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Structure of clusters and building blocks in amylopectin from developing wheat endosperm

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ABSTRACT

Changes in internal structure of amylopectin (AP) during wheat endosperm development were studied by isolating clusters and building blocks of AP from both large A-type and small B-type starch granules at different maturity stages up to harvest time at 49 days after anthesis (DAA). Clusters isolated from B-type granules had a degree of branching (DB) of 16.5–16.8% and were more tightly branched than those isolated from A-type granules (DB 15.7–16.2%). The degree of polymerization (DP) of the clusters increased in both types of granules during the pre-physiological maturity stage up to 28 DAA. Clusters at maturity were smaller with less branches and building blocks than at the end of the pre-maturity stage. It is suggested that this was due to a continuous trimming of the cluster structure after the active period of starch synthesis. Differences were evident between A- and B-type granules with regards to glucan trimming and the type of new chains produced.

1. Introduction

Amylopectin (AP) is a highly branched molecule with α-(1,4)-linked D-glucose chains and α-(1,6)-linked branches (Pérez & Bertoft, 2010). Branches of AP coalesce to form clusters, which are defined as groups of branches located within nine glucose residues from one another (Bertoft, 2007). External chains of clusters build up crystalline lamellae in the starch granules, whereas internal chains between the branches form the amorphous lamellae (Pérez & Bertoft, 2010). Tightly branched regions with internal chain lengths of ~1–3 glucose residues are called building blocks (Bertoft, 2007). These blocks occur in a range of sizes. The smallest consist of two chains with a degree of polymerization (DP) of 5–9 and the largest contain more than 10 chains (DP > 45); their relative abundance decreases with increasing block size. The number of building blocks in a cluster (NBbl) depends on the size of the cluster: in cereals the average NBbl is 5–6 (Bertoft, Källman, Koch, Andersson, & Åman, 2011; Bertoft, Koch, & Åman, 2012b). Inside the cluster, the building blocks are interconnected by longer segments with inter-block chain lengths (IB-CL) of ~5–8 glucose residues (Bertoft et al., 2012b). The internal organization of the chains inside the building blocks is unique to each starch. Building blocks from cereal starches (rye, oat, waxy maize, and rice) have higher numbers of the shortest internal B-chains (DP 3–7), resulting in a lower ratio of A:B chains in the building blocks than in starches from other sources. This discrepancy suggests that the conformation of chains in AP from cereals is mostly of the Haworth type, whereas that in other starches is of the Staudinger type (Bertoft et al., 2012b).

An understanding of the detailed structure of AP is important because the chain organization of the backbone in the amorphous lamellae has a profound impact on the granular architecture and functionality of the starch. Computer models of AP have been used to demonstrate the importance of chain lengths between branches for the initialization and stabilization of the arrangement of double helices (O'Sullivan & Perez, 1999). By correlating gelatinization parameters with the number of building blocks in clusters, the inter-block chain lengths, and the external chain lengths, Vamadevan, Bertoft, and Seetharaman (2013) found relationships between the structural parameters that revealed the importance of the internal structure of AP for the functionality of starch.

Both the internal chain length distribution and the internal chain organization of AP are important factors in the development of an understanding of AP biosynthesis. Nakamura (2002) modeled the starch biosynthesis process, highlighting glucan extension and branching in both crystalline and amorphous lamellae. It appears, however, that an examination of the unit chain distribution in AP

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alone is insufficient to explain all localized structural changes, such as chain elongation, glucan trimming and branching, or the locality (crystalline or amorphous lamellae) where they occur. The formation of the building block structure during starch biosynthesis is not yet understood, even though starches from a broad range of botanical sources have been used to study the structure and organization of AP constituents (cluster structure of AP, building block structure, and building block organization) (Bertoft, Koch, & Åman (2012a)). The changes in starch structure during development are due mainly to the expression pattern and substrate specificity of the different isoforms of starch biosynthetic enzymes (ADP-glucose pyrophosphorylase, starch synthases, starch branching enzymes, and starch debranching enzymes, DBE) involved in the development of endosperm starch (reviewed by Tetlow, 2011). Storage starches from different plant sources, including wheat endosperm (Kulp, 1973; Waduge, Xu, & Seetharaman, 2010; Waduge, 2012; Wei et al., 2010), corn endosperm (Li, Blanco, & Jane, 2007), rice endosperm (Briones, Magbanua, & Juliano, 1968; Murugesan, Hizukuri, Fukuda, & Juliano, 1992), and potato tubers (Liu, Weber, Currie, & Yada, 2003), show variations in structure with maturity.

Starch granules in wheat endosperm contains three size-classes at maturity: large A-type granules with a diameter >∼16 μm, small B-type granules with diameters of 5–16 μm, and very small C-type granules with diameters <5 μm (Bechtel, Zayas, Kaleikau, & Pomeranz, 1990). In practice, however, the size-distributions are overlapping considerably. A-type granules are initiated at ∼4 days after anthesis (DAA), B-type granules at ∼10–14 DAA, and C-type granules at ∼21 DAA (Bechtel et al., 1990; Parker, 1985). C-type granules contribute only very little to the total mass of starch and, because they are difficult to separate from the other small granules, they are included with the B-type granules in many studies (Tetlow, 2011). In a previous study (Kalinga et al., 2014) conducted on AP from developing wheat endosperm starches, it was found that certain structural parameters, such as the external or internal chain length and the relative number of A- and B-chains, of AP of both A- and B-type granules changes over time, suggesting that both internal (amorphous lamellae) and external (crystalline lamellae) chains are subjected to changes. The objective of this study was to provide further details of the evolution of the fine structure at the level of clusters and building blocks of AP in the developing wheat endosperm starch. Because A- and B-type starch granules of wheat have been reported to have different structural and functional properties (Ao & Jane, 2007), it is of interest to understand the differences in their AP building block structure.

2. Materials and methods

2.1. Materials

Wheat grains from Eastern hard red spring wheat (Hobson variety) cultivated in Ontario, Canada, in 2009 were harvested from four maturity stages: 7, 14, 28, and 49 days after anthesis (DAA) with a variation of ±3 days maturity within a spike (Peterson, 1965). Heads were tagged when 50% of the spikelets within the head were anthesised. Soon after harvesting, spikes were stored at −20 °C to prevent any enzyme activity until the starch was extracted.

