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THE STRUCTURE OF CLUSTERS FROM POTATO AMYLOPECTIN

Summary

The clusters from size-fractions of hydrolysed amylopectin from amylose-free potato starch (APP) were isolated by controlled α-amylolysis. The external chains were removed by further phosphorolysis and β-amylolysis, thereby transforming the APP and the clusters into ϕ,β-limit dextrins. The DP of the ϕ,β-LD of the clusters was rather uniform around 40-50. The unit chain composition was analysed by HPAEC-PAD and it was shown that the long internal B-chains, that interconnected the clusters in the amylopectin, had preferentially been cut into B-chains with DP 8-23. Smaller clusters possessed also increased amounts of the shortest B-chain with DP 3. The A:B-chain ratio decreased after α-amylolysis, showing that A-chains were not formed. The clusters were built up of only 4–6 chains and the density of branches was 11–13%. Very small, branched building blocks were also isolated from the APP and the clusters by an extensive α-amylolysis and analysed by GPC and HPAEC. The building blocks ranged between DP 5–30 and contained 2–5 very short chains (approx. CL 2-7). The predominating branched building blocks had DP 7–8 and were singly branched. The density of branches within building blocks was high (25%) and the clusters were at average composed of 2 or 3 building blocks.

Introduction

Amylopectin, the branched component of starch, is build up of numerous clusters [1-4] of short chains with a degree of polymerisation (DP) within the range 6–35. These chains are designated B1-chains (short chains carrying other chains through α-(1→6)-linkages) and A-chains (short chains not carrying other chains) [5]. Longer chains with DP approximately 35–60 (B2-chains) or longer (B3-chains) interconnect the clusters [6]. A large number of investigations on the unit chain profiles of different amylopectins have shown that they all possess this general pattern. However, starch granules with a B-crystalline X-ray diffraction pattern (mainly tuber and root starches) have somewhat longer average chain lengths (CL) and a clearly lower ratio of short chains to long chains than granules with A-crystallinity (mainly cereal starches) [7].

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When considering the extensive number of investigations on the unit chain profile of amylopectins, surprisingly little work has focussed on the actual branching pattern within the units of clusters and the mode of interconnection of the clusters. In fact, in order to really understand the fine structure of different amylopectins, these latter aspects are at least as important as the knowledge of the unit chain distribution. An enzymatic method, in which the α-amylase of *Bacillus amyloliquefaciens* plays the key role, was developed in my laboratory for the isolation of the units of clusters [8]. Shortly, the amylopectin macromolecule is initially rapidly fragmented into intermediate α-dextrins by the attack of the enzyme on long internal chains between the clusters [9]. When such internal chain segments no longer remain, the nine subsites around the active site on the enzyme will not be fully interacting with the substrate anymore [10]. As a result, the reaction rate decreases and the obtained clusters can be size-fractionated by methanol precipitation [11].

To study the branching pattern within the clusters, the external chains (that also have been partly attacked by the α-amylase) are largely removed with the enzymes phosphorylase and β-amylase [12]. In the remaining ϕ,β-limit dextrins (ϕ,β-LD) of the clusters all A-chains are found as maltosyl-stubs, whereas all B-chains are longer[9]. Thus, after a debranching and chromatographic analysis, the ratio of A- to B-chains can be estimated and the distribution of the internal parts of the B-chains is also obtained [12, 13]. The ϕ,β-LD can also be further hydrolysed by the α-amylase by a 100-fold increased enzyme activity. This extensive hydrolysis results in small building blocks (α-limit dextrins) of very tight branchings [14]. Therefore, it is also possible to study the size distribution of the building blocks found within the clusters.

The branching pattern of the clusters of amylopectin from an amylose-free potato is now reported. The results are compared with earlier studies on the amylopectin from waxy-rice [13, 14] and two maize mutants [15].

