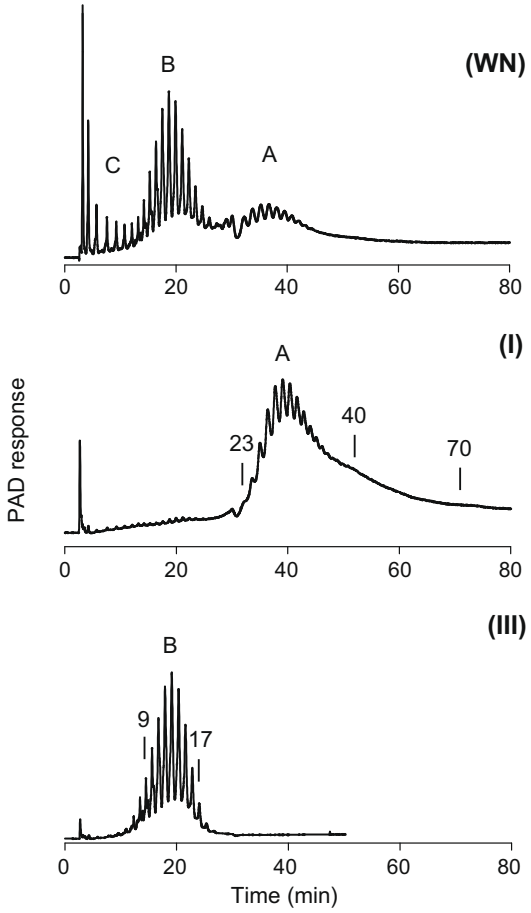

Figure 4. HPAEC–PAD chromatograms of waxy maize starch nanocrystals in solution (WN) and fractions I and III. Some DP are indicated.

3.2. Molecular composition of nanocrystals

The molecular components of the nanocrystal preparation (WN) were size fractionated on a column of Bio-Gel P-4 (Fig. 1). Two major populations of dextrins, labeled A and B, were of high and low DP, respectively, and corresponded to the fractions of branched and linear dextrins generally found in acid-treated starch granules.[19,25–28] In addition, a third population (C) of very low DP ($\leqslant$9) was detected. However, this population was found to be easily removed by extensive washing and was therefore not considered as being a part of the crystalline structure of WN. To obtain representative samples of populations A and B, the size-fractionated sample was pooled into four fractions (I–IV, Fig. 1). The fractions were analyzed by HPAEC–PAD, and their compositions were compared to those of the original sample. As fraction II was a mixture of populations A and B, and fraction IV contained population C, they were not further analyzed. Samples I and III, however, were representatives of populations A and B, respectively (Fig. 4).

Table 1 summarizes the composition of WN and fractions I and III. Peaks were distinguished in the HPAE chromatograms up to DP ~35 (Fig. 4), and higher values of DP were therefore approximations. Sample WN contained dextrins of DP 2–85 (DP 1 was not distinguished from disturbing impurities at void volume of the column). Population A was represented by dextrins at the DP range 23–85 and constituted 21 mol % of WN. Population B had DP 9–22 and was the major dextrin population (51 mol %). The $\overline{DP}$ of A was 29.7 and of B was 14.2, whereas the peak positions were at DP 26 and 13, respectively, which was in agreement with other investigations.[28,29] Population C with DP $\leqslant$8 represented only 5 wt %, but as it was not a crystalline structure in WN. The actual nanocrystals were considered to be composed of the dextrins at DP $\geqslant$9 where the $\overline{DP}$ was 18.8 and the relative constitution of populations A and B was 29 and 71 mol %, respectively.

Table 1
Composition of the nanocrystal preparation made from waxy maize starch (WN) and fractions I and III

	WN	I	III
Range of DP	2–85	2–85	2–22
$\overline{DP}$[a]	15.7 (18.8)	31.7 (32.8)	12.2 (12.8)
Wt % of WN	100	32	32
Mol % of A[b]	21	94	0.2
Mol % of B[b]	51	5	89
Mol % of C[b]	29	1	10

[a] Values in parentheses show the $\overline{DP}$ of the crystalline fraction at DP 9–85 in WN, 23–85 in I, and 9–22 in III.
[b] Populations of dextrins A at DP 23–85, B at 9–22, and C at $\leqslant$8.