2.2. Enzymes

α-Amylase (315 U/mL) from *Bacillus amyloliquefaciens* [(1,4)-α-D-glucan glucanohydrolase; EC 3.2.1.1], pullulanase (36.3 U/mg) from *Klebsiella planticola* (amylopectin 6-glucanohydrolase; EC 3.2.1.41), isoamylase (1000 U/mg) from *Pseudomonas* sp. (glycogen 6-glucanohydrolase; EC 3.2.1.68), and β-amylase (705 U/mg) from barley [(1,4)-α-D-glucan maltohydrolase; EC 3.2.1.2] were donated by Megazyme (Wicklow, Ireland). Phosphorylase *a* (∼25 U/mg) from rabbit muscle [(1,4)-α-D-glucan:orthophosphate α-D-glucosyltransferase; EC 2.4.1.1] was purchased from Sigma–Aldrich (Deisenhofen, Germany).

2.3. Starch isolation, granule separation, and amylopectin extraction

Starch isolation was conducted according to the method by Waduge et al. (2010). At 7 DAA only one size-distribution of granules existed, but at 14, 28, and 49 DAA large A-type and small B-type granules were separated by centrifugation in glycerol solutions (Waduge, 2012). The purity of separated A- and B-type granules was measured using a particle size analyzer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) and was >97% on volume basis. Amylopectin was extracted according to the method described by Klucinec and Thompson (1998) with minor modifications (Bertoft et al., 2011). The yields of AP varied between 45% and 64%. The purity of the AP was measured as described by Kalinga et al. (2013) and was >95% for all the samples.

2.4. Isolation of clusters and their φ,β-limit dextrins

Time course of α-amylolysis of AP for cluster preparation was conducted according to Kong, Corke, and Bertoft (2009). Clusters of amylopectin were then prepared based on the time-course experiment using pre-optimized conditions; 200 mg of AP (10 mg/mL) was hydrolyzed with α-amylase (0.09 U/mL) in a water bath at 25 °C for 90 min. The external chains of the clusters were removed by phosphorylase *a* and β-amylase (Kong et al., 2009). The resulting φ,β-limit dextrins (φ,β-LD) of the clusters were freeze-dried and the size distribution of the clusters was analyzed by gel permeation chromatography (GPC) on a column (1.6 cm × 90 cm) of Sepharose CL 6B (GE Healthcare, Uppsala, Sweden) as described elsewhere (Bertoft, 2007).

2.5. Chain length distribution of clusters

The unit chain distribution of the φ,β-LD of clusters was analyzed by means of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex ICS 3000, Synnyvale, CA, USA) on a CarboPac PA-100 column (4 mm × 250 mm), as described by Bertoft et al. (2012a), and PAD signals were converted to carbohydrate content as described by Koch, Andersson, and Åman (1998).

2.6. Building block preparation and structure

Building blocks were prepared from clusters by the method of Bertoft et al. (2012a). The size distribution of the building blocks was analyzed by GPC on a column (1.6 cm × 90 cm) of Superdex 30 (GE Healthcare, Uppsala, Sweden) with 0.05 M NaCl as an eluent at a rate of 1 mL/min. The column was calibrated using α-dextrins (Bertoft & Spoof, 1989). Fractions of 1 mL were collected, and the carbohydrate content was determined using phenol and sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.7. Statistical analysis

Statistical analysis was performed with SPSS 17.0 statistical software (SPSS Institute Inc., Chicago, IL, USA). All analyses were duplicated at least twice, and mean values are reported. A comparison of the means of the structural parameters of the clusters

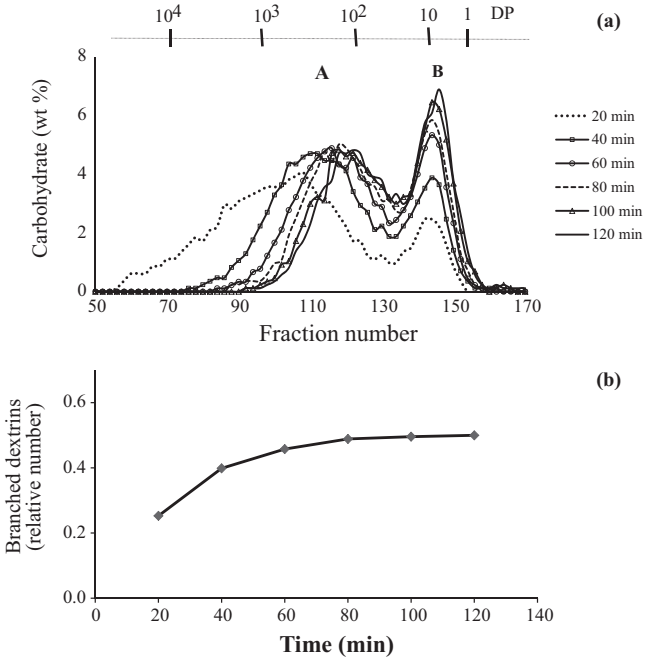

Fig. 1. Time course of α-amylolysis of amylopectin from 49 DAA A-type starch granules: (a) gel-permeation chromatography on Sepharose CL-6B showing two major groups of dextrins formed with DP > 40 (fraction A) and with DP < 40 (fraction B); (b) development of the relative number of branched dextrins in fraction A based on the total carbohydrate content in fraction A divided by the average DP of fraction A.

and the building blocks were conducted using one-way analysis of variance (ANOVA) and Duncan's test ($p < 0.05$).