Materials and methods

Starch from amylose-free potato (APP) was a kind gift from Lyckeby Stärkelsen, Sweden. α-Amylase of *Bacillus amyloliquefaciens* (EC 3.2.1.1), with an activity of 600 U/mg, was from Boehringer-Mannheim (Germany), β-amylase of barley malt (EC 3.2.1.2) from Megazyme International (Ireland), and phosphorylase *a* of rabbit muscle (EC 2.4.1.1) from Sigma (Germany). The debranching enzymes isoamylase of *Pseudomonas amyloderamosa* (EC 3.2.1.68) and pullulanase of *Klebsiella pneumoniae* (EC 3.2.1.41) were from Hayashibara Shoji Inc. (Japan).

Isolation of clusters. APP (10 g) was dissolved in 90% dimethylsulphoxide (DMSO), diluted in sodium acetate buffer (pH 6.5), and treated at 25°C with diluted α-amylase (0.03 U/mL) at a substrate concentration of 10 mg/mL as previously described

[12]. The reaction was stopped after 1h and branched intermediate α-dextrins were precipitated with five volumes of methanol. The precipitate was then dissolved in water and size-fractionated by the method of Bertoft and Spoof [11]. Four of the fractions, representing large (fraction L), medium (M), small (S), and very small (VS) dextrins, were collected for analyses in this study. Fractions L, M, and S were further treated preparatively with the amylase for 1.5 h as described above, and the products were again collected by precipitation in methanol (5 volumes). Fraction L was finally treated for an additional 3 h with the enzyme. After these treatments all fractions were comparatively resistant to further hydrolysis and were therefore considered as being composed of units of clusters.

Production of ϕ,β-LD. Preparative production of ϕ-LD by phosphorolysis [16] and further into ϕ,β-LD by β-amylolysis [12] was previously described. However, the products glucose 1-phosphate and maltose, respectively, were removed from the fractions of clusters on two PD-10 columns (Pharmacia) coupled in series and from APP by dialysis.

Debranching. APP or intermediate α-dextrins were debranched with isoamylase at pH 3.5, whereas ϕ,β-LD were debranched with pullulanase at pH 5.5 essentially as described elsewhere [13]. All samples were analysed by HPAEC-PAD as described below.

Production and analysis of building blocks. ϕ,β-LD (5 mg/mL) were treated with concentrated α-amylase (6 U/mL) for 3 h at 35°C. The reaction was stopped by boiling and the sample was then lyophilised. The dried sample (4 mg) was dissolved in hot DMSO (0.2 mL). One part of the sample was diluted in water and analysed on a column of Superdex 75 or by HPAEC. Another part (15 μL) was diluted to 1 mL with NaOAc buffer (pH 5.5), treated with pullulanase (1 μL) overnight at room temperature, and finally analysed by HPAEC.

Gel-permeation chromatography (GPC). Products from α-amylolysis of ϕ,β-LD were analysed on a column (1x90 cm) of Sepharose CL 6B (Pharmacia) as described by Bertoft *et al.* [14]. A column (1x90 cm) of Superdex 75 was eluted with 0.01 M KOH and used for the analysis of unit chains and building block profiles. Superdex 30 (1x90 cm) was eluted with water and was used for preparative isolation of size-fractions of building blocks or unit chains from APP. The columns were calibrated with samples of dextrins of known DP [11, 17].

High-performance anion-exchange chromatography (HPAEC). Ion-exchange chromatography was performed on Dionex series 4500i (USA) equipped with a BioLC gradient pump and pulsed amperometric detection (PAD). The main column and the guard column (CarboPac PA-100, Dionex) were eluted at 1 mL/min. The gradient included eluent A (150 mM NaOH) and eluent B (150 mM NaOH containing 500 mM

NaOAc). The sample (25 μL) was applied at 85% A and 15% B. Debranched samples of APP, α-dextrins, and ϕ,β-LD were then eluted with the following gradient: from 0–9 min a linear gradient of eluent B from 15–36%; 9–18 min from 36–45%; and 18–110 min from 45-100%. Building blocks and debranched blocks were eluted with a different gradient: from 0–15 min from 15–34%; 15–26 min from 34–40%; 26–52 min from 40–49%; and from 52–54 min from 49–100% of eluent B. For quantitative analysis the system was calibrated by the method of Koch *et al.*[18] using chains from debranched APP or building blocks from the ϕ,β-LD of APP prepared by fractionation on Superdex 75 or Superdex 30, respectively.