Sample I contained dextrins with a DP ranging from 2 to about 85 and $\overline{DP}$ 31.7 (Table 1). This sample contained only 6 mol % of molecules having a DP $\leqslant$22 and was therefore considered as pure enough to be representative of population A. The $\overline{DP}$ of dextrins with DP $\geqslant$23 was 32.8 (Table 1) and was slightly higher than the corresponding value (29.7) of population A before the fractionation of WN, because a part of the smaller dextrins of this population was obtained in fraction II.

Sample III contained dextrins with a DP range from 2 to 22 and a $\overline{DP}$ of 12.2. (Fig. 4) Since the smallest dextrins with DP 2–8 rep-

resented population C and only constituted 10.4 mol % of sample III, they were deleted from further calculations. Thus, the $\overline{DP}$ of the dextrins that were representative of group B in sample III was 12.8 (Table 1). In this case the value was somewhat smaller than the actual $\overline{DP}$ of group B in WN (which was 14.2) because some of the larger dextrins of B were obtained in fraction II.

3.3. Composition of sample III (DP ⩽22)

When sample III was subjected to β-amylolysis, large peaks of both maltose (DP 2) and maltotriose (DP 3) were obtained (Fig. 5). Maltotriose is the practically resistant end product of linear chains with an odd number of residues. As shown in Figure 5, every second chain possessed an odd number of residues, and thus the amount of maltotriose represented half of the molar amount of linear dextrins in the sample. The formation of β-limit dextrins (β-LD) at DP ⩾4 showed the presence of branched dextrins in sample III (Fig. 5). The calculation of the molar number of maltotriose and β-LD indicated that sample III was composed of roughly 2/3 (62 mol %) linear chains and 1/3 (30 mol %) branched dextrins (Table 2). The distribution of β-LD was bimodal and was divided into two populations: DP 4–8 (β-$\overline{DP}$ 5.5) and DP 9–22 (β-$\overline{DP}$ 13.4), which represented 21 mol% and 9 mol % of sample III, that is, 2/3 and 1/3 of the branched dextrins, respectively (Fig. 5). The first, major group of the branched dextrins was obviously sensitive to β-amylolysis (because of the lower DP), and most probably the branch (or branches) was found near the reducing end. The second, minor group of branched dextrins was apparently resistant to β-amylase because the β-$\overline{DP}$ (13.4) remained similar to the $\overline{DP}$ of sample III (12.8). This showed that the branch point was positioned at, or close to, the non-reducing end.

After debranching by isoamylase and pullulanase (Fig. 5), the range of DP was 2–22, with $\overline{DP}$ 11.3 (Table 2). The estimated average number of chains per molecule (NC), in sample III was 1.1. The number was close to 1.0 and suggested that the majority of the dextrins in sample III were not branched, that is, in contrast to the detected branched molecules discussed above. However, the debranching reaction was not complete as shown by the successive β-amylolysis, which resulted in the formation of small amounts of limit dextrins with DP ⩾4 (Fig. 5). On a molar basis these d,β-LD (β-LDs of debranched sample) represented 14% and implied that sample III contained branched dextrins that were resistant to the debranching enzymes.

The DP range (4–22) of the d,β-LD was similar to that of the β-LD (the β-limit dextrins obtained without previous debranching). The molar percentage of the part of the d,β-LD having a DP 4–8 was 7 mol %. By comparison with the β-LD, it was deduced that the first group of β-LD at DP 4–8, which constituted 21 mol %, could be further subdivided into two groups: dextrins sensitive to debranching enzymes (21–7 = 14 mol %) and dextrins resistant to the debranching enzymes (7 mol %). Similarly, the molar percentage of d,β-LD with DP 9–22 (8 mol %) was compared to that of the corresponding group of β-LD (9 mol %). Since the molar amount of the groups was almost the same, it was concluded that the branched dextrins that possessed a β-DP between 9 and 22 were not only resistant to the attack by β-amylase, but also resistant to the debranching enzymes.

