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Structural heterogeneity in waxy-rice starch

Eric Bertoft*, Qin Zhu, Helena Andtfolk, Marina Jungner

Department of Biochemistry and Pharmacy, Åbo Akademi University, BioCity, P.O. Box 66, FIN-20521 Turku, Finland

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Abstract

Alpha-amylase of *B. amyloliquefaciens* was used for the structural characterization of the amylopectin from waxy-rice starch. Fractions of α-dextrins with a degree of polymerization (d.p.) < 5000 were isolated from amylopectin hydrolysates after 1 and 3 h. ϕ,β-Limit dextrins were prepared by successive phosphorolysis and beta-amylolysis of the fractions and these were analysed by a second alpha-amylolysis. Based on the hydrolysis pattern, the limit dextrins were divided into two major groups, A and B, which possessed units of clusters of d.p. 100–200 and 90–130, respectively. An extensive alpha-amylolysis resulted in characteristic distributions of dextrins with d.p. < 80 which represented branched building blocks. Type A dextrins possessed more larger building blocks with d.p. ~ 40, but less intermediate and small blocks, than type B. The ϕ,β-limit dextrin of the original amylopectin had a distinct distribution enriched in small building blocks. A model is proposed in which the two types of dextrins originate from regular and less regular structural domains of the amylopectin fraction within the starch granules.

Keywords: Amylopectin; Waxy-rice starch; *B. amyloliquefaciens*

1. Introduction

The unit chain distribution of the branched amylopectin component of different starches was extensively investigated by debranching enzymes. Two major groups of chains, short and long, are universally found (Hizukuri, 1985) and are generally accepted to be organized into clusters (Manners, 1989). It was suggested that the long chains interconnect two or more individual clusters (Hizukuri, 1986), but so far details of the organization of the clusters into a macromolecular structure is very limited. Information on this level should improve our understanding of the synthesis of starch and its organization into granules (Smith et al., 1997), which is the physical form of the starch in plants (Jane et al., 1994).

The research in our laboratory has focused on searching for units of clusters in amylopectins of different origins. For this purpose the alpha-amylase of *Bacillus amyloliquefaciens* (earlier referred to as the liquefying type of *B. subtilis*) was used. The enzyme contains nine subsites unevenly distributed around the catalytically active site (Robyt and French, 1963; Thoma et al., 1970). The enzyme attack is fast when all subsites become filled with α-D-glucosyl residues (Robyt and French, 1963). The initial attack on amylopectin occurs therefore at external chains, that are hydrolysed preferentially into maltohexaose (Robyt and French, 1963), and independently at longer internal chain segments between the branches (Bertoft, 1989b). As a result the hydrolysis mixture contains branched intermediate α-dextrins among which individual clusters should be found.

The intermediate α-dextrins can be size-fractionated by precipitation in methanol (Bertoft and Spoof, 1989) and the residual external chains can then be reduced into resistant maltosyl- and glucosyl-stubs by successive treatment with phosphorylase and beta-amylase (Bertoft, 1989b). If these ϕ,β-limit dextrins are further hydrolysed by the alpha-amylase, only attack at the internal chains will occur. Because a unit cluster does not possess longer internal chains, the hydrolysis rate of the remaining short chains is comparatively slow and can be used for the identification of the clusters. Though the exact size and composition of the units of clusters are difficult to define, the amylopectin of waxy-maize starch was suggested (Bertoft, 1989a; Bertoft, 1991b) to contain clusters of a degree of polymerization (d.p.) ~ 150 or smaller (as limit dextrins), whereas the amylopectin from waxy-barley (Bertoft and Åvall, 1992) had units of d.p. 65–85. The amylopectins from both smooth pea (Bertoft et al., 1993b) and potato (Zhu and Bertoft, 1996) possessed cluster sizes around d.p. 30–70. In this work the cluster structure of waxy-rice starch (WRS), that contained only amylopectin, was investigated.

* Corresponding author. Tel.: + 358-2-215-4272; Fax: + 358-2-215-4745.