3. Results

3.1. α-Amylolysis of amylopectin from wheat starch

Mature endosperm starch was used as model for the estimation of the appropriate time of α-amylolysis for the production of clusters. The change in the molecular size distribution with time is shown in Fig. 1a. The α-amylolysis of wheat AP was similar to that previously reported for other starches (Bertoft, Zhu, Andtfolk, & Jungner, 1999; Kong et al., 2009; Zhu, Corke, Åman, & Bertoft, 2011) and produced a bimodal size distribution of dextrins. Fraction A typically represents clusters and groups of clusters and fraction B contains small fragments derived primarily from external AP chains (Bertoft, 1989). The hydrolysis induced an increase in the number of dextrin molecules over time, as shown in Fig. 1b. Initially, the hydrolysis reaction rapidly increased the number of dextrin molecules, but after ~80 min the changes with time became small because α-amylase, which contains nine subsites, reacts fast when all of the subsites are filled with D-glucosyl residues, but the reaction slows down considerably when this criterion is not met. The enzyme attacks simultaneously the external chains, resulting in the formation of predominantly maltohexaose (fraction B), and long internal AP chains, resulting in the release of clusters (found in fraction A) (Bertoft, 1989; Robyt & French, 1963). In this study, an enzyme activity of 0.09 U/mL was used, giving a near-zero increase in branched dextrin formation after 80 min of hydrolysis, which suggests a near-complete hydrolysis of the long internal chains (with ≥9 glucose residues) and the predominance of clusters of chains in fraction A. For all samples, 90 min was chosen for cluster preparation since no net formation of branched dextrins was observed beyond 90 min under the conditions of the time-course (Fig. 1b).

Table 1
Characterization of φ,β limit dextrins of clusters from amylopectin ($n = 2$).

Maturity stage	DP[1]	NC[2]	DB (%)[3]	CL[4]	ICL[5]
A-type granules					
7 DAA	85.0[a]	14.7[b]	16.2[abc]	5.8[b]	3.6[ab]
14 DAA	89.5[b]	15.1[b]	15.7[a]	5.9[c]	3.8[b]
28 DAA	87.6[b]	14.8[b]	15.7[a]	5.9[c]	3.7[b]
49 DAA	82.4[a]	14.2[a]	16.0[ab]	5.8[b]	3.6[ab]
B-type granules					
14 DAA	84.0[a]	14.9[b]	16.5[bc]	5.6[a]	3.4[a]
28 DAA	88.2[b]	15.8[c]	16.8[c]	5.6[a]	3.4[a]
49 DAA	82.9[a]	14.8[b]	16.7[c]	5.6[a]	3.4[a]

Note: Values followed by a different superscript in each column are significantly different ($p < 0.05$). DP = degree of polymerization; DAA = days after anthesis.
[1] Average size of the cluster as measured by GPC. Fractions containing <1% carbohydrates were excluded.
[2] Number of chains = DP/CL.
[3] Degree of branching = (NC−1)/DP × 100.
[4] Average chain length estimated by HPAEC.
[5] Internal chain length = [(CL−ECL) × NC]/(NC − 1) − 1, in which ECL = 1.5.

3.2. Characterization of φ,β-limit dextrins of clusters

In general, the φ,β-LDs of clusters (subsequently simply called "clusters") from all samples exhibited similar size distributions, of which only one representative sample (28 DAA A-type granules) is shown in Fig. 2a. The DP-range of the clusters was 15–680 at all maturity stages (fractions containing <1% carbohydrate were excluded). The average DP of the clusters (Table 1) increased from DP 85.0 to DP 89.5 when A-type granules were developing from 7 DAA to 14 DAA, and it remained unchanged thereafter until 28 DAA. At maturity (49 DAA), the size of the clusters decreased to DP 82.4. Similarly, when B-type granules were developing, the size of the clusters increased from DP 84.0 to 88.2 in the pre-physiological maturity stage, and then the size decreased to DP 82.9 when the granules reached maturity. A- and B-type granules at a certain maturity stage did not show differences in cluster size, except at 14 DAA when clusters in B-type granules were smaller than in the A-type granules.

By debranching the clusters and subsequent analysis of the internal unit chain distribution (Fig. 2b), additional parameters of the cluster structures were estimated and are summarized in Table 1. The average number of chains (NC) is another parameter that expresses the size of the clusters (Bertoft et al., 2012a). NC of the A-type granules did not change during the pre-physiological stage; however, it decreased from 14.8 to 14.2 during the period between 28 and 49 DAA ($p < 0.05$). In B-type granules, NC increased from 14.9 to 15.8 when the granules developed from 14 DAA to 28 DAA, and it decreased to 14.8 by 49 DAA. The degree of branching in the clusters (DB) did not change significantly in either B- or A-type granules during granule development. However, B-type granules generally exhibited a higher DB than A-type granules ($p < 0.05$).

Table 1 shows the average chain length (CL) and internal chain length (ICL) of the clusters. CL slightly increased from DP 5.8 to DP 5.9 ($p < 0.05$) in A-type granules when they developed from 7 DAA to 14 DAA, and it decreased to DP 5.8 by 49 DAA. In contrast, CL was smaller in B-type granules (DP 5.6) and it remained unchanged during their development. ICL was also somewhat smaller in the B-type granules (DP 3.4) and, in both types of granules, ICL remained practically constant throught development ($p < 0.05$).

3.3. Unit chains in clusters

The internal unit chain profiles of clusters in A- and B-type granules from developing endosperm were similar (an example is shown in Fig. 2b together with the internal chain profile of the original amylopectin). Maltose (A-chains) and maltotriose