The molar proportions of the different types of dextrins found in sample III are summarized in Figure 6 and Table 2.

Two different percentages are given in Figure 6 for each group. The first percentage is relative to sample III and the second (value in italic) is relative to the nanocrystals. Ratios in bold are approximate and show the proportions of the sub-groups of the respective previous major groups. It was concluded that 2/3 of the dextrins in sample III were linear chains and 1/3 were branched dextrins. In addition, it was noted that altogether 15 mol % (7 + 8 mol %) of the dextrins were resistant to the debranching enzymes, which corresponded to as much as half of all the branched dextrins in sample III.

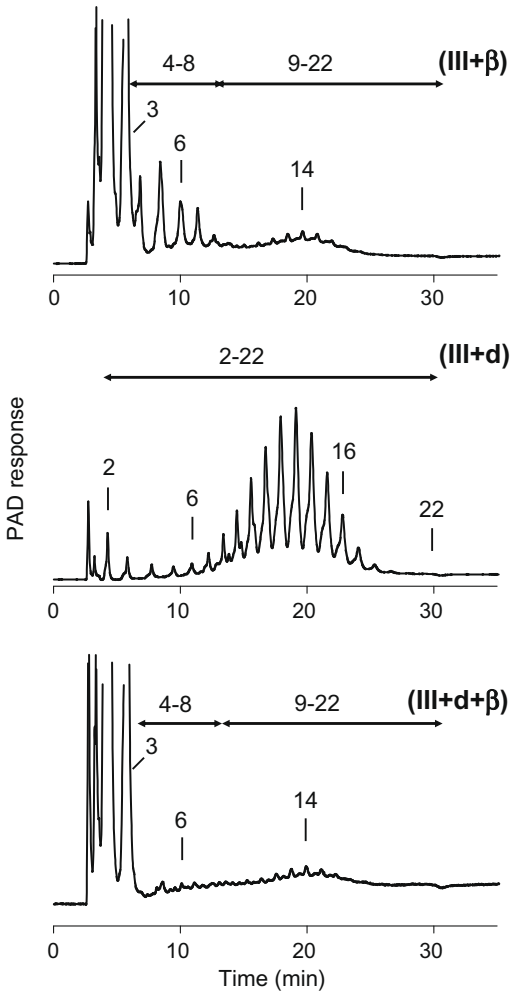

Figure 5. HPAEC–PAD chromatograms of sample III after enzymatic degradation by β-amylase (+β, debranching enzymes (+d), and β-amylase after debranching (+d+β). Some DP and fractions of DP ranges are indicated.

Table 2
Characterization of samples III and I

Sample	III	I
After β-amylolysis		
Range of DP of β-LD[a]	4–22	4–41
DP of β-LD	7.7	11.5
Linear dextrins (mol %)	62	4
Branched dextrins (mol %)	30	94
After debranching		
Range of DP	2–22	2–65
Average DP	11.3	15.8
Apparent NC	1.1	2.0
After debranching and β-amylolysis		
Range of DP of d,β-LD[b]	4–22	4–28
DP of d,β-LD	10.1	12.3
Linear dextrins (mol %)	92	73
Branched dextrins (mol %)	14	29

[a] Limit dextrin obtained by β-amylolysis of the sample.
[b] Limit dextrin obtained by β-amylolysis of the debranched sample.