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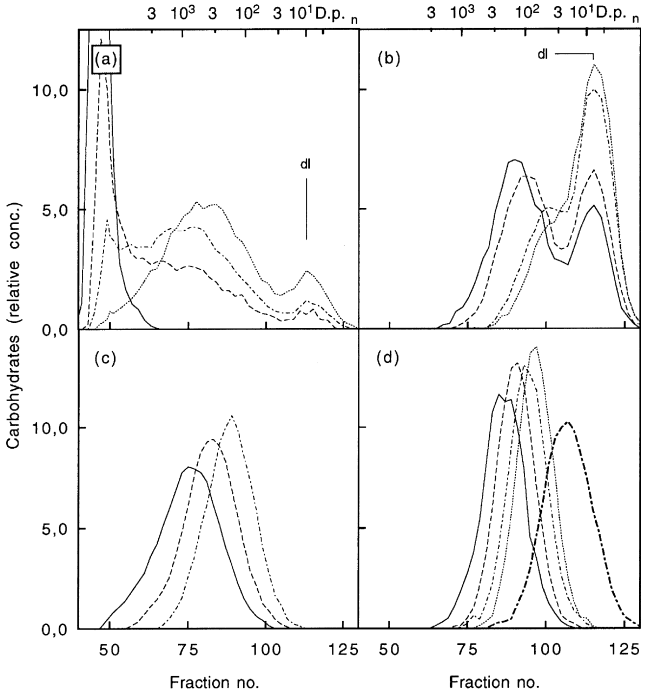

Fig. 1. Fractionation on Sepharose CL 6B of the hydrolysates of waxy-rice starch obtained after the action of alpha-amylase. (a) WRS before (—) and after hydrolysis for 0.25 (––), 0.5 (-·-) and 1 h (···); (b) WRS after hydrolysis for 3 (—), 5 (––), 24 (-·-) and 48 h (···); (c) fractions 2.1 (—), 7.2.2 (––) and 3 (-·-) obtained from the 1-h mixture by fraction precipitation; (d) fractions 10.1 (—), 10.2 (––), 11.1 (-·-), 11.2 (···) and 13 (-·-, bold) obtained from the 3-h mixture. The group of small dextrins that did not precipitate in methanol is shown by dI.

2. Experimental

2.1. Enzymes

Alpha-amylase of *Bacillus amyloliquefaciens* [(1 → 4)-α-D-glucan glucanohydrolase; EC 3.2.1.1] with an activity (Bertoft et al., 1993a) of 600 U/mg was purchased from Boehringer-Mannheim, whereas phosphorylase *a* from rabbit muscle [(1 → 4)-α-D-glucan:orthophosphate α-D-glucosyltransferase; EC 2.4.1.1], beta-amylase from sweet potato [(1 → 4)-α-D-glucan maltohydrolase; EC 3.2.1.2], and protease from *Streptomyces griseus* (type XIV) were from Sigma. Isoamylase from *Pseudomonas amyloderamosa* (glycogen 6-glucanohydrolase; EC 3.2.1.68) was obtained from Hayashibara Shoji Inc. The following enzymes were purchased from Boehringer-Mannheim and used in $NADP^+$-coupled reactions for determination of maltose and glucose 1-phosphate: α-D-glucosidase from yeast (α-D-glucoside glucohydrolase; EC 3.2.1.20), phosphoglucomutase from rabbit muscle (α-D-glucose 1,6-bisphosphate: α-D-glucose 1-phosphate phosphotransferase; EC 2.7.5.1), hexokinase from yeast (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1), and D-glucose 6-phosphate dehydrogenase from yeast (D-glucose 6-phosphate: NADP oxidoreductase; EC 1.1.1.49).

2.2. Starch isolation

Starch was isolated from waxy-rice (*Oryzae sativa* L., a gift from Associate Prof. Zhu Yanli, Beijing Medical College, China) and treated with protease as described by Morrison et al. (1984). The starch granules were then defatted by extraction overnight with hot aqueous 85% methanol in a Soxhlet apparatus, washed in ethanol, and finally dried in acetone.