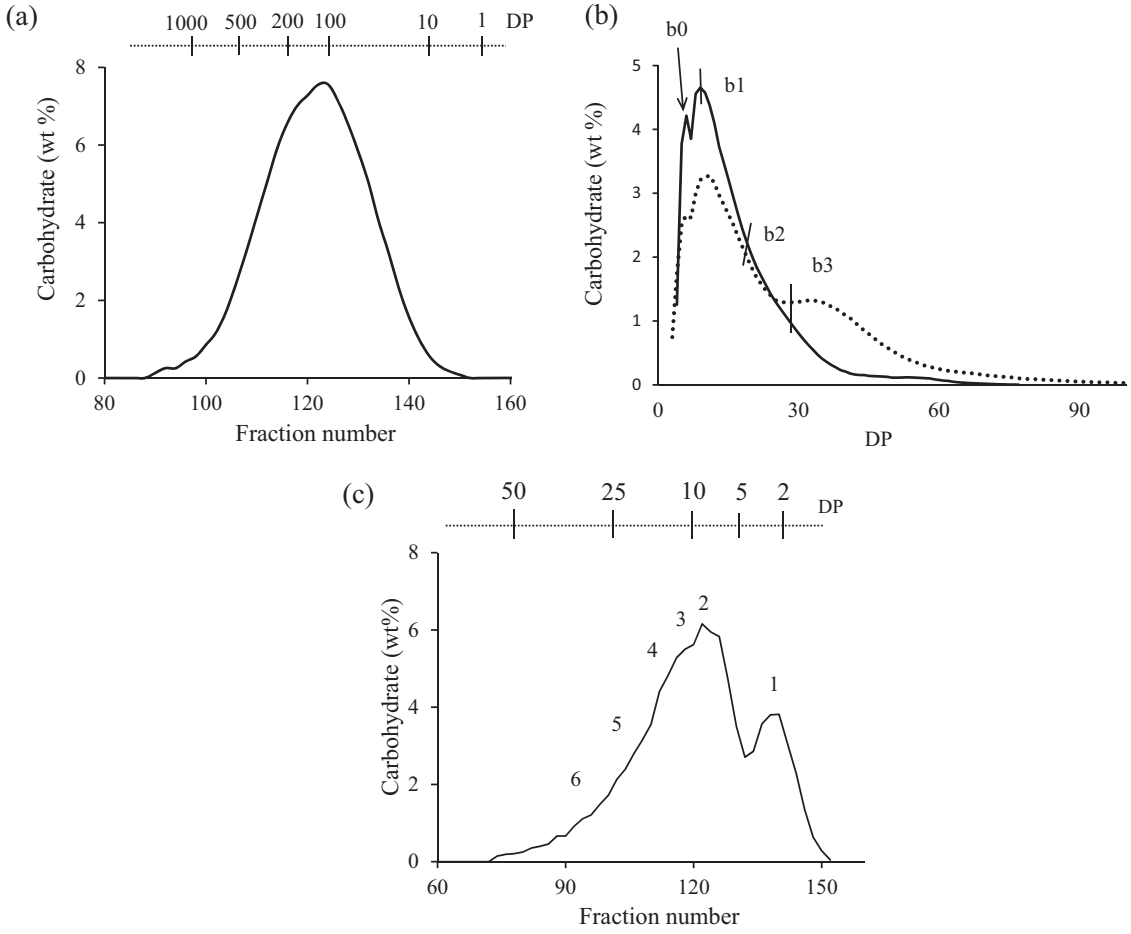

Fig. 2. Characterization of φ,β-limit dextrins of clusters isolated from amylopectin of A-type granules at 28 DAA. (a) Size distribution obtained by GPC on Sepharose CL-6B: (b) internal chain distribution obtained by HPAEC (only chains with DP ≥ 4 are shown) of clusters (—) and the original amylopectin (. . .): (c) size distribution obtained by GPC on Superdex 30 of building blocks in clusters. Categories of chains in clusters are indicated in (b) and groups of building blocks are indicated by numbers in (c).

(mixture of A- and the shortest internal B-chains) (Bertoft et al., 2012a) were excluded from the chromatograms. α-Amylolysis drastically reduced the proportion of long chains (DP ≥ 25), and the increasing population of short B-chains showed signature profiles with peaks around DP 6 and DP 9.

The relative number of different chains with DP 2, 3, and ≥4 are listed in Table 2. In clusters from A-type granules, the relative number of chains at DP 2 (A-chains) exhibited a pattern similar to that in the original amylopectins (Kalinga et al., 2014), i.e., the number of A-chains decreased slightly during the pre-physiological maturity stage up to 28 DAA and then increased at the maturity stage. In B-type granules, the number of A-chains decreased with increasing maturity. The mixture of A- and B-chains with DP 3 showed few significant changes. Chains of DP ≥ 4, which represent B-chains, also showed only minor changes between maturity stages: In A-type granules, B-chains in both the original AP (Kalinga et al., 2014) and in clusters showed similar change patterns (Table 2), i.e., during pre-physiological maturity, B-chains increased to the highest number at 28 DAA (40.0%) and then decreased to 38.1% by 49 DAA. In clusters of B-type granules, no significant change in B-chains was evident during granule development. In general, clusters in A-type granules included more chains with DP ≥ 4 than did B-type granules, except at 49 DAA when no difference was observed.

When α-amylase cleaves long internal B-chains in AP, specific categories of B-chains are formed, which were assigned with small letters as b0-, b1a-, b1b-, b2-, and b3-chains (Bertoft et al., 2012a). It is, however, not possible to distinguish the newly formed b-chains in the isolated clusters from B-chains that pre-existed in the parent amylopectin (Bertoft et al., 2012a). The b-chains of the clusters are defined in Fig. 2b, and their relative molar distributions are given in Table 2. In general, only very small differences of the chain categories were detected both between the granule populations and the maturity stages. However, at 28 DAA the clusters from A-type granules exhibited a maximum of b0- (DP 4–6) and b1a-chains (DP 7–10), whereas in B-type granules b0-chains were found in largest number at 49 DAA (11.1%). The number of b1b-chains (DP 11–18) was almost similar to that of b1a-chains, whereas the number of b2-chains (DP 19–27) was comparatively small and b3-chains (DP ≥ 28) occurred in almost trace amounts. In general, at all maturities, b1b-, b2-, and b3-chains were somewhat more abundant in the clusters from A-type granules than from B-type granules.

3.4. Building block composition of clusters

Extensive hydrolysis of clusters with α-amylase releases small, tightly branched areas in clusters, which are known as building blocks. Linear dextrins with DP 1–6 are released from segments located between the branched blocks. These linear dextrins are further hydrolyzed into glucose, maltose, and maltotriose by treatment with β-amylase, a procedure that results in a better size separation of the linear products from the branched building blocks (Bertoft et al., 2012a). The building block composition of developing wheat endosperm starches exhibited almost identical profiles and therefore only the building block composition of clusters isolated

Table 2
Relative molar distribution (%) of different chain categories in clusters isolated from amylopectin ($n = 2$).