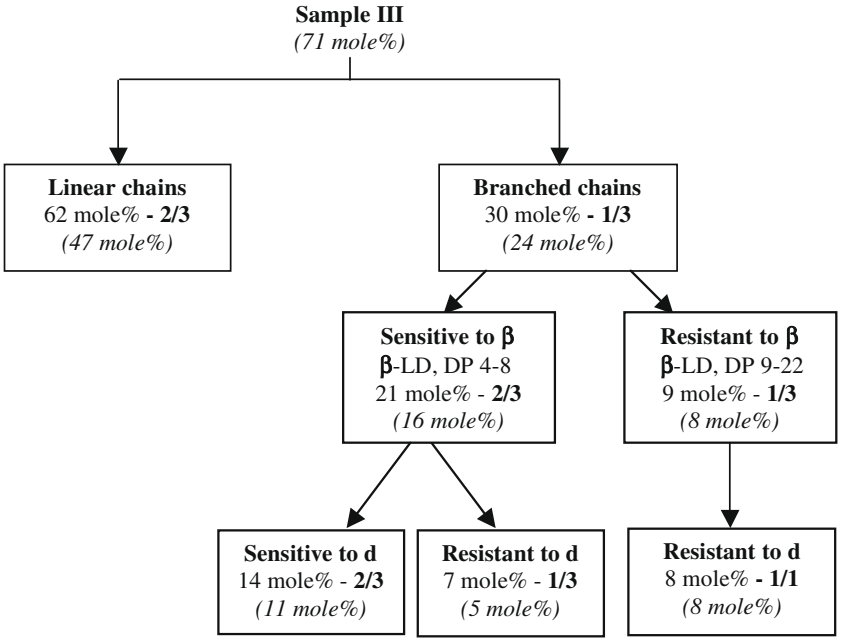

Figure 6. Summary of the enzymatic analysis of sample III. The first percentages are relative to sample III. The ratios in bold refer to the previous sample. The percentages in italics are relative to starch nanocrystals.

3.4. Composition of sample I (DP ⩾23)

Sample I represented population A in the original WN sample that constituted 29 mol % of the dextrins in the starch nanocrystals. After β-amylolysis of the sample (Fig. 7), a large peak of maltose was obtained but only an insignificant peak of maltotriose (4 mol % of sample I, i.e., 1.7 mol % of the nanocrystals), indicating that sample I practically lacked linear chains (Table 2). It was also noted that after β-amylolysis, the entire molecular-weight distribution of sample I shifted toward lower DP, suggesting that all molecules were sensitive to β-amylase attack. The large peak of maltose was therefore the result of the β-amylase attack on the external chains of the branched molecules. The relative amount of maltose, commonly called the β-limit value, was 66 mol %. As in sample III, the β-LD in sample I possessed two distinct size groups. Their DP ranges were different, however: 4–13 and 14–41 in sample I, as compared to 4–8 and 9–22 in sample III, respectively. Quantitatively, the β-LDs of smaller DP represented 65 mol % or 2/3 of sample I and thus the other group constituted 1/3 of the sample (Fig. 8).

After debranching of sample I with pullulanase and isoamylase (Fig. 7), the range of DP became 2–65, the $\overline{DP}$ was 15.8, and the apparent number of chains *e* was equal to 2. The distribution of debranched dextrins could be divided into two populations: DP 2–22 and DP 23–65. The proportion of the latter was 24 mol %. Since the DP range of this population was similar to the original distribution of dextrins, it probably corresponded to branched dextrins resistant to the debranching enzymes. Indeed, after successive β-amylolysis of the debranched sample, 29 mol % remained as d,β-LDs, which showed that the debranching was not complete. As for sample III, the d,β-LDs in sample I covered the same DP range as the β-LD and were distinguished as two subgroups. About half of the d,β-LDs possessed the DP range 4–13, the other half had DP ⩾14. Since 29 mol % of the dextrins were resistant to the debranching enzymes, each group represented roughly 14–15 mol % of sample I. The molar percentages of branched dextrins that were sensitive to debranching enzymes were obtained by difference.