Results and discussion

A selected series of intermediate α-dextrins was obtained after 1 h of hydrolysis of the potato amylopectin and analysed on Sepharose CL-6B. Large dextrins in fraction L possessed an average DP of 279 and represented 7.1% of all branched dextrins from the hydrolysis mixture (Table I).

Table I

Characterisation of amylopectin and fractions of α-dextrins.

Sample	Yield (%)	α-Amyl. time[1] (h)	Total time[2] (h)	DP[3]	CL[4]	ECL[5]	ICL[6]
APP		0	0	-	21.2	13.7	6.5
L	7.1	1.0	1.0	279	15.1	8.3	5.8
L-II		1.5	2.5	111	13.4	6.9	5.5
L-III		3.0	5.5	60	10.5	4.3	5.2
M	10.5	1.0	1.0	104	16.1	8.3	6.8
M-II		1.5	2.5	65	13.9	6.5	6.4
S	3.9	1.0	1.0	87	15.5	7.9	6.5
S-II		1.5	2.5	58	13.9	7.5	5.4
VS	7.2	1.0	1.0	37	16.0	9.0	6.0

[1]Hydrolysis with α-amylase in each successive step.
[2]The sum of the successive steps.
[3]From GPC on Sepharose CL-6B.
[4]From HPAEC after debranching with isoamylase.
[5]ECL = CL x (% ϕ,β-limit/100) + 1.5.
[6]ICL = CL - ECL - 1.

The DP of the other fractions (M, S, and VS) decreased to DP 37. When the fractions were treated further with the α-amylase, the smallest dextrins (fraction VS) were practically resistant to the enzyme (Fig. 1), which suggested that they represented small clusters from the amylopectin. Dextrins in fractions M and S decreased only slightly in

size by a second amylase treatment for 1.5 h (giving fractions M-II and S-II) and clusters were therefore probably to a large extent already present in these fractions after the initial 1 h treatment. The dextrins in fraction L were, however, readily attacked by the second amylase treatment, which showed that they represented groups of interconnected clusters. The dextrins from the second treatment (L-II) could even be hydrolysed a third time for an additional 3 h into fraction L-III before the hydrolysis rate became very low and clusters were obtained (Fig. 1). The average chain length (CL) decreased from 21.2 in the original APP to between 10.5 and 16. As shown below, this was mainly due to the attack at external chains by the enzyme [10], rather than the attack at longer chain segments between the clusters.

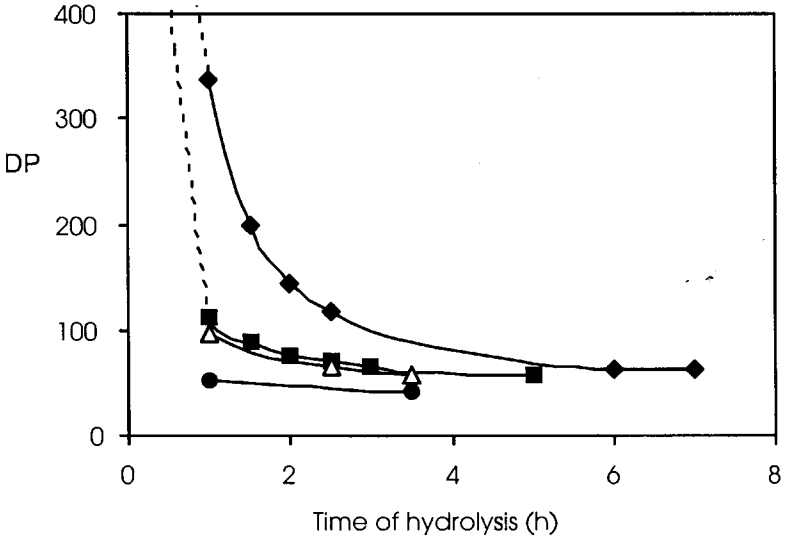

Fig. 1. DP as a function of time during α-amylolysis of fractions L (◆), M (■), S (△), and VS (●). The DP was estimated from GPC on Sepharose CL-6B excluding dextrins of DP < 20.

