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Composition of clusters and their arrangement in potato amylopectin

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Abstract

The cluster structure of amylose-free potato starch was investigated in detail. Groups of clusters (structural domains) were produced by α-amylolysis and size-fractionated by methanol precipitation. The domains were then further hydrolysed with α-amylase until free clusters were released. In the form of ϕ,β-limit dextrins the clusters possessed mainly DP around 70 and 50–55, but a minor group of very small clusters were of DP 25. In order to release the clusters, the α-amylase hydrolysed long B-chains into shorter B1b-chains. In the initial stages of hydrolysis small, branched fragments were released, possibly from branched structures outside the clusters. In domain structures composed of two clusters a B2-chain was involved in their interconnection, but when the number of cluster increased, the number of long B2- and B3-chains was apparently higher than predicted from the generally accepted cluster model. The two-directional backbone model offered a possible solution for the interconnection of groups involving several clusters.

Keywords: Potato amylopectin starch; Amylopectin structure; Cluster structure; Cluster interconnection; α-Amylolysis

1. Introduction

Amylopectin is the major macromolecular component of starch and is responsible for the architecture of the starch granules. It is composed of a large number of short chains of approximately 6–35 α-D-glycosyl residues connected by (1 → 4)-linkages. The chains are divided into A-chains, which do not carry other chains, and B1-chains, which carry other chains through (1 → 6)-branches (Peat, Whelan, & Thomas, 1952). In the generally accepted model by Hizukuri (1986), the short chains form clusters that are interlinked through long B2- and B3-chains with a degree of polymerisation (DP) of approximately 40–50 and 60–80, respectively. The proportion of short to long chains depends on the plant species and varies typically from roughly 5 ∼ 13 on a molar basis (Hanashiro, Tagawa, Shibahara, Iwata, & Takeda, 2002).

Within the starch granules the external segments of the clusters form double helices that crystallize into either A- or B-polymorphs (Imberty, Buléon, Tran, & Pérez, 1991). A typical representative of the latter type is potato starch (Buléon, Bizot, Delage, & Multon, 1982; McPherson & Jane, 1999; Vermeylen, Goderis, Reynaers, & Delcour, 2004) and it was shown that B-type starches generally possess somewhat longer average chain lengths (CL) and a higher proportion of long chains than A-crystalline samples (Hizukuri, 1985). The crystals form approximately 5–6 nm thick lamellae that alternate with 3–4 nm thick amorphous lamellae, in which most of the branches of the clusters are found (Jenkins, Cameron, & Donald, 1993; Sanderson, Daniels, Donald, Blennow, & Engelsen, 2006). Stacks of these structures build up semi-crystalline granular rings, often called "growth rings", that alternates with apparently completely amorphous rings. Details of these structures, like the presence of blocklets (Atkin, Abeysekera, Cheng, & Robards, 1998; Baker, Miles, & Helbert, 2001; Gallant, Bouchet, & Baldwin, 1997) or super-helices (Oostergetel & van Bruggen, 1993; Waigh, Donald, Heidelbach, Riekel, & Gidley, 1999), the latter only proposed for potato starch so far, is still under debate.

Despite a rather good knowledge and understanding of the participation of the clusters in the fine structure of the

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starch granules, the knowledge about the fine structure of the clusters themselves and their actual mode of interconnection remains comparatively poor. Clusters were so far isolated from comparatively few samples by using endo-acting enzymes. Possibly because of different action patterns, the reported sizes of the clusters differ. Thus, clusters in potato amylopectin were estimated to be of uniform size with DP (in the form of limit dextrins, in which most of the external chain segments have been removed) around 140 (corresponding to M_n 23,000) when using the maltotetraose forming amylase from *Psuedomonas stutzeri* (Finch & Sebesta, 1992), but of three different sizes from DP 40 to 140 when using cyclodextrin glycosyltransferase (Bender, Siebert, & Stadler-Szöke, 1982), or from 33 to 70 when using the α-amylase of *Bacillus amyloliquefaciens*, which corresponds to roughly 5–10 chains per cluster (Zhu & Bertoft, 1996). Other investigators used other methods and suggested that clusters in various starches are build up by 4.22 (Thurn & Burchard, 1985), 22–25 (Hizukuri, 1986), 2.1–12.9 (Hanashiro et al., 2002), or 18–34 chains (Gallant et al., 1997). This suggests an obvious lack of definition for what is meant by a cluster, which necessarily leads to arbitrary definitions based on the methods of the analyses in use. When using the α-amylase of *B. amyloliquefaciens* (also named "liquefying amylase" of *Bacillus subtilis*), we defined a cluster as a dextrin that is easily released from the amylopectin by endo-attack at internal chain segments, but comparatively resistant to further hydrolysis (Bertoft, 1986).

Our studies using the *B. amyloliquefaciens* amylase have shown that clusters from waxy types of cereals (typically low in amylose content) of A-crystalline starches (maize, barley, and rice) are of several sizes from DP 34 ~ 200 (Bertoft, 1989a; Bertoft & Åvall, 1992; Bertoft, Zhu, Andtfolk, & Jungner, 1999), and are larger than those found in normal potato starch of the B-type (Zhu & Bertoft, 1996). A similar difference was found when mutants of maize possessing A- and B-crystallinity (*wxdu* and *aewx*, respectively) were compared (Gérard, Planchot, Colonna, & Bertoft, 2000). In all cases most of the long B-chains were absent in the isolated clusters, which is in accordance with the present model for the interconnection of the clusters (Hizukuri, 1986). Clusters from waxy rice were found to be interconnected to form domains of different fine structures, either within or between the amylopectin macromolecules (Bertoft et al., 1999), and some degree of domain structure was also suggested for amylopectin from normal potato starch (Zhu & Bertoft, 1996).

The aim of the present investigation was to achieve a thorough analysis of the composition of clusters and their unit chain composition in amylose-free potato starch. The mode of the interconnection of the clusters was also of interest, especially in the light of our recently proposed two-directional backbone model, in which this aspect is fundamentally different from the classical model (Bertoft, 2004). The strategy was to isolate domain structures (groups of clusters) from the amylopectin macromolecule by an initial, short α-amylolysis, and from these samples finally isolate the clusters by a continued enzymatic hydrolysis.

2. Materials and methods

2.1. Starch sample and enzymes

Potato amylopectin starch (PAPS) was a kind gift from Lyckeby Stärkelsen, Sweden, and contained only amylopectin. α-Amylase of *B. amyloliquefaciens* [(1 → 4)-α-D-glucan glucanohydrolase; EC 3.2.1.1] was purchased from Boehringer–Mannheim (now Roche), Germany, and had an activity (Bertoft, Manelius, & Qin, 1993) of 600 U/mg. Rabbit muscle phosphorylase *a* [(1 → 4)-α-D-glucan:orthophosphate α-D-glucosyltransferase; EC 2.4.1.1], specific activity 24 U/mg, was from Sigma, Germany, and barley β-amylase [(1 → 4)-α-D-glucan maltohydrolase; EC 3.2.1.2] with a specific activity of ca 1400 U/mg was from Megazyme, Ireland. Isoamylase from *Pseudomonas amyloderamosa* (glycogen 6-glucanohydrolase; EC 3.2.1.68), activity 71,000 U/ml, and pullulanase from *Klebsiella pneumoniae* (amylopectin 6-glucanohydrolase; EC 3.2.1.41), activity 404 U/ml, were purchased from Hayashibara Shoji Inc., Japan. All enzyme activities (except for α-amylase) were given by the suppliers.

2.2. Production of fractions of α-dextrins

PAPS (10 g) was dissolved in 90% dimethylsulphoxide (DMSO, 200 mL) on a boiling water bath for 15 min and then stirred at room temperature for 4 days. The solution was then diluted with water (700 mL) and the temperature adjusted to 25 °C before a solution (100 mL) of α-amylase (0.3 U/mL) in 0.01 M sodium acetate buffer, pH 6.5, was added. The reaction was stopped after exactly 1 h by the addition of 5 M KOH (25 mL). A small aliquot (0.2 mL) was taken for analysis by gel-permeation chromatography (GPC) as described below. The pH was adjusted to 11 with 1.5 M HCl and 5 volumes of methanol was added. A precipitate was allowed to settle overnight and was recovered by centrifugation (30 min at 1800*g*) to give sample I, which represented intermediate α-dextrins obtained by a first, initial stage of α-amylolysis (Fig. 1). The methanol in a part (70 mL) of the supernatant fraction (S-I) was evaporated (Büchi Rotavapor R-3000). Because the sample contained DMSO, it could not be lyophilised and was therefore stored in a refrigerator until further analyses.