2.3. Production of α-dextrins

WRS (16 g) was dissolved in 90% Me_2SO (320 mL) on a boiling water bath and then stirred for two days at room temperature. The solution was then diluted with water and treated at 25°C (pH 6.5) with alpha-amylase using a final concentration of 0.03 U of enzyme and 10 mg substrate/mL as described previously (Zhu and Bertoft, 1996). At 1 and 3 h the reaction was stopped in large aliquots (800 mL) with 5 M KOH (20 mL). Intermediate α-dextrins in the aliquots

Table 1
Characterization of waxy-rice amylopectin and fractions of α-dextrins

Sample	α-Amylolysis (h)	Yield[a] (%)	β-Limit (%)	D.p.[b]	C.l.	E.c.l[c]	I.c.l.[d]	N.c.[e]
Amylopectin	0	—	54	—	16.8	11.0	4.8	—
2.1	1	36.6	51	451	13.5	8.9	3.7	33.4
7.2.2	1	4.1	49	227	12.0	7.9	3.3	18.9
3	1	12.8	49	122	12.9	8.3	4.1	9.5
10.1	3	10.4	38	163	9.4	5.6	3.0	17.3
10.2	3	9.1	38	110	9.1	5.4	3.0	12.1
11.1	3	3.7	42	70	8.6	5.6	2.4	8.1
11.2	3	3.2	45	56	8.9	6.0	2.4	6.3
13	3	4.9	63	12	7.8	6.9	1.7	1.5

[a] Yields from original mixtures at 1 and 3 h of alpha-amylolysis.
[b] From gel-permeation chromatography on Sepharose CL-6B (Fig. 1c and d).
[c] E.c.l. = c.l. × (% β − limit/100) + 2.
[d] I.c.l. = [(c.l. − e.c.l.) × n.c.]/(n.c. − 1) − 1, or if n.c. is large, i.c.l. ≈ c.l. − e.c.l. − 1.
[e] Number of chains = d.p./c.l.

Table 2
Yield and composition of ϕ,β-limit dextrins produced from waxy-rice amylopectin and fractions of α-dextrins

Sample	ϕ,β-Limit (%)	Yield[a] (%)	D.p.[b]	Groups of dextrins[c]
Amylopectin	56	76	—	—
2.1	55	86	272	bIa (550), cVIa (350), cVa (200)
7.2.2	54	85	139	cVb (175), cIVb (130), cIIIb (90), cIIb(65)
3	53	83	66	cIIIb (80), cIIb (60), cIb (45)
10.1	43	73	110	cVa (200), cIVa (150), cIIIa (100)
10.2	44	79	75	cIIIa (100), cIIa (75)
11.1	48	79	47	cIIb (65), cIb (50)
11.2	51	54	34	cIb (45), d(33)
13	70	40	16	d(20–15)

[a] Yield based on ϕ,β-limit.
[b] From gel-permeation chromatography on Sepharose CL-6B (Fig. 2a and b).
[c] The d.p. of the groups shown in parenthesis were estimated from Fig. 2.

were precipitated with five volumes of methanol into mixtures I and II, respectively, washed with methanol, then acetone, and air-dried. Mixtures I and II were then fractionated into a series of precipitates of different size-distributions using increasing methanol–water ratios from 0.5 : 1 to 5 : 1 as described by Bertoft and Spoof (1989).

2.4. Characterization of amylopectin and α-dextrins

A stock solution of WRS was made by dissolving in 90% Me_2SO (10 mg/mL) and then diluting four times, whereas α-dextrins were directly dissolved in hot water (2.5 mg/mL). An aliquot was diluted twice and the molecular-weight-distribution was analysed on a column of Sepharose CL 6B as described later. For determination of beta-amylolysis limit, the stock solution (0.4 mL) was diluted to 0.9 mL and then 0.1 M NaOAc buffer (0.1 mL, pH 4.8) and beta-amylase (5 μL) was added. After incubation overnight, the maltose formed was analysed enzymically using $NADP^+$-coupled reactions (Bertoft, 1989b; Beutler, 1984). Phosphorolysis-beta-amyloylsis limit was measured as described earlier (Bertoft, 1989b), with the modification that the stock solution was diluted to 2 mg/mL before addition of phosphorylase *a*. The total carbohydrate was measured with phenol–H_2SO_4 (Dubois et al., 1956). For the estimation of average chain length (c.l.), 0.1 M NaOAc buffer (0.05 mL, pH 3.5) and isoamylase (2 μL) was added to an aliquot (0.5 mL) of the stock solution. After incubation for 4 h at 25°C, the mixture was boiled and the reducing value determined (Waffenschmidt and Jaenicke, 1987). The completeness of the debranching reaction was confirmed with successive beta-amylolysis (Bertoft, 1991b). The c.l. was calculated as total carbohydrate/reducing value. The average external chain length (e.c.l.) and internal chain length (i.c.l.) were calculated from the beta-amylolysis limit value (Bertoft, 1989b).