The external chains were next removed by successive phosphorolysis and β-amylolysis from APP and the fractions containing the clusters (fractions L-III, M-II, S-II, and VS). The remaining ϕ,β-limit dextrins represented therefore the internal structures, or branching zones of the clusters (BZC) [15]. Their DP ranged from 31–48 (Table II) and was comparable to that found for the double mutant *aewx* from maize [15]. As both starches were of B-crystalline type, this suggests a common cluster size of such starches and is in contrast to the several A-crystalline starches earlier investigated [14-16, 19], in which the clusters tend to be larger and the DP-range is much broader.

Table II

Characterisation of the ϕ,β-LD of amylopectin and fractions containing clusters.

Sample	ϕ,β-Limit (%)	DP[1]	CL[2]	NC[3]	Branch density[4]	A:B[5]
APP	57	-	9.1	-	11.0	1.3
L-III	26	40	7.8	5.1	12.8	0.9
M-II	36	46	8.9	5.2	11.2	0.8
S-II	43	48	7.9	6.0	12.7	1.0
VS	47	31	8.5	3.7	11.8	0.8

[1]From GPC on Sepharose CL-6B.
[2]From HPAEC after debranching with pullulanase.
[3]Number of chains = DP/CL.
[4]Density of branches (%) = (1/CL) x 100.
[5]Molar ratio of A-chains to B-chains.

The ϕ,β-LD of the APP and the clusters were debranched and analysed by HPAEC-PAD. From the difference of the CL-values before and after limit dextrin production, the ϕ,β-limit values were calculated (Table II) and finally these were used for estimation of the average lengths of the external and internal chains [9] of the samples (Table I). The APP sample possessed similar values to those we earlier found for amylopectin from a normal potato starch [12], though the CL and ECL were slightly lower compared to other reports [20]. The effect of the α-amylolysis on the ECL-values was clearly seen, but the ICL decreased only little (Table I). This could be explained by the fact that the long internal chain segments between clusters, that the enzyme easily attacks are rather few compared to the very short internal chains found within the clusters. The average number of chains (NC) in the clusters was approx. 4-6 and the branch density 11–13% (Table II), which again was similar to that found for *aewx*-maize starch [15]. In A-crystalline starches from *wxdu*-maize [15] and *wx*-rice [13] both the NC and the density of branches were higher (approx. 6-18 and 13-19%, respectively).

In a ϕ-LD the A-chains have been reduced into maltotetraosyl-stubs [21] and after successive β-amylolysis only a maltosyl-stub remains [9]. This is the case with all normal amylopectins, in which chains shorter than DP 6 are not found [22, 23]. In the α-dextrin samples, however, a part of the A-chains (5–15%) had been reduced into maltotriosyl-stubs, in addition to trace amounts of maltosyl-stubs (not shown). Such very short chains are resistant to both phosphorylase and β-amylase, and the maltotriose formed after the debranching will therefore be a mixture of maltotriose from a part of the A-chains and the shortest possible B-chains. It was therefore necessary to subtract the maltotriose originating from A-chains from the maltotriose peak of the

debranched ϕ,β-LD clusters in order to obtain a correct estimation of the ratio of A:B-chains. In APP, the ratio was 1.3 (Table II) and it was clearly lower (0.8–1.0) in the isolated clusters. This suggested that the long B-chains between clusters mainly were cleaved into two new B-chains by the α-amylase [12]. The result was different from that found earlier for amylopectin from a normal potato starch, for which an increased A:B-ratio was found after α-amylolysis [12]. It remains unclear if this was due to true differences between the two samples or if it was a result of the improved resolution obtained with HPAEC compared to GPC that was used in the earlier investigation.