The precipitate (sample I) was dissolved in hot water. A small amount of unsolubilised material was removed by centrifugation before the carbohydrate concentration (measured by phenol–sulphuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956)) was adjusted to 10 mg/mL. Sample I was then subjected to fractional precipitation in methanol (Bertoft & Spoof, 1989) as described in the scheme in Fig. 1. An initial addition of 0.5 volumes of methanol did not give any precipitate, but successive

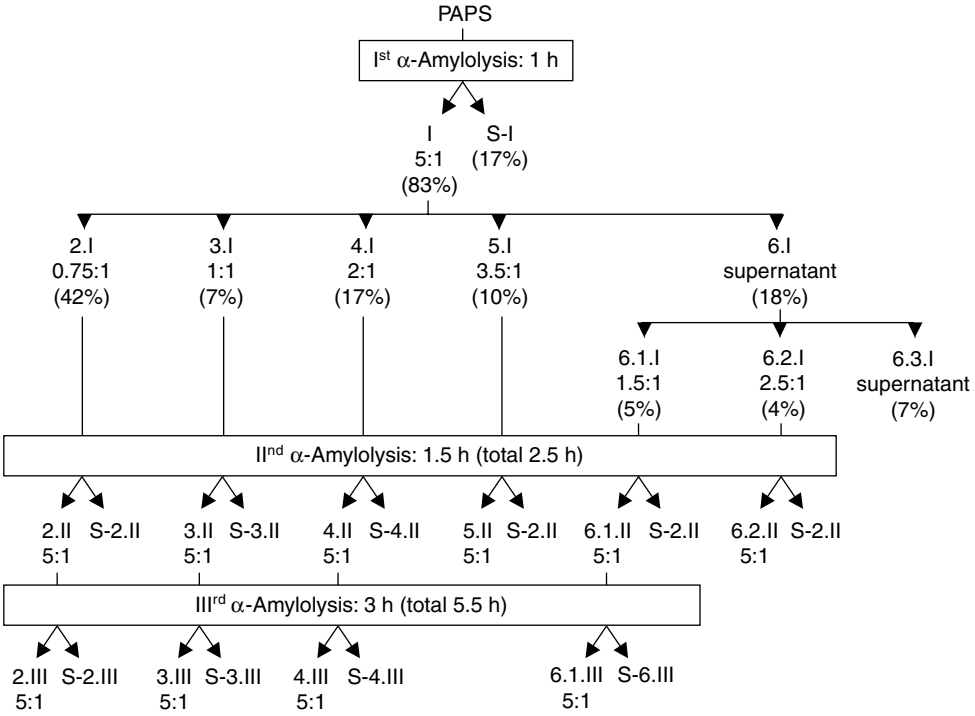

Fig. 1. Scheme showing the isolation of intermediate α-dextrins from potato amylopectin starch after enzymic treatment for 1 h. Sample I was size-fractionated by precipitation in methanol with the methanol-water ratios shown. Yields are shown in parenthesis. The samples were further subjected to a second (II) and third (III) stage of hydrolysis and collected as a precipitate and supernatant (designated S) fraction, respectively.

additions gave samples 2.I–5.I. The residual supernatant fraction (sample 6.I) was concentrated to 10 mg/mL and sub-fractionated into samples 6.1.I–6.3.I. The precipitates (samples 6.1.I and 6.2.I) were washed in methanol (5 volumes) and dissolved in water, whereas the methanol of the final supernatant fraction (6.3.I) was evaporated, and the samples were finally lyophilised.

Based on small-scale α-amylolysis experiments, samples 2.I–6.2.I were dissolved in water (10 mg/mL) and subjected to a second α-amylolysis (under identical conditions as described above) for 1.5 h. The dextrins were precipitated in 5 volumes of methanol (Fig. 1) to give samples 2.II–6.2.II and the supernatants were collected to give samples S-2.II–S-6.2.II. A third stage of α-amylolysis was conducted for a further 3 h to give samples 2.III, 3.III, 4.III, and 6.1.III and their supernatant fractions, respectively.

2.3. Production of ϕ,β-limit dextrins

Samples of α-dextrins (2 mg/mL) were dissolved in hot water. To 1 volume of the solution was added 0.1 volume of 1.1 M sodium phosphate buffer (pH 6.8), 0.05 volume of 2.8 mM EDTA (disodium salt), and 0.25 volumes of a freshly prepared solution of phosphorylase *a* in water (1 U/mL). The mixture was stirred overnight at room temperature, then boiled and the volume reduced to 20% by rotary evaporation before the protein (enzyme) was removed by centrifugation (15 min at 1800*g*). The phosphorolysis dextrins were separated from glucose-1-phosphate and the buffer salt on two PD-10 columns (Sephadex G-25, Pharmacia) coupled in series. The carbohydrate concentration was adjusted to 2 mg/mL and the phosphorolysis was repeated to give limit dextrins (ϕ-LD). To the purified solutions of ϕ-LD (containing typically 3–5 mg/mL) was added 0.33 volumes of 0.01 M sodium acetate buffer (pH 6.0) and β-amylase to a concentration of 4 U/mg dextrins. After incubation overnight at room temperature, the enzyme was destroyed by boiling, the volume reduced to approximately 30%, and the maltose removed by filtration through the PD-10 columns two times. The ϕ,β-LD was then lyophilised. Preparative production of ϕ,β-LD from the original amylopectin sample (PAPS) was performed similarly, but the products were purified by dialysis instead of column chromatography.

2.4. α-Amylolysis on analytical scale

PAPS or ϕ,β-LD of PAPS (2 mg) was dissolved in hot 90% DMSO (50 mg/mL) and stirred 2 days before diluted in hot water to 11.1 mg/mL. α-Dextrins and their ϕ,β-LDs were dissolved directly in hot water to the same concentration. Nine volumes of the sample were mixed with 1 volume of α-amylase (0.3 U/mL) diluted in 0.01 M NaOAc buffer, pH 6.5, to give similar final conditions as in the preparative experiments described above. Incubation took place at 25 °C. At intervals, aliquots (0.1 mL) were taken and the reaction stopped by addition of 5 M KOH (2.5 μL).

2.5. Molecular weight distribution

Samples of dextrins or α-amylolysis mixtures (from either preparative or analytical scale experiments) were diluted to ~3 mg/mL. An aliquot (0.2 mL) was treated with 5 M KOH (0.02 mL), applied on a column (1 × 90 cm) of Sepharose CL 6B (Pharmacia, Sweden), and eluted with 0.5 M KOH at 0.5 mL/min. Fractions (0.5 mL) were analysed for carbohydrates with the phenol–sulphuric acid reagent (Dubois et al., 1956). The column was calibrated with dextrins of known DP (Bertoft & Spoof, 1989).

2.6. Analysis of chain length

PAPS or ϕ,β-LD of PAPS (2 mg) was dissolved in hot 90% DMSO (50 μL) and stirred 2 days before diluted in hot water (400 μL). α-Dextrins and their ϕ,β-LD were dissolved directly in water (450 μL) on a boiling water bath. PAPS and α-dextrins were then treated with a solution of 0.01 M NaOAc, pH 3.5 (50 μL), and isoamylase (1 μL), whereas the samples of ϕ,β-LD were treated with 0.01 M NaOAc buffer, pH 5.5 (50 μL), and pullulanase (1 μL). All incubations took place at room temperature overnight and were interrupted by heating. The debranched samples were analysed by high-performance anion-exchange chromatography (HPAEC).

2.7. Composition of supernatant fractions

Samples of the supernatants (1 mg/mL), obtained after methanol precipitation of α-dextrins, were analysed by HPAEC. Aliquots of the samples (0.45 mL) were also treated with 0.01 M NaOAc buffer, pH 6.0 (50 μL), and β-amylase (1 μL). After incubation at room temperature overnight boiling stopped the reaction.

2.8. High-performance anion-exchange chromatography (HPAEC)

All samples were diluted in water to suitable concentration before filtration (pore size 0.45 μm) and injection (25 μL) to the HPLC system (series 4500i, Dionex, USA) equipped with a BioLC gradient pump and pulsed amperometric detection (PAD). The column (Carbo-Pac PA-100, 250 × 4 mm, combined with a guard column) was eluted at 1 mL/min with a mixture of eluent A (150 mM NaOH, 85%) and eluent B (150 mM NaOH containing 500 mM NaOAc, 15%) from 0 to 1 min, whereafter the sample was injected. Debranched samples were eluted with a gradient made by increasing the proportion of eluent B as follows: from 1–10 min from 15% to 36%; 10–19 min from 36% to 45%; 19–111 min from 45% to 100%; and 111–113 min from 100% to 15% (return to the start mixture). Supernatant samples were eluted with the following gradient: from 1 to 16 min eluent B from 15% to 34%; 16–27 min from 34% to 40%; 27–53 min from 40% to 49%; 53–55 min from 49% to 100%; and 59–61 min from 100% to 15% (return to the start mixture). For samples containing a lot of maltose (β-amylase treated), the PAD signal was recorded at a 10× less sensitive output range (3000 nA instead of 300 nA) for an initial period.