Maturity stage	DP 2[1]	DP 3[2]	DP ≥ 4[3]	b0[4]	b1a	b1b	b2	b3
A-type granules								
7 DAA	51.2^{bc}	10.6^{ab}	38.2^{ab}	10.0^{bc}	11.7^{a}	11.1^{ab}	3.9^{b}	1.5^{bc}
14 DAA	51.2^{bc}	9.9^{ab}	38.9^{bc}	9.5^{a}	11.8^{a}	11.8^{d}	4.1^{c}	1.7^{c}
28 DAA	49.1^{a}	10.9^{b}	40.0^{c}	10.5^{d}	12.4^{b}	11.7^{cd}	3.8^{b}	1.6^{c}
49 DAA	52.2^{bc}	9.6^{a}	38.1^{ab}	9.8^{ab}	11.5^{a}	11.3^{bc}	3.9^{bc}	1.6^{c}
B-type granules								
14 DAA	53.0^{c}	9.8^{ab}	37.3^{a}	10.0^{abc}	11.5^{a}	10.8^{a}	3.5^{a}	1.5^{bc}
28 DAA	52.5^{bc}	10.0^{ab}	37.5^{ab}	10.3^{cd}	11.7^{a}	10.6^{a}	3.5^{a}	1.4^{ab}
49 DAA	50.7^{ab}	10.9^{b}	38.3^{ab}	11.1^{e}	11.9^{ab}	10.7^{a}	3.4^{a}	1.3^{a}

Note: Values followed by a different superscript in each column are significantly different ($p < 0.05$). DP = degree of polymerization; DAA = days after anthesis.
[1] a-Chains.
[2] Mixture of a- and b-chains.
[3] b-Chains.
[4] Categories of b-chains: b0 = DP 4–6, b1a = DP 7–10, b1b = DP 11–18, b2 = DP 19–27, b3 = DP ≥28.

Table 3
Building block structures of clusters of amylopectin from developing wheat endosperm starch ($n = 2$).

Maturity stage	Linear dextrins[1]		Branched blocks[2]			NBbl[3]	DBbl (%)[4]	IB-CL[5]
	Mole (%)	DP	Weight (%)	Mole (%)	DP			
A-type granules								
7 DAA	51.7^{a}	2.2^{b}	82.4^{c}	48.3^{b}	11.0^{b}	6.4^{ab}	7.5^{a}	6.4^{a}
14 DAA	55.4^{ab}	2.1^{ab}	80.0^{ab}	44.6^{ab}	10.2^{a}	7.0^{d}	7.8^{b}	6.6^{ab}
28 DAA	57.2^{b}	2.0^{ab}	79.7^{a}	42.8^{a}	10.5^{ab}	6.6^{c}	7.6^{ab}	6.7^{b}
49 DAA	52.7^{a}	2.1^{ab}	81.8^{bc}	47.3^{b}	10.7^{ab}	6.3^{ab}	7.6^{ab}	6.4^{a}
B-type granules								
14 DAA	55.2^{ab}	2.0^{ab}	81.3^{abc}	44.8^{ab}	10.6^{ab}	6.4^{ab}	7.7^{ab}	6.4^{a}
28 DAA	53.9^{ab}	2.1^{ab}	81.3^{abc}	46.1^{ab}	10.6^{ab}	6.7^{c}	7.6^{ab}	6.4^{a}
49 DAA	57.5^{b}	1.8^{a}	81.4^{abc}	42.5^{a}	10.9^{ab}	6.2^{a}	7.5^{a}	6.5^{ab}

Note: Values followed by a different superscript in each column are significantly different ($p < 0.05$). DP = degree of polymerization; DAA = days after anthesis.
[1] Fragments with DP 1–3.
[2] Dextrins with DP ≥5.
[3] Average number of branched building blocks = $(Wt\%_{Bbl}/100) \times (DP_{cluster}/DP_{Bbl})$.
[4] Density of building blocks = $NBbl/DP_{cluster} \times 100$.
[5] Inter block chain length = $(mole\%_{linear\ dextrins} \times DP_{linear\ dextrins})/mole\%_{Bbl} + 4$.

from A-type granules at 28 DAA is shown as a representative sample in Fig. 2c. The building block profile was composed of regions representing the linear dextrins (group 1 with a peak at DP ~ 2) and the branched building blocks, which consist of groups 2, 3, and 4 and contain 2, 3, and 4 chains, respectively. The large building blocks of groups 5 and 6 with DP ≥ 20 contain multiple branches (Bertoft et al., 2012b). Group 2, which showed a peak at DP ~ 8, was most common, followed by group 3. Building blocks of group 4 and larger were found in successively lower quantities in all samples. The results suggested only minor differences among the maturity stages of developing wheat endosperm starch.

The compositions of the linear dextrins and branched building blocks in the clusters are summarized in Table 3. The quantity of linear dextrins in A-type granules increased continuously during the pre-physiological maturity stage up to 28 DAA, and then it decreased ($p < 0.05$). In B-type granules these changes were insignificant. Branched building blocks accounted for 79.7–82.4% of the cluster by weight. The average size of the branched building blocks changed slightly with maturity. In A-type granules, the size of the building blocks at 7 DAA was DP 11.0, which decreased to DP 10.2 at 14 DAA ($p < 0.05$) and remained almost constant thereafter. The DP of branched building blocks in B-type granules remained nearly unchanged throughout grain development.

The building block structure of the clusters is also summarized in Table 3. The average number of branched building blocks in a cluster (NBbl) ranged from 6.2 (49 DAA B-type granules) to 7.0 (14 DAA A-type granules). The density of branched building blocks (DBbl) in the clusters increased slightly when A-type granules developed from 7 DAA to 14 DAA but remained constant afterwards. No change in DBbl was observed during B-type granule development. The inter-block chain length (IB-CL) corresponds to the average chain length between two adjacent building blocks (Bertoft et al., 2012a). In the current study, a significant ($p < 0.05$) increase in IB-CL occurred when A-type starch granules developed from 7 DAA to 28 DAA (from 6.4 to 6.7), followed by a decrease back to 6.4 at 49 DAA. In B-type granules, IB-CL remained constant throughout granule development.