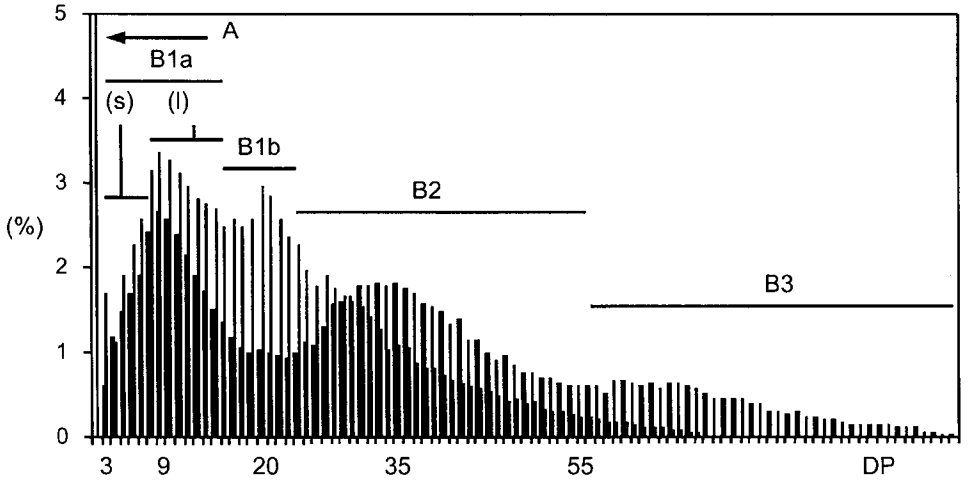

Fig. 2. Bar graph of the chain length distribution of the ϕ,β-LD of APP (black bars) and clusters (sample S-II; light bars). Areas of peaks in HPAEC were corrected to carbohydrates by weight. Groups of different chains are shown. All A-chains are shown as maltose (DP 2). Because peaks at DP >55 were not actually resolved, the bars for B3-chains were only roughly estimated by comparison with GPC of the debranched samples.

The distribution of the internal B-chains of the ϕ,β-LD was analysed by HPAEC (Fig. 2). The long chains of APP were divided into B2- and B3-chains [6] and, on the basis of the division of chains in *wx*-rice starch [13], the short B-chains were sub-divided into B1a- and B1b-chains. The former group was further sub-divided into very short chains [B1a(s)] and somewhat longer chains [B1a(l)]. It was interesting to notice, that the profile of the shortest B1a(s)-chains was different from that of the *wx*-rice starch [13], which possessed a peak at DP 5, rather than at DP 7 (Fig. 2). This indicated that the mode of tightly clustered branching in the two starches was different. From the example given in Fig. 2 (fraction S-II) it is clearly seen that, in order to release the clusters, the α-amylase cleaved the long internal chains, which mainly resulted in the production of B1b-chains together with some B1a(l)-chains. The profiles

of the other clusters were similar (though not identical) to that of fraction S-II. However, the clusters produced from fraction L did not show any increase in maltotriose, whereas increasing amounts were obtained for clusters from fractions M, S, and VS (only S-II shown). This suggested that the clusters, though fairly similar regarding their sizes and unit chain composition, possessed minor differences regarding their mode of interconnection.

The analysis by GPC of the size-distribution of building blocks after an extensive α-amylolysis was first described for *wx*-rice starch and its clusters [14]. Interestingly, the size-profiles could be divided into two types that possibly originated from different structural domains of the starch granules. The DP of the branched building blocks ranged from 5 to approx. 40, and the average DP was 13–17 [14], which was similar to that later found for the amylopectin from *wxdu*-maize [15]. The size-distribution of the building blocks of APP obtained by GPC is shown in Fig. 3.