3. Results and discussion

3.1. Production and fractionation of intermediate α-dextrins

The molecular weight distribution of α-dextrins obtained after 1 h of α-amylase treatment of potato amylopectin starch (PAPS) is shown in Fig. 2a. Two major fractions had been formed and were isolated as sample I (precipitate in methanol) and sample S-I (supernatant) in yields of 83 and 17 wt%, respectively. By earlier investigations on

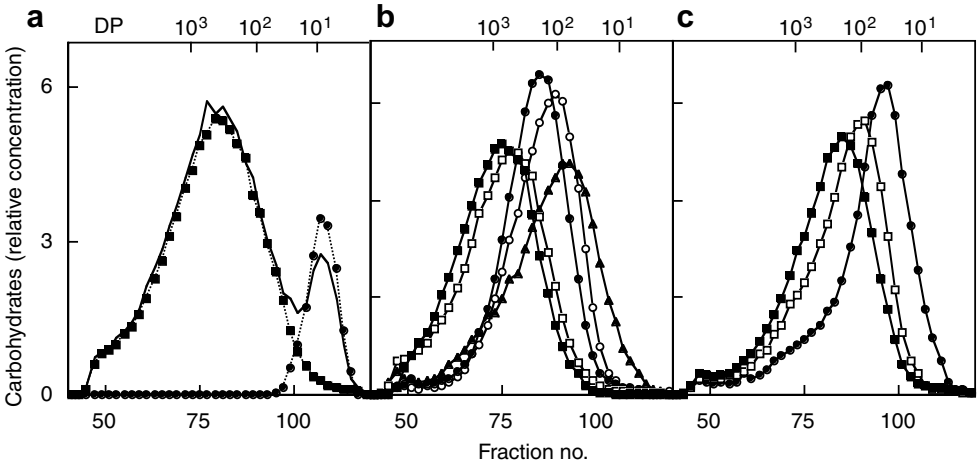

Fig. 2. Fractionation of intermediate α-dextrins on Sepharose CL 6B. (a) The hydrolysate after 1 h of α-amylolysis before (whole line) and after fractionation into sample I (■) and the supernatant S-I (●); (b) fractional precipitation of sample I into 2.I (■), 3.I (□), 4.I (●), 5.I (○), and 6.I (▲); (c) fractional precipitation of 6.I into 6.1.I (■), 6.2.I (□), and 6.3.I (●).

amylopectin from different starches (Bertoft, 1991; Bertoft et al., 1999; Zhu & Bertoft, 1996), it was known that the dextrins contained in sample I were branched, and represented clusters or groups of clusters from PAPS. Sample I was fractionated by methanol precipitation (Bertoft & Spoof, 1989) into a series of size-categories labelled 2.I–6.I (Fig. 2b) in a total yield of 94%, of which 2.I represented a major part (42%, Fig. 1). The samples possessed rather broad molecular weight distributions and the separation at the high molecular weight side was poor, especially when comparing fraction 6.I with the others. Fraction 6.I was therefore further sub-fractionated into 6.1.I, 6.2.I, and 6.3.I and, as shown in Fig. 2c, a separation was achieved at the low molecular weight side but not at the larger side (approx. DP > 300). It is interesting to notice that the similar phenomenon was found for amylopectin from normal potato starch (Zhu & Bertoft, 1996), whereas no such problems arose with waxy maize (Bertoft & Spoof, 1989) and waxy rice samples (Bertoft et al., 1999). Possibly, some special structural feature of a fraction of the amylopectin macromolecules from potatoes gives rise to this altered behaviour in methanol. The average DP of the fractions, as estimated from the gel-permeation chromatograms, ranged from 37 to 480 (Table 1). The peak positions were higher, however, ranging from DP 43–830.

The supernatant fraction S-I eluted from the column of Sepharose CL 6B mostly at DP < 20 (Fig. 2a). S-I was also analysed by HPAEC-PAD (Fig. 3a). Linear dextrins, which are known to be produced mainly by an attack by the α-amylase at the external chains of amylopectin (Bertoft, 1989a; Robyt & French, 1963), were represented by major peaks at DP 5–12, Minor peaks that eluted between the linear dextrins, and are known to represent branched dextrins (Jodelet, Rigby, & Colquhoun, 1998), were also present at DP 8–18. The branched nature of several of the dextrins was confirmed qualitatively in the form of β-limit dextrins (β-LD) at DP 4–6 (Fig. 3b) (Linear dextrins are reduced into maltose and maltotriose by β-amylase.). Because these very small, branched fragments were formed already at the initial stages of α-amylolysis, it is reasonable to believe that they originated from long chain segments between (or otherwise outside) the clusters of branches.

Each fraction (except 6.3.I, which was comparatively resistant) was further hydrolysed by the α-amylase in one or two steps (representing a total of 2.5 and 5.5 h of hydrolysis, Fig. 1), until the hydrolysis rate was very low. At this stage the samples should contain single clusters (Bertoft, 1989a). Some examples of the obtained mixtures are shown in Fig. 4. From each sample a precipitate formed in methanol was collected. The supernatants were also collected and analysed by HPAEC. Those obtained from the fractions containing larger dextrins (2.I–5.I, Fig. 3c and d) possessed linear dextrins mostly at DP 5–12, suggesting a continuous attack at external chains. However, linear dextrins at DP 2–4 were also formed, especially at stage III. Such small fragments were probably produced as a result of the comparatively long incubation times, when the

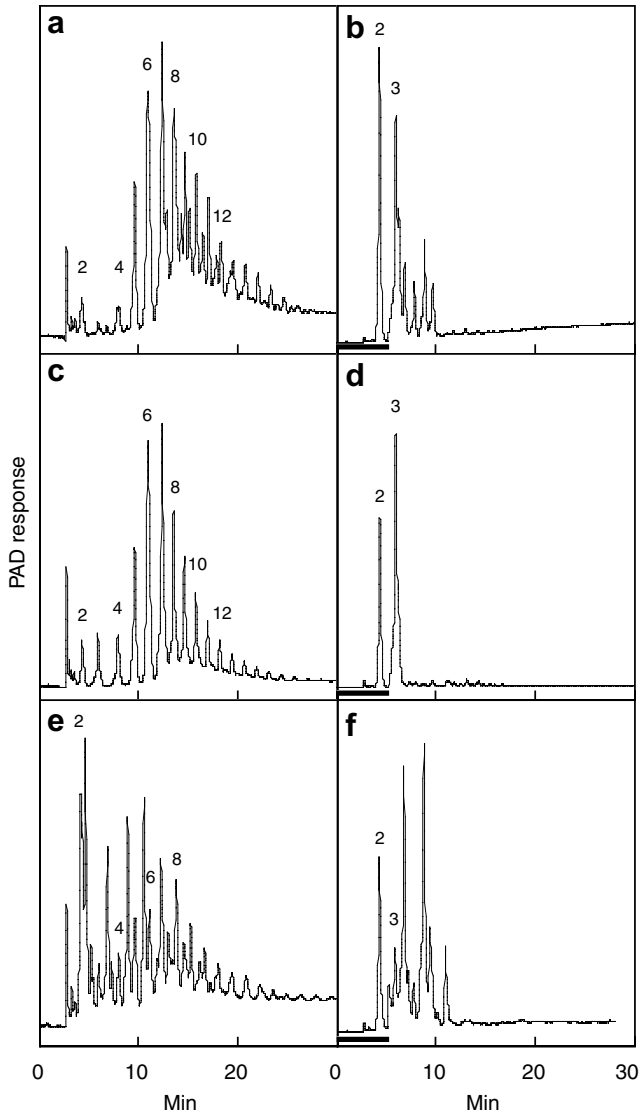

Fig. 3. Analyses of supernatant fractions with HPAEC-PAD of (a) S-I, (b) S-I after β-amylolysis, (c) S-3.II, (d) S-3.II after β-amylolysis, (e) S-6.2.II, and (f) S-6.2.II after β-amylolysis. Numbers show DP of linear dextrins. Thick bar in (b), (d), and (f) shows the time interval at which the PAD response was recorded at 10× lower sensitivity.

enzyme slowly attacks and shorten the already released dextrins at DP 5–7 (Robyt & French, 1963). The amounts of branched dextrins were, however, very small compared to those obtained after the initial attack (sample S-I, Fig. 3a and b), suggesting that easily released branches (outside clusters) were absent.