Table 4
Relative molar distribution (%) of groups of building blocks in clusters isolated from amylopectin ($n = 2$).

Maturity stage	Group 2	Group 3	Group 4	Group 5	Group 6
A-type granules					
7 DAA	56.0^{a}	24.9^{c}	9.6^{b}	7.7^{bc}	1.8^{c}
14 DAA	60.8^{c}	23.3^{abc}	8.4^{a}	6.5^{a}	1.0^{a}
28 DAA	59.2^{bc}	23.8^{abc}	8.8^{ab}	6.9^{ab}	1.3^{ab}
49 DAA	57.0^{ab}	24.5^{bc}	9.6^{b}	7.6^{bc}	1.3^{ab}
B-type granules					
14 DAA	60.1^{c}	23.0^{ab}	8.3^{a}	7.1^{abc}	1.6^{bc}
28 DAA	59.0^{bc}	23.5^{abc}	8.8^{ab}	7.3^{abc}	1.5^{bc}
49 DAA	58.9^{bc}	22.4^{a}	8.8^{ab}	8.0^{c}	1.9^{c}

Note: Values followed by a different superscript in each column are significantly different ($p < 0.05$). DAA = days after anthesis.
Building blocks in group 2, 3 and 4 contain 2, 3 and 4 chains, respectively, in group 5 approximately 5–7 chains, and in group 6 more than 7 chains.

The relative molar distribution of the groups of branched building blocks in the clusters is provided in Table 4. The relative distribution of the different groups did not change during B-type

granule development. In A-type granules, however, significant differences were observed during pre-physiological maturity, primarily when the granules developed from 7 DAA to 14 DAA, when group 2 increased from 56.0% to 60.8%. Instead, groups 4, 5, and 6 decreased, whereas the relative molar amount of group 3 building blocks remained practically indifferent. Later, group 2 decreased slightly to 57% at 49 DAA, whereas groups 4 and 5 tended to increase. The abundance of each group of building blocks was rather similar in B-type granules as in A-type granules, with the exception of group 6, which was generally found in slightly higher quantities in the B-type granules. However, in the newly developed A-type granules at 7 DAA, group 6 resembled the quantity found in B-type granules.

4. Discussion

In this study the development of amylopectin structure was analyzed with respect to two major growth stages: grains harvested from 7 to 28 DAA were considered to be at pre-physiological maturity, and grains harvested at 49 DAA were considered to be at maturity. Starch is synthesized during the pre-physiological maturity stage, whereafter only very little net synthesis occurs (Jenkins, Meredith, & Loney, 1975). Therefore, starch analyzed up to 28 DAA represents mixtures of starch from very early stages and starch laid down at later stages, and it is not possible to know if the structure at the later stages represents a homogenous sample or if it is a mixture of different structures from early and later stages. The latter is probably true since the endosperm represents a mixture of cell types of varying physiological age (Olsen, 2001) and any structural changes that were detected would suggest that the real differences in the structure of starch laid down at the later stages were actually much more pronounced than measured. Differences that were detected after 28 DAA suggested, however, that changes occurred within the existing starch.

4.1. Clusters in amylopectin

The structural changes in AP extracted from endosperm starches at different maturity stages have been discussed elsewhere (Kalinga et al., 2014). It was observed that the AP fine structure of both A- and B-type granules was subject to change during development until its physiological maturity. This study examined the internal structure of AP in developing endosperm at two different structural levels: clusters and building blocks. It is assumed that, when amylopectin is hydrolysed with α-amylase from *B. amyloliquefaciens*, long chains of AP are cleaved, leaving relatively resistant clusters built up by short chains (Bertoft et al., 2012a). The average size of the clusters isolated from AP of developing wheat endosperm (between DP 82.4 and 89.5; Table 1) was larger than that previously reported for clusters isolated from several other botanical sources; only Andean yam bean starch was reported so far to have clusters of similar average size (Bertoft et al., 2012a). Andean yam bean (Bertoft, Piyachomkwan, Chatakanonda, & Sriroth, 2008) and wheat starch at any maturity stage (Waduge, 2012) were both reported to have A-type crystallinity. In general, starches that exhibit A-type crystallinity (e.g., cereal storage starches) possess relatively large clusters compared to starches with B-type crystallinity (e.g., potato and yam starch) (Bertoft et al., 2012a).

In both A- and B-type wheat granules, the size and the number of chains (NC) of the clusters increased during pre-physiological maturity, but decreased at maturity. At the pre-physiological maturity stage, there is an ample supply of sugars as well as an increased expression of starch synthases (SS), ADP-glucose pyrophosphorylase (AGPase), debranching enzymes (DBE) (Shewry et al., 2009), and starch branching enzymes (SBE) (Morell, Blennow, Kosar-Hashemi, & Samuel, 1997; Shewry et al., 2009). Apparently, the coordinated response of these enzymes produces larger clusters with additional branches. At maturity, when net synthesis of starch ceased, NC decreased in both types of granules, which could be attributed to a continuous glucan trimming, in which the DBEs remove unfavorably positioned glucan chains (Ball et al., 1996). Clusters isolated from A-type granules at different maturity stages had longer average CL and ICL than clusters isolated from B-type granules, whereas NC and DB were lower (Table 1). This indicated that clusters in B-type granules had a denser structure with more branches than in A-type granules.

4.2. Unit chain composition of the clusters

The unit chain composition of isolated clusters showed small variations with developmental stage as well as between A- and B-type granules (Table 2). The amount of maltotriose was considerably higher in the clusters (~10%) than in their original AP (~2 mole%) (Kalinga et al., 2014), a typical pattern previously observed with other starches. Chains with DP 3 are believed to be produced by α-amylolysis as a result of a general preferential mode of cluster interconnection (Bertoft & Koch, 2000; Bertoft et al., 2012a; Kong et al., 2009). During the development of the starch granules, only minor changes occurred in the amount of maltotriose, indicating that the pattern of cluster interconnection remained similar during wheat endosperm development.