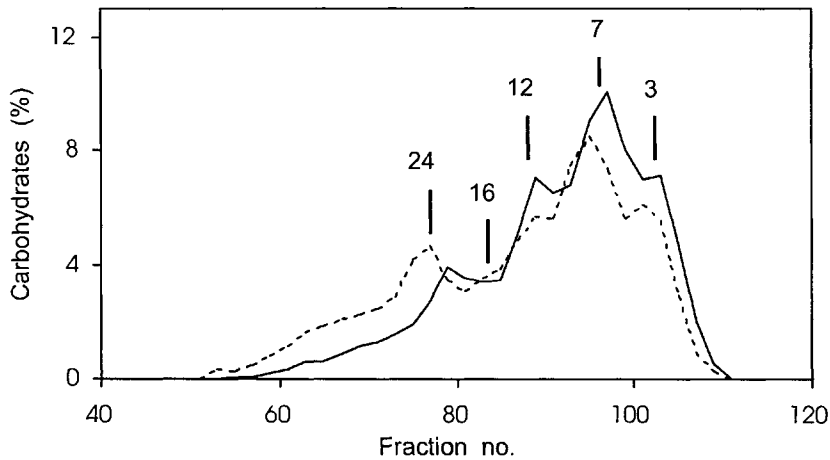

Fig. 3. Fractionation on Superdex 75 of building blocks obtained by extensive α-amylolysis of the ϕ,β-LD of APP (——) and clusters (fraction S-II; ----). DP values are indicated.

The major fraction of blocks had a DP of 6-8 and only small amounts of blocks with DP >14 were found. This was similar to the mainly B-crystalline *aewx*-maize sample [15] and suggested a common, small building block structure for this type of starches. The profile of building blocks was also analysed with HPAEC (Fig. 4), by which a large number of individual peaks representing building blocks up to DP 19 were resolved.

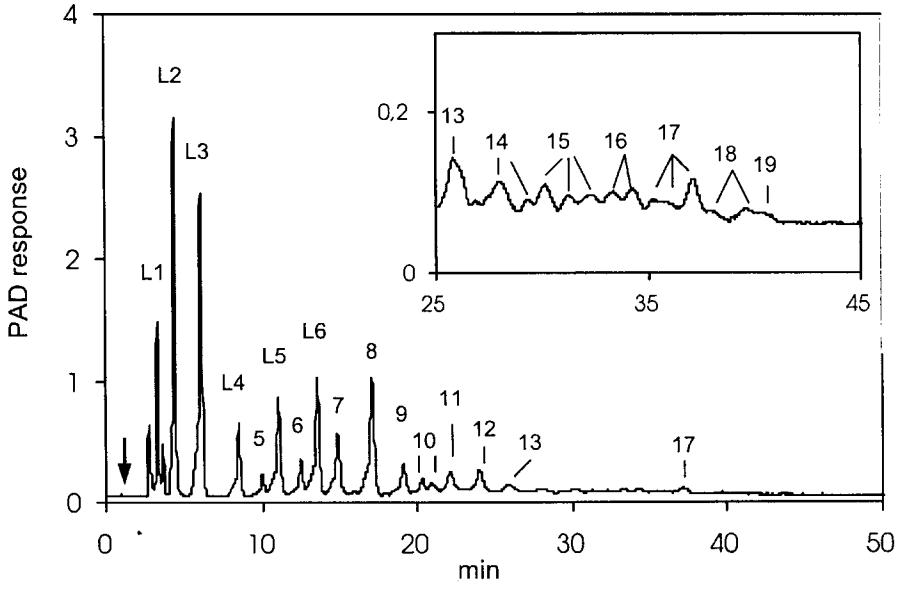

Fig. 4. Fractionation by HPAEC-PAD of building blocks obtained by extensive α-amylolysis of the ϕ,β-LD of APP. DP values are indicated. L indicate linear dextrins. Sample injection and start of the gradient is shown by arrow. Inset is a magnification of the chromatogram.