Supernatants obtained from dextrins of low DP (S-6.1.II, S-6.1.III, and S-6.2.II) possessed, however, a lot of branched dextrins (Fig. 3e). Surprisingly, the dextrins were apparently completely resistant to debranching (not shown), but susceptible to β-amylase attack, which resulted in large amounts of β-limit dextrins (Fig. 3f). The rate of the α-amylolysis of these samples (especially 6.1.II and 6.2.I) was slow and the major shift in molecular weight distribution occurred at high DP where the material was degraded (Fig. 4b). This resulted in a more homogenous

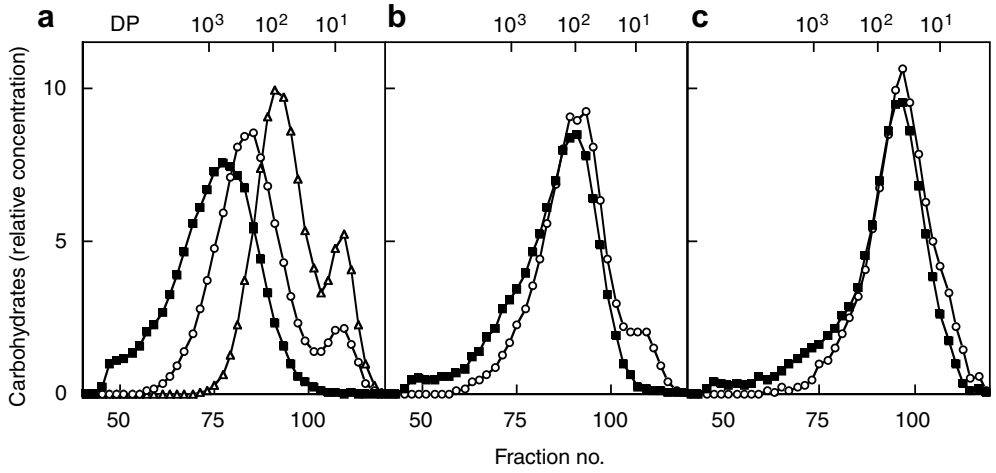

Fig. 4. Gel-permeation chromatography on Sepharose CL 6B of hydrolysates obtained at the second (1.5 h) and third stage (3 h) of α-amylolysis. (a) Sample 3.I before treatment (■) and at the second stage (○) and sample 3.II after treatment to the third stage (△); (b) sample 6.2.I before treatment (■) and at the second stage (○); (c) sample 6.3.I before (■) and after α-amylolysis for 2.5 h (○).

distribution curve, whereas the peak-DP position remained practically constant. The same was true for sample 6.3.I, which only was treated with the α-amylase on an analytical scale a second time (Fig. 4c). It is thus possible that the high-DP material gave rise to the complex mixture of branched dextrins in the supernatants, suggesting a special type of structure that supports the unusual behaviour in methanol discussed above. It should be noted that this material only represented a minor fraction (roughly 2 wt%) of the whole amylopectin. Nevertheless, it might be of importance for the understanding of the structure of potato amylopectin. It is therefore interesting to notice that a small fraction (2–3 wt%) of intact PAPS, but not waxy maize starch, was resistant to debranching by pullulanase (Bertoft, 2004), which also suggested the presence of a minor, different structure. As already mentioned, the peak-DP position in the low molecular weight samples was almost constant during the hydrolysis, suggesting the presence of single clusters. It is therefore also possible that some of the branched dextrins obtained in the supernatants arose from clusters that began to slowly decompose.

3.2. Characterisation of ϕ,β-LD by α-amylolysis

Each of the fractions obtained as precipitate in methanol after α-amylolysis was treated successively with phosphorylase and β-amylase to remove the external chain stubs that remained after the α-amylase attack. This made the structure of the branched dextrins more uniform. The molecular weight distributions of the altogether 17 samples are shown in Fig. 5. The shape of the distribution profiles was similar to that of the respective α-dextrin, suggesting an even attack on the whole material by the exo-acting enzymes. The average DP-values were reduced and the peak-DP values ranged from 25 in sample 6.3.I to 380 in 2.I (Table 1).

The fractions, as well as the ϕ,β-LD of the original amylopectin, were all further treated with α-amylase to follow the hydrolysis rate and thereby obtaining the size of the clusters of each sample (Zhu & Bertoft, 1996). As a summary of the results (not shown) it was concluded that samples 2.III, 3.III, and 4.III obtained from the third stage of α-amylolysis, and samples 5.II, 6.1.II, and 6.2.II from the second stage, contained free clusters. In addition a major part of sample 6.2.I contained clusters because the peak-DP position was insensitive to the α-amylolysis. The peak-DP reflected the size of the major part of the clusters in each fraction (Table 1) and from the yields of each fraction (Fig. 1) it can be estimated that approximately 50% of the clusters in PAPS were of DP ~70 and 40% of DP 50–55 (in the form of limit dextrins). Finally, a group of clusters with a small size of only DP 25 was found in sample 6.3.I. However, this was a minor type that only represented <10% of all the clusters in PAPS. Thus, the amylopectin possessed comparatively small clusters with a rather uniform size-distribution in accordance with that found for amylopectin from normal potato and from an *aewx* maize starch, both of the B-crystalline type, in contrast to samples of A-crystalline types that earlier were investigated (Bertoft, 1991; Bertoft et al., 1999; Bertoft & Åvall, 1992; Gérard et al., 2000; Zhu & Bertoft, 1996). The uniform size-distribution of the clusters, which suggests a regular structure of the crystalline lamellae inside starch granules, provides structural support to the calorimetric investigation by Fredriksson, Silverio, Andersson, Eliasson, and Åman (1998), who concluded that the crystalline regions in potato starch granules are more homogenous than in many other starches. It might also partly explain the strong radial orientation, and thus organisation, of the polymer chains found in potato starch granules (Buléon et al., 1997).

A detail of interest was that the clusters of DP 70 were mostly represented in sample 2.I, i.e., in domains originally isolated as the high molecular weight fraction, whereas

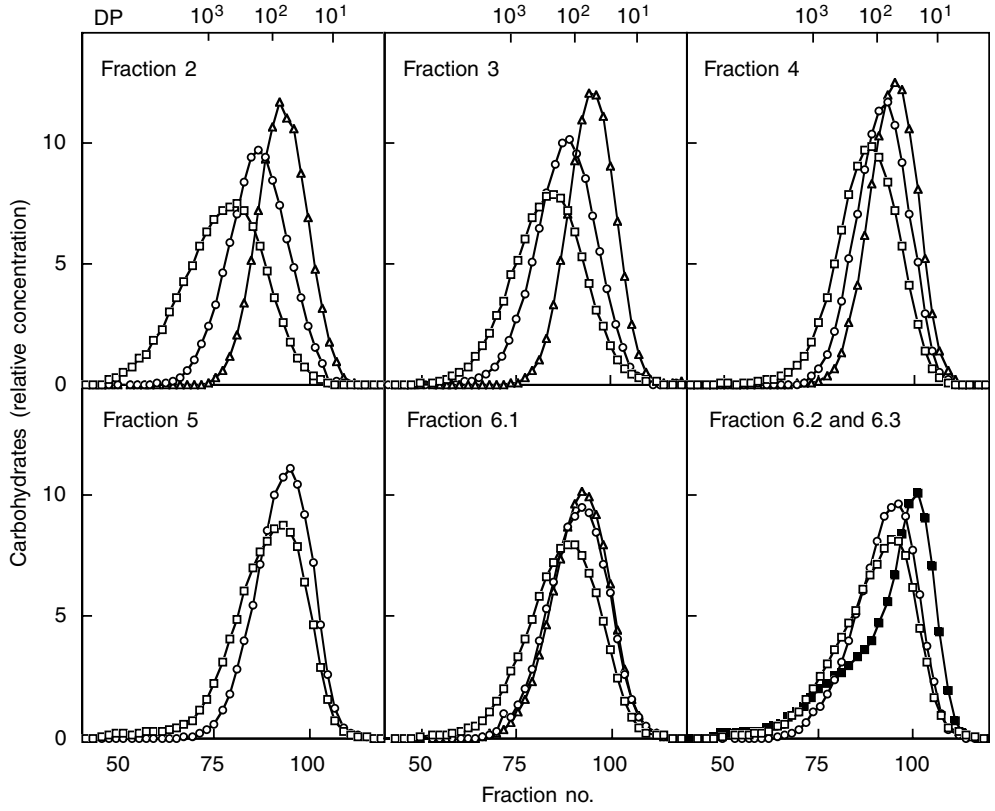

Fig. 5. Molecular weight distribution on Sepharose CL 6B of ϕ,β-limit dextrins of the α-dextrin fractions obtained at stage I (□), stage II (○) and stage III (△) of α-amylolysis. Sample 6.3 (■) was only represented by stage I.