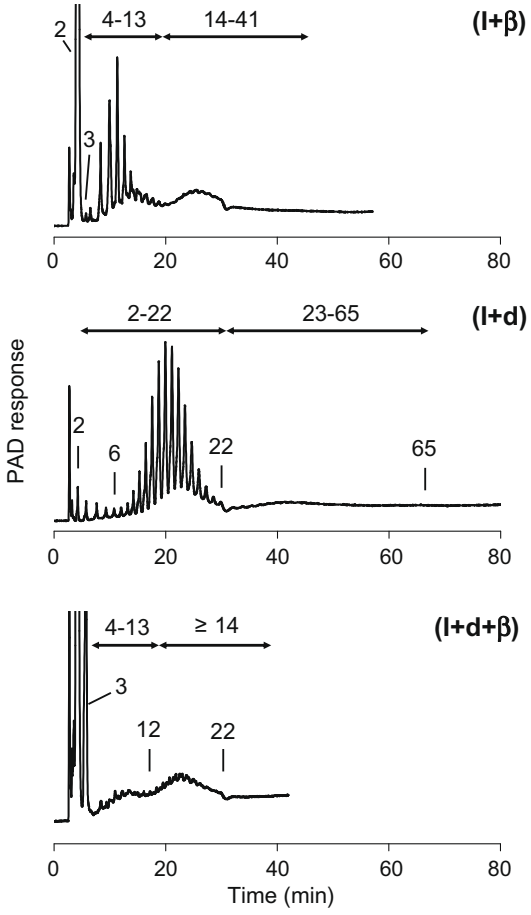

Figure 7. HPAEC–PAD chromatograms of sample I, after enzymatic degradation by β-amylase (+β, debranching enzymes (+d), and β-amylase after debranching (+d+β). Some DP and fractions of DP ranges are indicated.

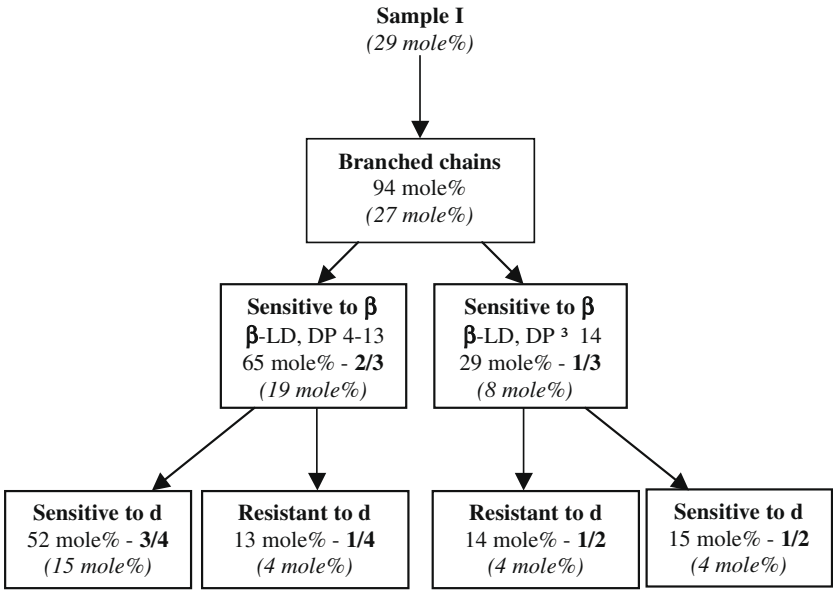

Figure 8. Summary of the enzymatic analysis of sample I. The first percentages are relative to sample I. The ratios in bold refer to the previous sample. The percentages in italics are relative to starch nanocrystals.

The different groups of dextrins in sample I and their relative proportions are reported in Figure 8 and Table 2. To summarize, sample I contained only branched molecules with one or several branch points as shown by the fact that practically all dextrins remained as limit dextrins after β-amylase attack. However, contrary to sample III, no dextrins were completely resistant but were distinguished as more sensitive (2/3 of the molecules) or less sensitive (1/3) to the attack. Each of these groups contained dextrins that also showed resistance to the action of isoamylase and pullulanase. Altogether, about 1/3 of sample I (15 + 15 mol %) was either completely or partially resistant to the debranching enzymes.