Because chains with DP 3 in clusters are mixtures of a- and b-chains, it is difficult to quantify the accurate proportion of the two chain categories. Only chains with DP 2 were therefore considered to represent pure (or "true") a-chains, and chains with DP ≥ 4 were considered "true" b-chains. When starch granules developed from the pre-physiological maturity stage to the maturity stage, the number of true a-chains increased in A-type granules but decreased in B-type granules. This finding suggested that the mode of branching by SBE, or alternatively trimming by DBE, that occurs in A- and B-type granules is different.

The true b-chains (b0, b1a, b1b, b2, and b3) newly formed during the isolation of the clusters by α-amylase are believed to play a role in interconnecting the clusters and they are therefore remnants of inter-cluster segments of the parent AP molecule (Bertoft et al., 2012a). The major types of inter-block chains within the clusters are b1- and b2-chains, which for the most part correspond to BS_{major}-chains (DP ~8–25) in the original AP. The smallest type of B-chains is b0-chains (to which principally also the b-chains with DP 3 belong). These chains are likely completely embedded in the building blocks and lack inter-block segments. The b1-chains probably contain one inter-block segment and are sub-divided into b1a- and b1b-chains: b1a-chains have DP of 7–10 and b1b-chains have DP from 11 to 18. The b2-chains (DP 19–27) and b3-chains (DP > 28) are involved in the interconnection of three and four building blocks and contain therefore two and three inter-block segments, respectively (Bertoft et al., 2012a).

Changes were observed in the number of b0-chains in clusters isolated from A- and B-type granules. In addition, a slight increase of b1a-chains in clusters of A-type granules was observed at 28 DAA. In contrast, the other b-chain categories, especially b2- and b3-chains, remained practically unchanged during both A- and B-type granule development for two plausible reasons: (1) inside clusters long b-chains remained unaffected during granule development, and glucan chain rearrangement (trimming) occurred primarily with short b-chains; (2) even though structural changes occurred in the long B-chains of AP, the clusters did not reflect these changes due to the removal of the affected segments during isolation of the clusters.

Table 5
Theoretical composition of chains and building blocks in average wheat clusters at 28 and 49 DAA and a comparison with the model of a cluster at 28 DAA in Fig. 3a.

Maturity stage	Number of chains[1]					Number of building blocks[2]			
	a	DP3	b0	b1	b2 + b3	Group 2	Group 3	Group 4	Group 5 + 6
Model									
28 DAA	7	1	2	3	1	4	1	1	1
A-type granules									
28 DAA	7.3	1.6	1.6	3.5	0.8	3.9	1.6	0.6	0.5
49 DAA	7.4	1.4	1.4	3.2	0.8	3.6	1.5	0.6	0.6
B-type granules									
28 DAA	8.3	1.6	1.6	3.5	0.7	4.0	1.6	0.6	0.6
49 DAA	7.5	1.6	1.6	3.4	0.7	3.7	1.4	0.5	0.6

[1] Based on NC and the relative distribution of chain categories in Tables 1 and 2, respectively.
[2] Based on NBbl and the relative distribution of groups of building blocks in Tables 3 and 4, respectively.

4.3. Building blocks in clusters

Minor differences were evident in the size distribution and structural characteristics of building blocks during endosperm development (Tables 3 and 4). Similar types of profiles (Fig. 1c) have previously been obtained for all other starches so far analyzed (Bertoft et al., 2010; Bertoft et al., 2011, 2012a; Bertoft, 2007; Kong et al., 2009; Zhu et al., 2011). The size distribution of building blocks in AP is, therefore, an ubiquitous feature of the AP cluster structure, but the chains that connect the building blocks (IB-CL) are related to the AP structure. In this study of developing wheat endosperm starches, IB-CL was between 6.4 and 6.7, which is characteristic of cereal and other starches of A-type crystallinity (Bertoft et al., 2012a).

In both types of granules, the NBbl of the clusters (Table 3) increased during pre-physiological maturity and decreased at maturity, indicating a direct correlation with cluster size and NC value (Table 1). Given that the occurrence of the other groups was mostly similar throughout development, the major reason for the fluctuations in the NBbl would appear to be that the relative number of group 2 (the smallest) building blocks changed. These results suggested the possibility that a trimming of the glucan chains in group 2 blocks caused the size of the cluster, as well as the NC, to decrease at maturity. However, small but interesting differences were also observed in group 6 building blocks. It was suggested that these building blocks are composed of two smaller building blocks with one or two chains attached to a longer internal segment of DP > 7, and they can be considered to be remnants of incomplete biosynthesis (Bertoft et al., 2012b). Between 14 DAA and 49 DAA, the relative number of group 6 building blocks was higher in B-type granules than in A-type granules, but it was highest in A-type granules at 7 DAA, when it in fact corresponded to the level found in B-type granules. Tetlow et al. (2004) discovered that the AP synthetic enzymes (SS I, SS II, and SBE II) form protein complexes that are detectable only after 10–15 DAA (Tetlow et al., 2008). This biosynthetic enzyme complex is believed to increase the efficiency of AP synthesis, which suggests that lack of the enzyme complex resulted in a greater number of large building blocks (group 6) at 7 DAA. The relatively large quantity of group 6 building blocks in B type granules is not clear.

4.4. A trimming model of clusters

Based on the data presented above (Tables 1–4), one can estimate the number of different chains and types of building blocks of a typical average cluster. This estimated data is presented in Table 5 for clusters in large and small granules at 28 DAA (physiological maturity) and at 49 DAA (mature starch). These calculations suggested only very small differences in the average structures of clusters from A- and B-type granules with regards to both groups of building blocks and types of unit chains. The major difference was only, in fact, that the clusters in B-type granules at average had one more chain, and this was due to one more A-chain than in clusters from A-type granules. Based on this, a general model for clusters in both A- and B-type granules at 28 DAA is proposed in Fig. 3a. The number of building blocks belonging to groups 2, 3, 4, and 5 are encircled and the categories of chains in the cluster are indicated. Group 6 building blocks was not included in the model because the numbers estimated for that group were extremely small (0.1), which indicated that it unlikely existed in more than every tenth cluster. A comparison of the numbers of building blocks obtained experimentally at 28 DAA with those calculated from the model are indicated in Table 5; the numbers show a fairly good correlation, considering that a single illustration represents the average structure of what is probably a very complex mixture of cluster structures. There is also a good agreement between the numbers of chains of different categories obtained experimentally and those in the model.