Table 1
Degree of polymerisation of fractions isolated after α-amylolysis and their ϕ,β-LDs

Sample[a]	α-Dextrin			ϕ,β-Limit dextrin	
	Time (h)[b]	DP[c]	Peak-DP[c]	DP[c]	Peak-DP[c]
2.I	1.0	480	830	246	380
2.II	2.5	126	297	99	170
2.III (c)	5.5	68	100	51	70
3.I	1.0	279	600	118	217
3.II	2.5	111	214	84	130
3.III (c)	5.5	60	93	40	50
4.I	1.0	138	210	87	135
4.II	2.5	71	125	59	72
4.III (c)	5.5	54	93	42	54
5.I	1.0	104	125	64	72
5.II (c)	2.5	65	93	46	54
6.1.I	1.0	124	214	78	115
6.1.II (c)	2.5	88	125	55	75
6.1.III	5.5	67	110	53	70
6.2.I (c)	1.0	88	100	54	55
6.2.II	2.5	58	80	48	55
6.3.I (c)	1.0	37	43	31	25

[a] (c) indicates fractions containing clusters based on the rate of α-amylolysis.
[b] The total time of successive hydrolysis steps by α-amylase.
[c] Values from GPC on Sepharose CL 6B.

domains of smaller sizes (samples 3.I–5.I) contained the smaller clusters of DP 50–55. This indicated that within the amylopectin macromolecule, or alternatively within or between granules, certain parts are enriched in larger clusters, whereas others mostly contain smaller clusters. However, it was noticed that both types of cluster sizes, and in addition the smallest clusters of DP 25, were found in the sub-fractions of sample 6.I. This particular fraction (supernatant after fractional precipitation) contained the possible minor component with altered behaviour in methanol already discussed. Whether this also suggested a certain domain structure including the latter material and clusters of several sizes has, unfortunately, to await future investigations.

3.3. Distribution and categories of chains

A comparison of the unit chain distribution of PAPS with those of the α-dextrins obtained at different times of hydrolysis (examples are given in Fig. 6a and b) showed that, as expected (Bertoft, 1991; Bertoft & Koch, 2000), the long chains of the amylopectin were preferentially attacked by α-amylase. Chains of intermediate sizes between the long and short chains were formed at DP 26–40. The enzymatic attack resulted also in the formation of short chains at DP 3–10. A peak at DP 6 and a minimum at DP 8 were characteristic of all dextrins and stages of hydrolysis. Though this coincided with the typical profile of the "fingerprint" A-chains of PAPS (Bertoft, 2004), there was probably no structural connection between the

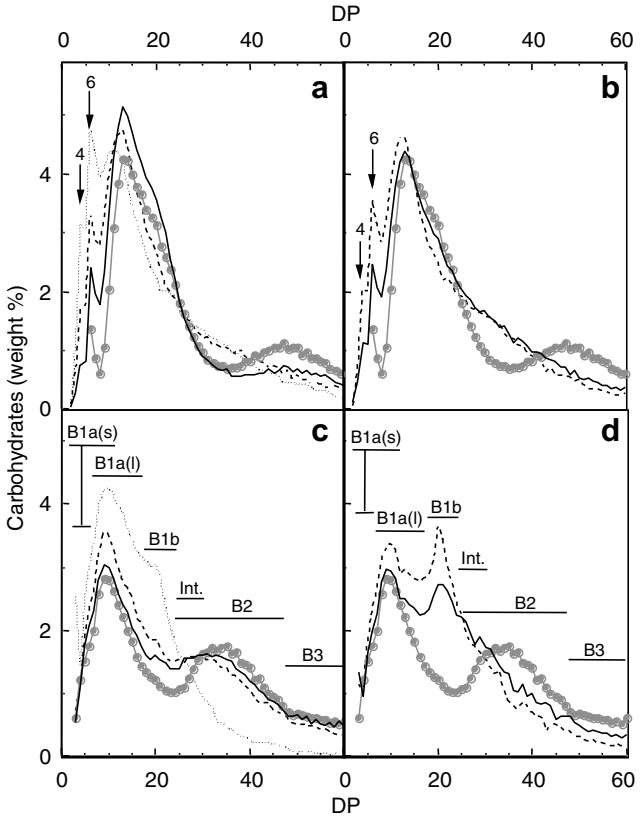

Fig. 6. Unit chain distribution obtained by HPAEC-PAD of (a) sample 2.I (———), 2.II (- - - -) and 2.III (· · ·) and (b) sample 5.I (———) and 5.II (- - - -). The positions for DP 4 and 6 are shown and the original chain distribution of PAPS (grey circles) is included for comparison. Only chains with DP ⩽ 60 are shown. In (c) and (d) the B-chain distribution of the ϕ, β-limit dextrins of samples 2 and 5, respectively, are shown and different B-chain categories are indicated. Chains of intermediate lengths (Int.) between B1b and B2 were formed by the α-amylase.

original chains at DP 6–8 (formed by starch synthesising enzymes) and the ones formed by the α-amylase attack. In addition, the α-amylase produced a characteristic profile of chains at DP 3–5, with a peak at DP 4 that tended to be more pronounced at later stages. Only trace amounts of DP 2 were detected. These typical patterns showed certain preferential modes of attack by the enzyme at the external chain segments. The shortest chains produced were probably mostly shorted A-chains, whereas chains at DP 5–8 could be mixtures of A- and B-chains.

The unit chain distribution profiles are difficult to interpret because of the simultaneous action of the α-amylase at the external chains and at the internal chains of the amylopectin. It is, however, possible to follow the later action from the unit chain composition of the ϕ,β-LDs (Fig. 6c and d), in which the external chains have been reduced so that all A-chains remain as maltosyl chain stubs and the external part of the B-chains is only a single residue (Bertoft, 1989b). Thus, the shortest possible B-chain, in which the internal chain length (ICL) is one residue (Umeki & Yamamoto, 1975), has a length (CL) of three residues in the ϕ,β-LD of amylopectin. With the LDs produced from the intermediate α-dextrins the situation became, however, more complicated because of the α-amylase attack at the external chains, which to some extent already produced very short external stubs (Fig. 6a and b). As illustrated in Fig. 7, an external chain length (ECL) of two residues makes the A- and B-chain resistant to the exo-acting enzymes. In this case the A-chain is represented by maltose after debranching, as expected for a ϕ, β-LD, but the B-chain remains as CL 4, instead of the expected CL 3 when ICL is only 1. As mentioned above, so short external chains were hardly found in the samples. When ECL is 3, both chains remain resistant to phosphorylase and only the B-chain can be attacked by β-amylase, with the result that both chains give rise to CL 3. Finally, if ECL is 4 both types of chains will give a correct product, even though the A-chain is not attacked by phosphorylase. Because the α-dextrins possessed chains with ECL 3, the chains at DP 3 obtained from the ϕ,β-LDs were mixtures of the A- and B-types. The amount of chains at DP 3 in the α-dextrins, which on a molar basis was quite high at stage II and III, was therefore added to the maltose amount obtained from the debranched ϕ,β-LDs and subtracted from the profile of the B-chains in Fig. 6c and d.

The B-chains of the ϕ,β-LDs were divided into categories on the basis of previous discussions (Bertoft, 2004; Bertoft & Koch, 2000). It was obvious that the endo-attack by α-amylase occurred at the long B2- and B3-chains and resulted mainly in the formation of chains corresponding to the lengths of B1b-chains, which is a sub-group of the short B1-chains at CL 18–24 (Fig. 6c and d). This type of chains was also produced by the α-amylase during degradation of waxy rice (Bertoft & Koch, 2000) and waxy maize amylopectin (Bertoft, 1991). Though B1b-chains were a major product, some chains corresponding to the shorter representatives of long B2-chains, and marked "intermediate" in Fig. 6, were also produced. A similar intermediate fraction was detected from waxy rice (Bertoft & Koch, 2000). Especially at later stages, chains corresponding to the clustered B1a were also formed. It should be observed that the chains discussed were formed by the α-amylase as a result of cleavage of longer chains, and it is therefore uncertain whether they should be considered as the same types of chains already found in the amylopectin, or as

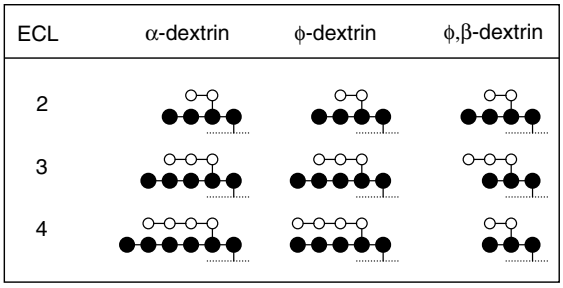

Fig. 7. Expected structure of ϕ- and ϕ,β-limit dextrins obtained from α-dextrins with very short external chains (ECL 2–4) in the form of A- (○) and the shortest possible B-chain (●).

new types with only the CL in common with the respective categories. The nomenclature and division into the different categories is therefore somewhat hazardous. Nevertheless it is useful for the discussion and, overall, it was interesting to notice that the pattern was quite similar to the amylopectins from A-crystalline samples (waxy maize and waxy rice) that were analysed in detail earlier.