4. Discussion

The molecular composition of the nanocrystals obtained by acid treatment of native starch granules followed the general pattern described by various authors.[27,29,30] Thus, sample III that, in this investigation, represented dextrin population B, possessed mostly linear chains with an average length of 12.2. The average DP of population B in the original WN sample was 14.2. In the double-helical structure this corresponds to a length of 5 nm and to the thickness of the crystalline lamellae inside the starch granules.

The molecular composition of sample III was, however, considerably more complicated because as much as 1/3 of the dextrins were in fact branched. The peak of the chains in the debranched sample remained at DP 13, suggesting that the branched dextrins were composed of one longer and one or two very short chains. Indeed, the appearance of very small dextrins at DP 2–6 in the mixture of the debranched sample (Fig. 5) showed that the branch chains were short. Several branches were resistant to the attack by debranching enzymes (both isoamylase and pullulanase). Probably, many of these branches consisted of only a single glucosyl residue, which is known to be too short a branch chain length to be attacked.[31] It is also known that an α-(1→6) linkage on the reducing glucosyl residue is resistant to hydrolysis.[32] Thus, several types of dextrin structures were possibly resistant to debranching, making them undetectable in the debranching experiment. However, the formation of d,β-LDs after the debranching proved their existence.

Sample I, which represented dextrin population A in the original WN sample, corresponded to the group of singly branched dextrins commonly found in acid-treated starches with a peak DP around 25–30. The apparent number of chains per dextrin was exactly 2, and the major group of chains released after debranching possessed a peak at DP 14 (Fig. 7), which, therefore, support the general view that two chains similar to population B are interlinked in population A.[26] This type of structure was further supported from the average length of the external chains (ECL) that can be calculated from the apparent chain length (CL, which corresponds to the DP of the debranched sample) and the β-amylolysis limit as ECL = CL × β-limit/100 + 2.[33] The ECL was 12.4, which corresponded to the DP of fraction III as well as to the ECL of native waxy maize starch.[34] Furthermore, it was possible to estimate the average internal chain length[33] (ICL = CL − ECL − 1) to 2.4. This was short compared to native waxy maize starch, in which ICL is 4.6[33] and suggested that longer internal chain segments were practically absent in the nanocrystals.

As for sample III, a large part of the dextrins in sample I was either only partially or not at all debranched. This means that the CL value obtained by debranching with isoamylase and pullulanase was apparent, and the dextrins were, to a large extent, multiply branched. Sample I contained low amounts of dextrins with DP values up to 85, and these were all probably multiply branched. Similar fractions of higher molecular weight have been previously described.[27] However, for reasons discussed below, the major group around DP 28 was probably also, to a large extent, multiply branched.

The β-LDs that were formed from sample I constituted two major types with lower DP ($\overline{DP}$ 7.6) and higher DP ($\overline{DP}$ 20.3), that is, more sensitive and less sensitive to β-amylase action, respectively (Fig. 7). The difference between the $\overline{DP}$ of sample I (32.8) and that of the latter group of β-LD (20.3) was 12.5, whereas the difference between the two populations of β-LDs was 12.7 (20.3 to −7.6). Both values corresponded to the ECL of sample I (12.4) and to $\overline{DP}$ of sample III (12.8). We deduce, therefore, that the basic structure of the dextrins in sample I was two interconnected chains of the types found in sample III. In about 1/3 of the dextrins (Fig. 8), one of the chains was external and open for the attack by β-amylase, whereas the other possessed one (or more) short branch chain