Linear dextrins of DP 1-6 represented fragments of the internal chains between the clusters of the amylopectin and between the building blocks within the clusters. The smallest branched building block had DP 5-7. Several of the larger blocks were represented by more than one peak, suggesting that they were found as singly and/or multiply branched molecular species. The PAD-response, which decreases with the DP of linear dextrins [22], was found to give a similar response for the branched dextrins. The relative molar amount of the branched building blocks of APP is shown in Fig. 5a. Blocks with DP 8 predominated and the distribution of building blocks of the clusters (not shown) was similar to that of the whole amylopectin sample (with the exception of fraction VS, in which DP 7 predominated). This suggested a rather high structural homogeneity among the clusters of APP, in contrast to the amylopectins of *wx*-rice[14] and maize mutants [15]. The average number of branched blocks within the clusters of APP was only between 2–3.

The mixtures of building blocks were also debranched with pullulanase and analysed by HPAEC. The relative molar amount of the chains from the branched blocks of APP was obtained by subtracting the pre-existing linear chains from the chromatograms (Fig. 5b).

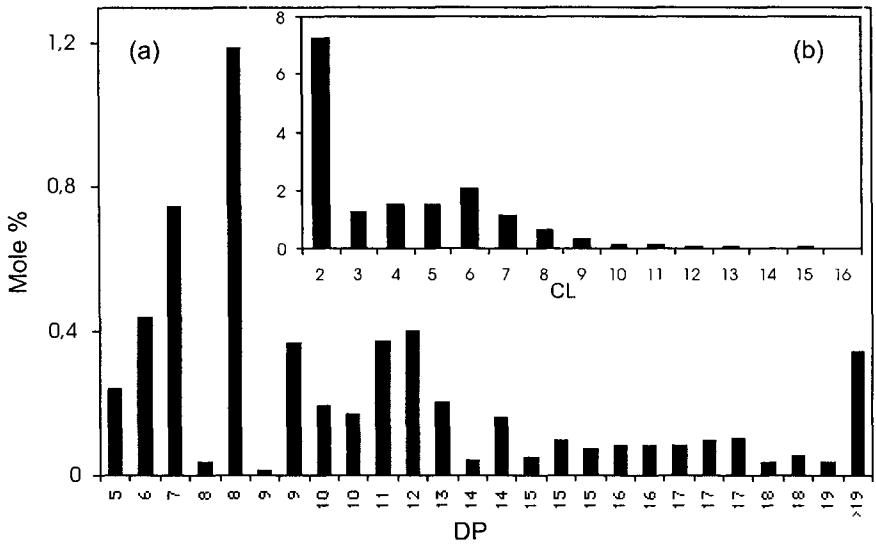

Fig. 5. Bar graph of the molar distribution of (a) branched building blocks from the ϕ,β-LD of APP and inset (b) the chains obtained after debranching. The linear dextrins obtained by the extensive α-amylolysis have been subtracted from the graphs.

The chains of the branched building blocks were very short and corresponded to the shortest chains in APP (A- and B1a(s)-chains). The structural characteristics of the branched building blocks are summarised in Table III. From the DP-values (approx. 12) and the CL (4) an average number of chains near 3 was obtained. If it is assumed that the ECL of the branched blocks (α-LD) was approx. 2, the ICL was also about 2 between the branches inside the blocks. The branching density was approx. 25%.

Table III

Characterisation of branched building blocks in amylopectin and its clusters.

Sample	DP[1]	CL[1]	ICL[2]	NC[3]	Branch density[4]
APP	10.8	4.0	2.2	2.7	24.9
L-III	11.8	3.9	1.9	3.0	25.4
M-II	12.6	4.3	2.5	2.9	23.3
S-II	11.8	4.1	2.1	2.9	24.6
VS	10.3	3.8	1.8	2.7	26.6

[1]From HPAEC.

[2]ICL = [((CL - ECL) x NC) / (NC - 1)] - 1.

[3]Number of chains = DP/CL.

[4]Density of branches (%) = (1/CL) x 100.

To characterise the branched building blocks further, a semi-preparative separation was performed by GPC on Superdex 30. Fractions representing increasing DP were collected, debranched, and finally analysed with the HPAEC-equipment. A linear relation between the DP and the number of chains was obtained (Fig. 6) and could be used to estimate the NC of the individual peaks of building blocks in Fig. 5a.