2.5. Production of ϕ,β-limit dextrins

Preparative production of phosphorolysis limit dextrins (ϕ-LD) (Bertoft, 1991b) and the successive beta-amylolysis was described earlier (Zhu and Bertoft, 1996), but the maltose formed from the ϕ-LD samples was removed on two PD-10 columns (Sephadex G-25, Pharmacia) coupled in series. The ϕ,β-LD were then lyophilized.

2.6. Alpha-amylolysis of ϕ,β-LD

The limit dextrins were dissolved in hot 90% DMSO (50 mg/mL) and diluted with water (5 ×) to 4 mL. A solution of alpha-amylase (0.21 mL) in 0.1 M NaOAc buffer (pH 6.5) was added to give a final enzyme concentration of 0.03 U/mL. Incubation took place at 25°C and aliquots were taken at intervals for analysis by gel-permeation chromatography. Extensive alpha-amylolysis was performed identically, but with a 100 × more concentrated enzyme solution to give 3 U/mL.

2.7. Gel-permeation chromatography

The samples were diluted to 1.2–1.5 mg of carbohydrates/mL in ~0.5 M KOH. Aliquots (0.2 mL) were eluted at 0.5 mL/min from columns (1.0 × 90 cm) of Sepharose CL-6B or Superdex 75 (Pharmacia) with 0.5 and 0.1 M KOH, respectively. Fractions (0.5 mL) were analysed for carbohydrates, using the phenol–H_2SO_4 reagent (Dubois et al., 1956). The columns were calibrated with dextrins of known molecular weight as described (Bertoft, 1991a; Bertoft and Spoof, 1989).

3. Results and discussion

3.1. Isolation of α-dextrin fractions

Gelatinized WRS was hydrolysed with a diluted alpha-amylase solution (0.03 U/mL) and the reaction was