at, or very close to, the non-reducing end thus blocking the β-amylase (resulting in the larger β-LDs with $\overline{DP}$ 20.3). We therefore conclude that these dextrins were carrying at least two branches. In 2/3 of the dextrins both chains were open to the attack by β-amylase, and the branch was mainly found at the reducing end side. A majority of these latter dextrins had a β-DP of 5–8 (Fig. 8) and were possibly only carrying a single branch point. The remaining was most probably multiply branched because of their comparatively large size (β-DP 9–14). Both populations of β-LD could further be divided into dextrins that were sensitive or (either completely or partially) resistant to the action of the debranching enzymes (Fig. 8). As discussed above, this type of dextrin may have a very short side chain (one glucosyl residue) and/or a chain branched at the reducing end. Thus, there were clear similarities to the theme of structures found in sample III.

Jane et al.[29] have shown that waxy maize starch granules contained several branches scattered into the crystalline lamellae and were found in population A. As previously discussed, this population of dextrins (represented by sample I) was indeed to a large extent multiply branched, and about 1/3 of the dextrins in population B (sample III) also carried at least one branch. From the quantitative data given in Figures 6 and 8, it was concluded that not less than one-half of all dextrins in the nanocrystals were branched molecules that represented the remnants of the clustered branches of amylopectin inside the semicrystalline rings of the granules. More precisely, they likely represented remnants of branched building blocks from which clusters are constructed.[11,13]

A tentative model of the building blocks inside clusters is drawn in Figure 9a. The internal chain length (ICL) between branches in adjacent blocks was experimentally estimated to 7–8 residues.[13] Larger blocks contain two or a few branches in close proximity to each other with very short internal chains (ICL 1–5), which is in agreement with results from computer models of two adjacent double helices.[15] Inside the starch granules, some blocks are found embedded in the amorphous lamella, whereas others are located at, or immediately above or below, the boundary between the amorphous and crystalline lamellae. The boundary itself is irregular on the Angström scale. When the granule is treated with acid, the amorphous parts are eroded, leaving a nanocrystal composed of double helices (Fig. 9b). Depending on the exact position of the branched building blocks, the two major groups of molecular components (A and B) remain in the crystalline structure. This gives rise to the different types of dextrins detected as resistant to the debranching enzymes and to β-amylase (Fig. 9c). However, the groups mainly consist of very similar molecular structures. Thus, 27 mol % of branched molecules were found in population A and an approximately equal amount (24 mol %) were found in population B (structures 2–4). Each of the latter was (most probably) forming a double helix with a linear chain. As the linear chains constituted ~47 mol % of the molecules, one-half (24 mol %) were combined with the branched dextrins of population B into double helices, whereas the other half formed double helices constituted of two linear chains.

The branched molecules (2) and (3) in population B are examples of possible dextrins being sensitive to β-amylase, but either sensitive or resistant to debranching enzymes, respectively. They are analogous to the molecular structures (5) and (6) in population A, and from the quantitative estimations it was clear that these structural types were divided approximately equally between populations A and B. The molecular structure (4) in population B carries a resistant branch at the non-reducing end side, making it resistant to β-amylase as well. The structure is analogous to structures (7) and (8) of population A, and, again, it was equally divided between the populations. In conclusion, the nanocrystals could be considered as possessing a principally homogenous and regular molecular structure.