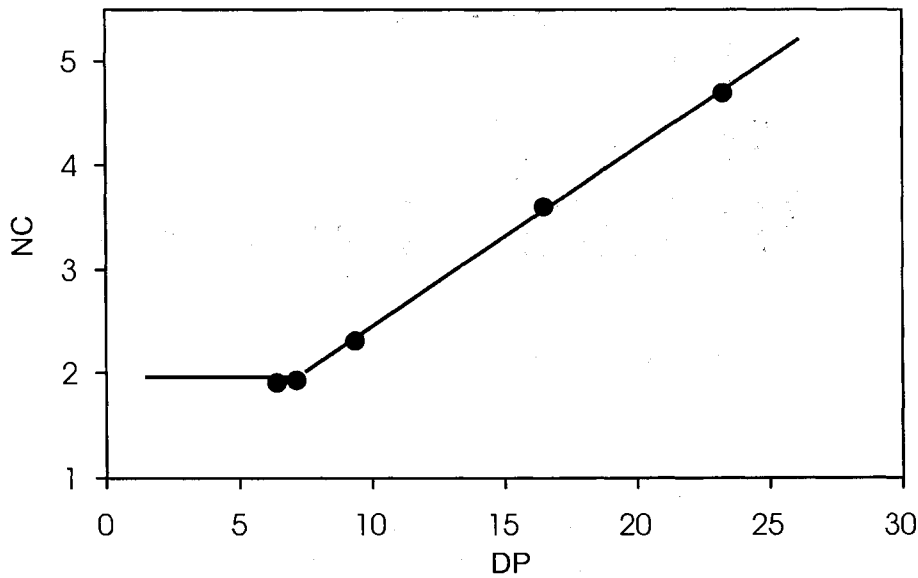

Fig. 6. Number of chains *versus* DP of size-fractions of branched building blocks from APP.

The major blocks of DP 7-8 contained only 2 chains, while the second abundant group of blocks at DP 11-12 mostly was composed of 3 chains. The larger building blocks with DP around 24 (Fig. 3) had 5 chains, whereas blocks with 4 chains were only little represented.

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STRUKTURA KLASTERÓW AMYLOPEKTYNY ZIEMNIACZANEJ

Streszczenie

Za pomocą kontrolowanej α–amylolizy amylopektyny z pozbawionej amylozy skrobi ziemniaczanej (AAP) otrzymano frakcje klasterów o różnych rozmiarach. Zewnętrzne łańcuchy usunięto następnie przez fosforolizę i β-amylolizę przeprowadzając w ten sposób AAP w ϕ,β-dekstryny. DP klasterów tych dekstryn był dość wyrównany mieszcząc się w granicach 40 do 50. Skład poszczególnych łańcuchów analizowano za pomocą HPAEC-PAD pokazując, że długie wewnętrzne łańcuchy B łączące ze sobą poszczególne klastery zostały pocięte głównie na łańcuchy B o DP 8–23. Mniejsze klastery również zawierały większą liczbę krótszych łańcuchów B o DP 3. Stosunek łańcuchów A:B po α-amylolizie obniżał się, co wskazywało, że łańcuchy A nie tworzyły się. Klastery tworzyły się tyko z 4 do 6 łańcuchów, a gęstość odgałęzień wynosiła 11 do 13%. Przez daleko idącą α-amylolizę wydzielono z AAP bardzo małe rozgałęzione fragmenty strukturalne, które zanalizowano za pomocą GPC i HPAEC. DP tych fragmentów wynosił od 5 do 30. Zawierały one 2 do 5 bardzo krótkich łańcuchów (przeciętnie 2–7). Przeważnie fragmenty te miały DP 7 do 8 oraz pojedyncze rozgałęzienia. Gęstość rozgałęzień była wysoka (25%), a klastery przeciętnie zawierały 2 lub 3 takie fragmenty.