The average chain length (CL) of the ϕ,β-LDs after the initial 1 h of α-amylase attack was similar to that of the ϕ,β-LD of the original PAPS sample (∼9) and was only little reduced after the successive treatments up to a total of 5.5 h (Table 2). The average internal chain length (ICL) was in all cases, except in fraction 2.III, somewhat longer (4.6–6.8) than reported for fractions from normal potato amylopectin (4.2–4.5) (Zhu & Bertoft, 1996). The ICL-values were, however, in the order of those found by molecular modelling of the segments preferentially required to interconnect and aline two double helices in the starch granules (O'Sullivan & Pérez, 1999). Though the changes in the CL and ICL-values were moderate, the decrease in the total internal chain length (TICL) of the B-chains was clear (from 16.7 to 10.4–13.3 in fractions containing clusters) and reflected the endo-attack at the internal chains.

The average number of chains (NC) decreased in all fractions as a result of the fragmentation of larger dextrins into smaller by the α-amylolysis (Table 2). There was a specific linear relation between the DP and NC at each step (I, II, and III) of α-amylolysis (Fig. 8), so that DP at a certain NC-value decreased with hydrolysis time (and the density of branching tended to increase). This should, at least partly, reflect the removal of branches outside true clusters and found as the small, branched dextrins in the supernatant fractions (Fig. 3). The regression lines in Fig. 8 crosses each other at NC ∼3.7, which suggests a theoretical minimum NC-value of the clusters and coincided with that found for the smallest clusters contained in sample 6.3.I. Fig. 8 was used to estimate the number of chains of the majority of the clusters obtained at the peak-DP values (from Table 1) of the fractions. This suggested that clusters of DP ∼70 were composed of ∼11 chains and those at DP 50–55 contained 6–7 chains (Table 2). The smallest dextrins in fraction 6.3.I possessed a peak-NC of 2.9 (Indeed, to be classified as cluster one expects NC to be ⩾3.). It is of interest to notice that at average the NC (6–11) of the isolated clusters tended to be larger than that suggested by the ratio of short to long chains (6.3) of the potato amylopectin (Bertoft, 2004). Thus it seems that the macromolecule possessed a surplus of long chains.

The degree of branching (DB) of the clusters ranged from 8.6 to 12.8. This was comparable to that of clusters of small sizes in a B-crystalline *aewx*-maize starch (Gérard et al., 2000), but somewhat lower than found in fractions from waxy rice (Bertoft & Koch, 2000), and supported the conclusion that clusters in B-crystalline samples are less densely branched than those in A-crystalline types (Gérard et al., 2000).

The distribution and the categories of B-chains found in the fractions that represented isolated clusters are shown in Fig. 9. A general trend was that clusters isolated from fractions that in stage I of α-amylolysis represented dextrins of larger size (fraction 2.I and 3.I) possessed somewhat less long B-chains, but also a less pronounced peak of B1b-chains. In the series of sub-fractions obtained from fraction

Table 2
Characterisation of ϕ,β-limit dextrins

Sample[a]	CL[b]	ICL[c]	TICL[d]	NC[e]	Peak-NC[f]	DB[g]
PAPS	9.0	6.5	16.7	–	–	11.1
2.I	9.0	6.5	15.4	27.5	42.7	10.8
2.II	8.1	5.6	13.6	12.1	21.1	11.3
2.III (c)	6.8	4.3	10.4	7.5	10.9	12.8
3.I	8.4	5.9	14.1	14.1	24.4	11.1
3.II	8.1	5.6	13.9	10.3	16.1	11.2
3.III (c)	7.8	5.3	12.1	5.1	7.1	10.4
4.I	9.3	6.8	15.1	9.3	15.3	9.6
4.II	8.1	5.6	13.5	7.3	8.8	10.7
4.III (c)	7.4	4.9	11.8	5.7	7.9	11.1
5.I	9.3	6.8	14.4	6.9	8.2	9.2
5.II (c)	8.9	6.4	13.3	5.2	6.5	9.1
6.1.I	8.5	6.0	14.2	9.2	13.0	10.5
6.1.II (c)	8.4	5.9	13.9	6.6	9.2	10.2
6.1.III (c)	7.1	4.6	11.5	7.4	10.9	12.2
6.2.I (c)	8.7	6.2	13.6	6.2	6.3	9.7
6.2.II (c)	7.9	5.4	12.9	6.0	6.7	10.5
6.3.I (c)	8.5	6.0	12.6	3.7	2.9	8.6

[a] (c) indicates fraction containing clusters based on the rate of α-amylolysis.
[b] Average chain length obtained by HPAEC-PAD of debranched samples.
[c] Average internal chain length = CL − ECL − 1, in which ECL is 1.5 in ϕ,β-LD.
[d] Total internal chain length of the B-chains obtained by HPAEC-PAD.
[e] Number of chains = DP/CL (in which DP is from Table 1).
[f] Number of chains at peak-DP (Table 1) calculated with the regression lines in Fig. 8.
[g] Density of branches (as percent) = (NC − 1)/DP × 100 [in which (NC − 1) equals the number of branches].

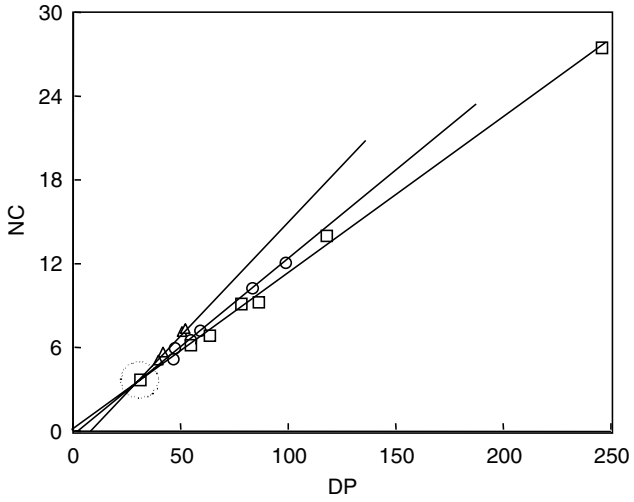

Fig. 8. The average number of chains versus the degree of polymerisation of ϕ,β-limit dextrins at stage I (□), II (○), and III (△) of α-amylolysis. Lines cross at NC 3.7 (encircled).

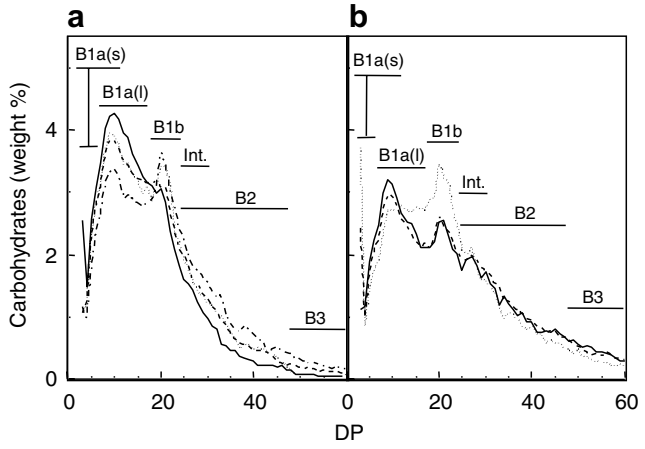

Fig. 9. B-chain distribution obtained by HPAEC-PAD of the ϕ,β-limit dextrins of clusters from PAPS. (a) Samples 2.III (———), 3.III (- - - - - -), 4.III (· · ·), and 5.II (–·–); (b) samples 6.1.II (———), 6.2.I (- - - - -), and 6.3.I (· · ·). Only chains with DP ⩽60 are shown. Different B-chain categories are indicated, of which "Int." are chains intermediate between B1b and B2. A-chains in the form of DP 3 were subtracted from the peak at DP 3 (see Section 3 and Fig. 8).