The cluster size and number of chains (Table 1) were larger at the end of pre-physiological maturity (28 DAA) than at maturity. The number of the small building blocks with only two chains (group 2) was also larger at 28 DAA (Tables 4 and 5), which suggested a trimming of the chains during the period between 28 DAA and 49 DAA. Such trimming was originally suggested by Ball et al. (1996) based on the fact that phytoglycogen, which is a highly branched glucan polymer in isoamylase deficient plants (e.g., *su2* maize), does not crystallize and form granules as normal amylopectin does. Ball et al. (1996) considered phytoglycogen a pre-cursor of amylopectin, which has to be trimmed, i.e., some short chains must be removed by the debranching enzymes (DBE) in order to produce the normal amylopectin structure. So far, however, no further structural evidence has been provided to show exactly how the trimming occurs. The changes in cluster structure observed in this work possibly suggested, for the first time, a more detailed hint based on structural analyses of how this trimming actually might happen.

Two places in Fig. 3a are highlighted with bold arrows where an a-chain is attached close to the non-reducing end of a b1- and b3-chain. Possibly, such positions are unfavorable and contribute to defects in the crystalline lamellae because the chains cannot form double helices. Therefore, the a-chains will be removed by glucan trimming conducted by DBE. Removal at place 1 results in the structure illustrated in Fig. 3b, in which the building block of group 2 has disappeared and the b1-chain has been transformed into an a-chain. The result is similar to the experimentally calculated structure of the clusters in A-type granules at 49 DAA (Table 5), in which the major difference between 28 and 49 DAA is a reduction in the number of building blocks of group 2 and in the number of b1-chains, whereas the number of a-chains is practically indifferent. Removal of the a-chain at place 2 also removes the small building block, but it only results in a reduction of the number of a-chains,

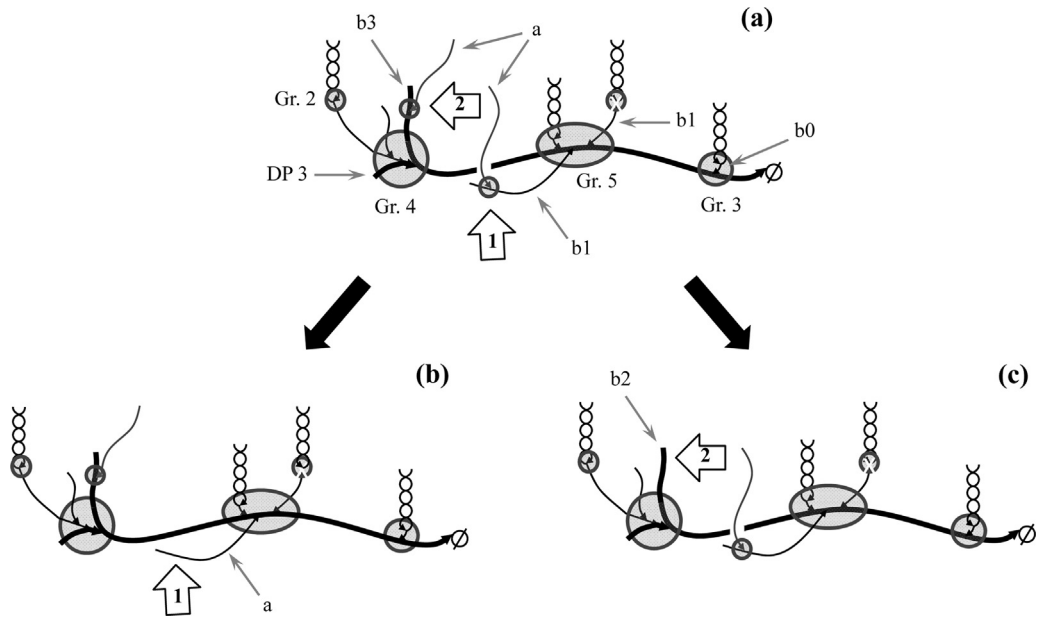

Fig. 3. Models of clusters in wheat amylopectin: (a) the building block structure of an average cluster at 28 DAA based on numbers given in Table 5. Building blocks of different groups are encircled and gray arrows shows examples of different chain categories. Many external chains form double helices that will crystallize in the starch granule. Bold lines indicate chains forming the backbone of the cluster and the chain with DP 3 (alternatively a b0-chain) presumably connects to another cluster in the amylopectin molecule. Bold arrows 1 and 2 highlights hypothetical positions that are trimmed by debranching enzymes before maturity. (b) Trimming at position 1 results in less building blocks of group 2 and the transformation of a b1-chain into an a-chain. (c) Trimming at position 2 results in less building blocks of group 2 and a reduction of a-chains.

whereas the number of long b-chains (b2- and b3-chains) is unaffected. This event resembles the calculated numbers for the average clusters at 49 DAA in B-type granules. Thus, the first example could represent a major type of trimming in A-type granules, whereas the second example might be more common in B-type granules.

5. Conclusions

The structure of clusters isolated from AP changes as a function of the maturity stage of wheat endosperm development. Throughout development, there are only small differences between clusters in A- and B-type starch granules; however, the clusters in B-type granules are more tightly branched than in A-type granules. Clusters initially increase in size in both types of granules. Marked changes in cluster characteristics occur between the end of the pre-physiological maturity stage (28 DAA) and the maturity stage (49 DAA): cluster sizes become smaller, and the number of chains and building blocks are lower in clusters at 49 DAA. The reduction in the number of building blocks at post-physiological maturity is due mainly to the lower number of building blocks that contain two chains (group 2). A trimming activity is a possible cause of the modifications after the pre-physiological maturity period.

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