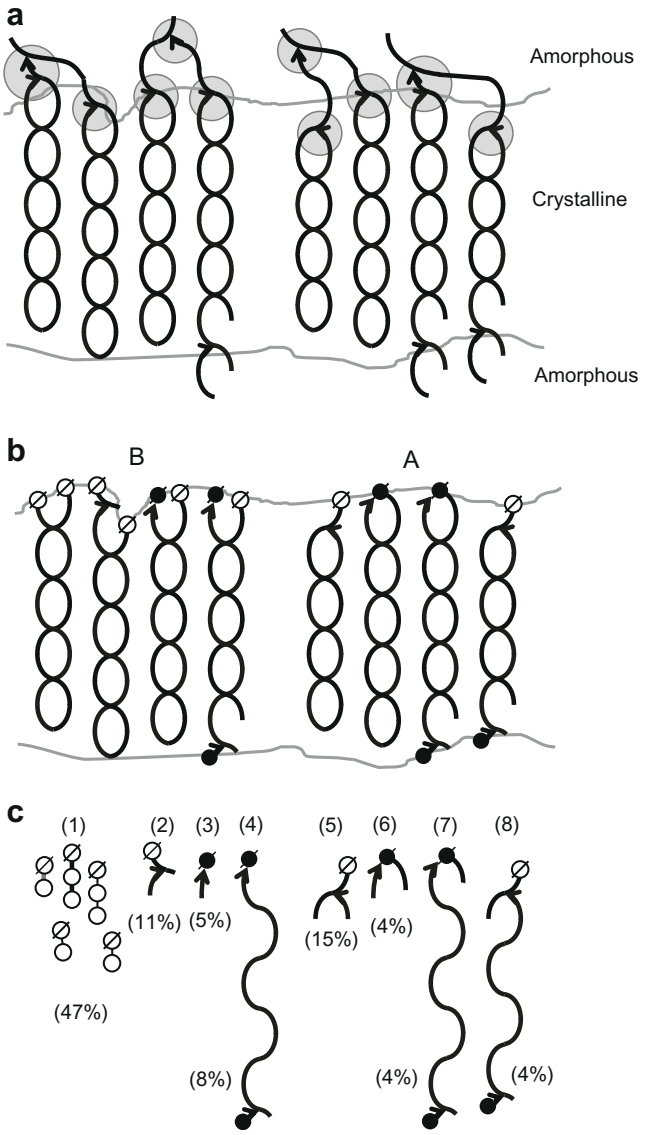

Figure 9. (a) A hypothetical model of branched building blocks (encircled in light gray), being constituents of the larger structural units of clusters, as they are found in the amorphous and crystalline lamellae of the starch granule. Double helices extend from the blocks into the crystalline lamella. The branches are symbolized by arrows and chains of (1→4)-linked glucosyl residues by solid lines. The borders between the lamellae are indicated by irregular light gray lines. (b) The molecular composition of nanocrystals formed after acid treatment of the granule. Examples of possible structures found in populations A and B are shown. Ø symbolizes a reducing-end residue and the filled symbol a symbolizes reducing residue involved in a (1→6) linkage being resistant to debranching enzymes. ● symbolizes a single residue in a branch also resistant to debranching. (c) Dextrins of different principal types (1–8) formed from the structures in (b) by β-amylolysis. The molar proportions found in the nanocrystals are indicated. Structure (1) symbolizes maltose and maltotriose that are formed from linear dextrins of population B. The glucan chains in branched dextrins are reduced into short segments, unless the enzyme is blocked by a branch close to the non-reducing end. The structures of β-limit dextrins are therefore divided into more sensitive (2, 3, 5, and 6), less sensitive (7 and 8), or resistant (4) to β-amylase.

5. Conclusions

The aim of the present work was to fully characterize the molecular content of A-type nanocrystals prepared by mild sulfuric acid hydrolysis of waxy maize starch granules. Several populations of dextrins have been found which correspond to different structural motifs. One of these has $\overline{DP}$ 14.2 which, in the double-helical structure, corresponds to a length of 5 nm and to the thickness of

the crystalline lamellae within the starch granule. This clearly indicates that the nanocrystals correspond to the crystalline lamellae present in native starch granules. As the nanocrystals were described by parallelepipedal blocks with a length of 20–40 nm and a width of 15–30 nm, this would indicate that between 150 and 300 double-helical components are making up these crystalline domains. Further analysis indicates that roughly half of the dextrins in the nanocrystals are branched molecules, which is far more than previous investigations suggested. We also conclude that they are equally distributed between populations A and B of high and low molecular weights, respectively. Taking into account the length of these branches and the thickness of the platelets, it is likely that the majority of the branching points are found at the reducing-end surface of the nanocrystals, whereas the rest are located at the non-reducing side.

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