6.I the proportion of long B-chains was highest. The result was unexpected because these samples contained clusters obtained from a fraction representing mostly smaller dextrins. It is possible that these long chains were derived from the minor material of larger DP and different structure that the fractions possessed, and discussed above, rather than being integrated chains of the clusters. The amount of the shortest B-chains with DP 3 was very high in fraction 6.3.I that contained the smallest clusters and was obtained already at the initial stage of hydrolysis. Elevated amounts of this DP were also detected in clusters from fraction 2.III and 6.2.I. This could suggest a certain mode of interconnection of these clusters to the rest of the macromolecule as discussed earlier in the paper concerning waxy rice amylopectin (Bertoft & Koch, 2000).

From the molar distribution of chain categories in the limit dextrins, it was possible to estimate the molar ratio of A:B chains. In the original macromolecule the ratio was 1.3, but in all fractions obtained after α-amylase attack the ratio was lower (Table 3), which could be explained as an increase of the proportion of short B-chains as a result of the cleavage of longer B-chains or/and as a loss of A-chains. By a theoretical reconstruction of the categories of chains, it was earlier suggested that some A-chains, namely the shortest "fingerprint" A-chains and the category of long A-chains found in PAPS, were not part of the clusters, but found elsewhere in the amorphous parts of the macromolecule (Bertoft, 2004). The theoretical ratio of the clustered A-chains to B-chains in PAPS was therefore lower (1.0) and close to the ratios found for the fractions containing clusters (0.8–1.1) in this investigation. Thus it is possible that some of the A-chains indeed were lost during the isolation of the clusters. These A-chains should be found among the branched, small dextrins remaining in the supernatants (Fig. 3). Because only the

Table 3
Some molar ratios of chains in ϕ,β-LD of PAPS and α-dextrins[a]

Sample[b]	A:B	A:BS	A:B1a(s)	B1a(s):B1a(l)+B1b
PAPS[c]	1.3	1.7	4.5	0.6
$PAPS_{clusters}$[d]	1.0	1.3	3.5	0.6
2.I	1.1	1.4	3.8	0.6
2.II	1.1	1.3	3.8	0.5
2.III (c)	1.0	1.1	2.7	0.7
3.I	1.1	1.3	3.6	0.6
3.II	1.2	1.4	3.9	0.6
3.III (c)	0.9	1.1	3.1	0.5
4.I	1.0	1.2	3.7	0.5
4.II	1.1	1.3	3.9	0.5
4.III (c)	1.0	1.1	3.3	0.5
5.I	0.9	1.1	3.0	0.5
5.II (c)	0.8	0.9	2.8	0.5
6.1.I	1.0	1.3	3.5	0.6
6.1.II (c)	1.1	1.3	3.5	0.6
6.1.III (c)	1.1	1.2	3.3	0.6
6.2.I (c)	0.9	1.1	2.6	0.7
6.2.II (c)	1.0	1.2	3.2	0.6
6.3.I (c)	0.8	0.9	2.3	0.7

[a] A-chains were estimated as the amount of maltose obtained after debranching and correction with A-chains in the form of maltotriose, BS are short chains of DP 3–24, B1a(s) are very short and correspond to "fingerprint" B-chains (B_{fp}) at DP 3–7, B1a(b) have DP 8–17, and B1b have DP 18–24.
[b] (c) indicates fractions containing single clusters.
[c] The ratios consider all types of A-chains in PAPS.
[d] The ratios consider only those A-chains that were suggested to participate in clusters by Bertoft (2004).

short chains build up the clusters, different ratios of the short chains give a characterisation of the structure of the clusters and are also given in Table 3. The ratio of all A-chains to the short B-chains (BS) in PAPS was 1.7, but if only the clustered A-chains are considered it was 1.3. The latter value was only slightly higher than found in the fractions of clusters (0.9–1.2). An explanation could be the fact that in the macromolecule some of the long chains also participated in the cluster structure (because they were interconnecting the clusters), whereas in the isolated clusters they were cleaved into BS-chains, thereby decreasing the ratio. The ratio of A-chains to the shortest sub-category of the B1-chains (B1a(s)), in PAPS characterised as "fingerprint" B-chains (Bertoft, 2004), showed similar trends as A:BS, whereas the relative proportion of the shortest B1a(s) to the rest of the BS-chains (B1a(l) and B1b) was more or less constant. Overall, the composition of chains in the fractions of isolated clusters agreed largely with that previously predicted from a reconstruction of chains in PAPS (Bertoft, 2004). The ratio of A:BS in the clusters was close to 1, which is expected when each double helix of the cluster is formed from one A- and one B-chain.

3.4. The mode of interconnection of clusters and their composition of unit chains

From the knowledge of the relative molar distribution of chains in the fractions and the number of chains of the major part of the dextrins at the peak-DP (peak-NC

in Table 2), it was possible to estimate the constitution of chains of isolated dextrins (Table 4). Thus, the large dextrins obtained after the initial stage of α-amylolysis in sample 2.I, which possessed a peak-DP at 380, were build up from ~43 chains, of which ~36 chains were very short of the types A and B1a. A majority of these chains should be involved in the crystalline lamellae in the starch granules. There was further ~2 chains of the B1b-type, 3 or 4 B2 and only 1 B3-chain. The typical cluster obtained from sample 2.I after additional hydrolysis (sample 2.III) had DP 70 and a total NC ~11. As expected, there were practically no long chains left in the isolated cluster. The degree (or density) of branches (DB) in the cluster was 14.1%, which was higher than of the original dextrins in sample 2.I (11.0%) and showed that longer internal chain segments between clusters had been removed by the α-amylase. Similar trends as for sample 2.I–III were seen for the other samples (Table 4). An interesting detail was that the number of B1b-chains in all of the isolated clusters was only 0.5–0.7, which suggested that this type of chain was found in only every second cluster at average. The clusters obtained in samples 6.1.II–6.2.II possessed somewhat elevated number of B2-chains (0.4–0.6 chains/cluster) but, as discussed above, these chains were probably not involved in the ''normal'' structure. The degree of branches of the clusters in samples 2.III, 3.III, and 4.III was higher (12.2–14.1%) than in 5.II, 6.1.III, and 6.2.III (10.2–11.3%) regardless similar DP (50–70), which suggested that the clusters of the potato amylopectin had slightly different internal structures. The latter, less dense type was precipitated by larger volumes of methanol, which indicated that precipitation in alcohol is not only dependent on the size of the dextrins, but also on their fine structure. A similar conclusion was made by Frigård, Andersson, and Åhman (2002). The very small clusters in sample 6.3.I contained in practice only short A and B1a-chains in approximately equal number. The low NC (2.5–3) and DB (7.6%) suggested that the chains connected to a central B1a-chain in the dextrin were comparatively outspread along the latter chain.

The larger dextrins obtained in stage I by 1 h α-amylolysis represented larger structural domains composed of more than one cluster. As shown in Fig. 3b, very little branches were lost from the fractions containing two or more clusters when they were attacked by the α-amylase. The number of cluster units contained in the larger dextrins was therefore estimated by comparison of their NC-values and the NC-value of the single cluster from each series of fractions (2–6.2, Table 4). Samples 2.II, 3.II, and 4.I were estimated to represent dextrins with approximately two clusters. In these particular samples the number of B3-chains was practically zero, whereas the number of B2-chains was 1.0–1.5. According to the existing theory, B2-chains are involved in the interconnection of two clusters (Fig. 10) (Hizukuri, 1986), for which the result thus provided experimental support. In all three samples there appeared to be only one B1b-chain, suggesting that it was found in only one of the two clusters.

Table 4
Composition of chains and clusters in ϕ,β-LDs at peak-DP

Sample[a]	Peak-DP[b]	Chain category[c]				DB[d]	No. of clusters[e]	IC-DP[f]
		A+B1a	B1b	B2	B3			
2.I	380	36.4	1.9	3.5	0.9	11.0	3.9	27
2.II	170	18.4	1.0	1.5	0.2	11.8	1.9	19
2.III (c)	70	9.9	0.7	0.3	0	14.1	1	–
3.I	217	21.1	1.2	1.7	0.4	10.8	3.4	14
3.II	130	14.0	0.8	1.0	0.2	11.6	2.3	7
3.III (c)	50	6.2	0.5	0.3	0	12.2	1	–
4.I	135	12.6	1.1	1.3	0.3	10.6	1.9	17
4.II	72	7.6	0.5	0.6	0.1	10.8	1.1	–
4.III (c)	54	7.0	0.6	0.3	0	12.8	1	–
5.I	72	6.8	0.6	0.7	0.1	9.6	1.3	–
5.II (c)	54	5.4	0.6	0.4	0.1	10.2	1	–
6.1.I	115	11.0	0.9	0.9	0.2	10.4	1.3	–
6.1.II (c)	75	7.9	0.6	0.6	0.1	10.9	1	–
6.1.III (c)	70	7.8	0.6	0.5	0	11.3	1	–
6.2.I (c)	55	5.3	0.4	0.5	0.1	9.6	1	–
6.2.II (c)	55	5.8	0.5	0.4	0	10.4	1	–
6.3.I (c)	25	2.5	0.2	0.2	0	7.6	1	–

[a] (c) indicates fractions containing single clusters.
[b] Adapted from Table 1.
[c] Number of chains at peak-DP.
[d] Degree of branching at peak-DP = (peak-NC − 1)/peak-DP × 100.
[e] Peak-NC/peak-NC of a single cluster. Peak-NC values are given in Table 2.
[f] Average inter-cluster degree of polymerisation = ($DP_{fraction}$ − $DP_{cluster}$ × No. of cluster)/No. of clusters.

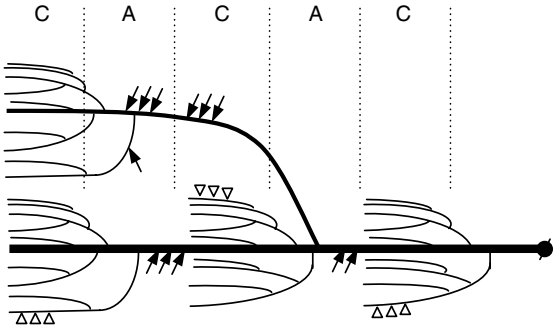

Fig. 10. A dextrin from amylopectin containing four clusters organised according to the principles of the classical model by Hizukuri (1986). The clusters of short chains (thin lines) are interconnected through 1 B2- (bold line) and 1 B3-chain (very bold line), but alternatively they can also be linked through 2 B2-chains. Arrows show possible places of endo-attack by the α-amylase at long internal segments, which releases smaller groups or single clusters. Very small arrows remind about the simultaneous exo-attack that results in short, linear dextrins collected in the supernatants after methanol precipitation. Multiple endo-attack results also in small linear and, in addition, some branched fragments. The reducing end (Ø) and the crystalline (C) and amorphous (A) lamellae inside starch granules are indicated.

Dextrins of fraction 3.I contained 3.4 clusters apparently interconnected by two long chains (1.7 B2 + 0.4 B3-chains) (Table 4). As illustrated in Fig. 10, the model by Hizukuri (1986) predicts that three clusters should be interconnected by either 2 B2-chains or 1 B3-chain, and thus the fraction was possibly a mixture of both alternatives. Sample 2.I represented a domain structure containing four clusters. The number of B1b-chains was two, again indicating that they were found in every second cluster, and the number of long chains interconnecting the clusters was ~4.5, of which ~1 was a B3-chain and 3–4 were B2-chains. As seen in Fig. 10, the number of the long B-chains was higher than predicted from the classical model to interconnect four clusters (only 2–3 long B-chains were expected to be found).

An interesting parameter is the inter-cluster degree of polymerisation (IC-DP, Table 4), which expresses the average size of the segments between the clusters. Due to a multiple attack pattern of the α-amylase against the inter-cluster segments, the DP of the resulting cluster products is smaller than only a single attack would result in. The average size of the segments was therefore estimated from the DP of the large domain level dextrins and the DP of their individual clusters as follows:

$$\text{IC-DP} = (\text{DP of domain} - \text{DP of cluster} \times \text{No. of clusters})/\text{No. of clusters}. \quad (1)$$

It should be noted that, unfortunately, the estimation was rather inexact of several possible reasons, such as the comparatively rough estimation of peak-DP on GPC and the degree of multiple attack on internal segments depending on hydrolysis time and/or the exact structure in these areas. Nevertheless, the result suggested that IC-DP between two clusters was of the order 7–19 residues (Table 4), which is compatible with the classical cluster model. However, when the sample contained several clusters the IC-DP tended to increase, so that in sample 2.I with 4 clusters it was roughly 27 residues at average. This is not predicted by the classical model, in which IC-DP is expected to be constant regardless the number of clusters involved.

Thus it appears that when analysing higher structural levels of the potato amylopectin, in which several clusters are involved, an alternative to the generally accepted model of interconnection seems necessary to find. In the two-directional backbone model (Bertoft, 2004) the clusters are connected to a backbone composed of the long chains. This alternative model was used to interconnect four clusters with 1 B3 and 3 B2-chains in the amylopectin of PAPS in accordance with the result for sample 2.I (Fig. 11). Note that in this model the backbone is amorphous and the number of chains that build up the backbone is flexible and depends on the particular type of starch. Thus, in potato amylopectin that contains a high number of long chains, the number of chains in the backbone could be comparatively high, including both the long A-chains and the shortest "fingerprint" A-chains (Bertoft, 2004). Therefore, a number of branches are not included into clusters but exist in the backbone and are released by α-amylase already at the initial stages of attack and found as the small, branched fragments in the supernatant of sample S-I (Fig. 3). It is also likely that the small clusters contained in sample 6.3.I, also released at the initial stages, were from easily attacked areas in the backbone (Fig. 11). By the attack at the chains of the backbone, several A-chains would be lost and explains the lower ratio of A:B-chains in the fractions of α-dextrins compared to the original amylopectin. Hizukuri and Abe (1993) analysed the skeletal, or inner core, structure of potato amylopectin and found that it was composed of B-chains with DP ranging from 17 to 47 (in the form of glucoamylase limit dextrins). Interestingly, the chains carried both A- and

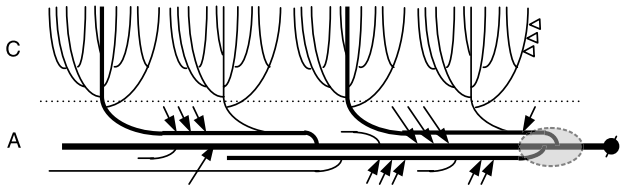

Fig. 11. A dextrin from amylopectin containing four clusters organised according to the principles of the two-directional backbone model by Bertoft (2004). The clusters of short chains (thin lines) are interconnected through 3 B2- (bold lines) and 1 B3-chain (very bold line) found in the amorphous backbone. A B2-chain forms a part of every second cluster, whereas the other clusters are connected to the backbone through a short B1b-chain. Arrows show possible places of endo-attack by the α-amylase at long internal segments, which releases smaller groups or single clusters. Multiple endo-attack results in small linear and, especially at very initial stages, in branched fragments originating from very short "fingerprint" A-chains and long A- and B-chains. Small clusters with only few branches originate also from the backbone (encircled) and were collected in sample 6.3.I. Very small arrows remind about the simultaneous exo-attack. The reducing end (Ø) and the crystalline (C) and amorphous (A) lamellae inside starch granules are indicated.

B-chains and the span length between the branches was estimated to about 10 residues regardless the length of the core chain. This description of the core structure is perfectly compatible with the result in this investigation and the backbone model. The model in Fig. 11 offers also an explanation to the sharp increase in the size of the inter-cluster segments when large structural domains were studied. Two clusters are interconnected through one B2-chain and form a small unit (such as those found in e.g., sample 2.II) with IC-DP in the order of 15–19 residues. Two such units are, however, interlinked through a B3-chain in the backbone. Because several other chains are bound to the B3-chain (with possibly a span length around 10, as found by Hizukuri & Abe (1993)) the IC-DP increases considerably even though the additional chains are not directly participating in the actual inter-linkage of the clusters.

It was interesting to notice that in all fractions apparently only every second cluster at average contained a B1b-chain. The structural reason for this is not obvious, but one suggestion is included in Fig. 11. In every second case a B1b-chain connects the cluster to the backbone, whereas in the other cases a B2-chain contributes with its non-reducing end to the cluster. Because the fission of B2-chains resulted in the production of B1b-chains, one has to assume that the free clusters containing B1b-chains were those in which the B2-chains originally participated, whereas free clusters lacking B1b-chains were actually having such chains originally, but after the enzymatic attack they were converted into smaller chains (B1a).

4. Conclusions

Clusters of PAPS were of rather homogenous size in the DP-range 50–70 and 7–11 chains. Differences in the degree of branching indicated differences in the fine structure of the clusters. A minor group of very small clusters of DP 25 contained only three chains, but it was uncertain if they should be considered as true clusters participating in the crystalline structures of the starch granules. Some other branches in PAPS were apparently also not involved in the clusters, but probably existed as single branches in the amorphous areas. Two clusters were apparently interconnected through a B2-chain as predicted in the classical model. However, on a larger structural level, when several clusters were involved, the mode of interconnection was more complicated. The large amount of long chains compared to the number of clusters, and the large number of glucosyl residues found at inter-cluster areas can be explained by the two-directional backbone model.

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