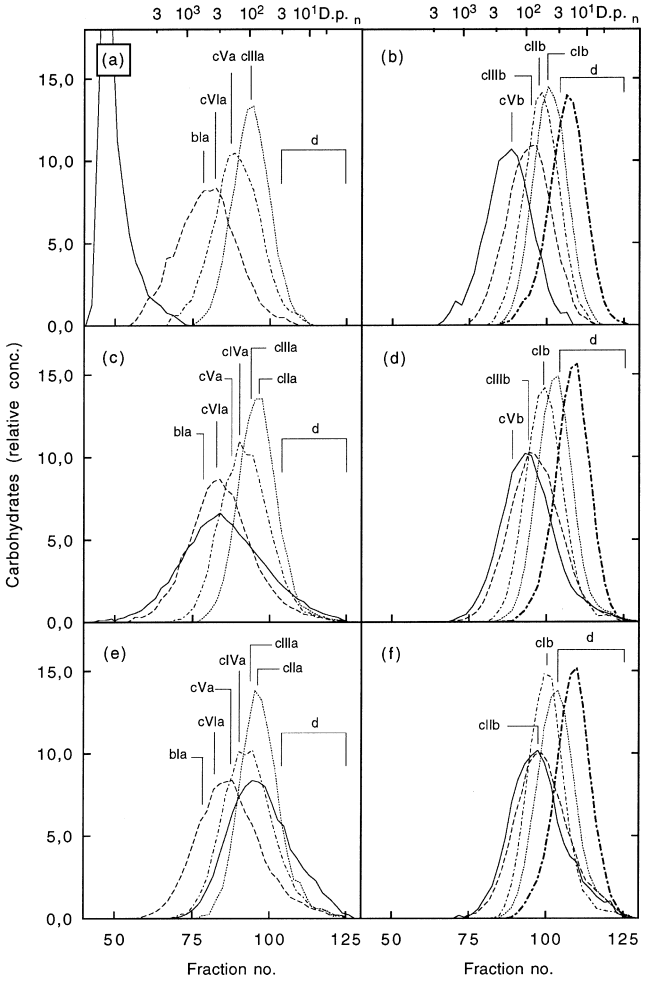

Fig. 2. Fractionation on Sepharose CL 6B of ϕ,β-LD before and after alpha-amylolysis: (a) and (b) the samples before treatment with alpha-amylase; (c) and (d) the hydrolysis mixtures after 1 h; (e) and (f) the hydrolysis mixtures after 5 h. In (a), (c) and (e): WRS (—), fractions 2.1 (––), 10.1 (-·-) and 10.2 (···). In (b), (d) and (f): fractions 7.2.2 (—), 3 (––), 11.1 (-·-), 11.2 (···) and 13 (-·-, bold), Groups of ϕ,β-LD are traced by bIa-d.

followed by gel-permeation chromatography on a column of Sepharose CL 6B. The hydrolysis was similar to that of the amylopectins from waxy-maize (Bertoft, 1989a) and potato (Zhu and Bertoft, 1996). Initially it was very fast and dextrins of all sizes were produced (Fig. 1a). All of the high-molecular-weight materials that eluted at the void volume of the gel had been hydrolysed into a broad range of intermediate dextrins with a d.p. from 20 to 5000 after 1 h. A group of low-molecular-weight materials (dI) that was produced simultaneously included mostly maltohexaose from the external chains of the amylopectin (Bertoft, 1989a; Robyt and French, 1963).

The material in fraction dI continued to increase at a nearly similar rate during the subsequent four hours of the reaction, whereas the hydrolysis rate for the larger intermediate dextrins decreased (Fig. 1b). At 3 h the chromatogram possessed a comparatively narrow distribution of dextrins with a peak around d.p. 100–200. After an additional 2-h hydrolysis the peak had only decreased to d.p. 80–100, showing that long internal chain segments no longer existed. A prolonged hydrolysis for 24 and 48 h showed that most of the materials obtained at 5 h were still hydrolysable and did not represent limit dextrins.

The 1- and 3-h mixtures were used for a preparative precipitation in methanol : water (5 : 1) giving two main samples that were without the low-molecular-weight material in fraction dI (not shown). Mixture I was obtained from the 1-h sample and mixture II from the 3-h sample in yields of 79% and 63%, respectively. This represented $\sim$ 90% of the dextrins with d.p. $>$ 20 in both alpha-amylolysis mixtures.

Mixtures I and II were then subjected to fractional precipitation with increasing methanol : water ratios giving fractions of different size-distributions of the intermediate α-dextrins. Some of these fractions were chosen for further studies. Fractions 2, 7, and 3 were from the 1-h hydrolysis mixture and fractions 10 and 11 were precipitates from the 3-h mixture. Fraction 13 was obtained from the supernatant of the latter after the methanol : water ratio had been increased to 5 : 1. Fractions 2 and 7 were further sub-fractionated to give 2.1 and 7.2.2, respectively, whereas fractions 10 and 11 were sub-fractionated into 10.1, 10.2, 11.1 and 11.2. The molecular-weight-distributions of these final preparations are shown in Fig. 1c and d, and the yields are given in Table 1.

3.2. Characterization of WRS and α-dextrin fractions

The average d.p. of the selected fractions from mixture I ranged from 122 to 451, and the selected precipitates from mixture II possessed d.p. from 56 to 163 (Table 1). Thus, the larger α-dextrins in the latter series were of similar sizes to the smaller dextrins of the former series. Fraction 13 contained low-molecular-weight dextrins with an average d.p. of 12. The average c.l. of the waxy-rice amylopectin was 16.8, which was slightly shorter than the earlier reported number average values of 18.9–21 (Akai et al., 1971; Hanashiro et al., 1996). The β-limit value of 54% was within the range found for amylopectins in general (Manners, 1989). The e.c.l. was calculated to be 11.0 and the i.c.l. was 4.8. Both values were shorter than those found for most other amylopectins (Manners, 1989) and suggested a highly branched structure of WRS.

As a result of the alpha-amylase attack, the c.l. of the isolated α-dextrins was lower (Table 1). After 1 h the c.l. had decreased to $\sim$13 and after 3 h to $\sim$9. As shown earlier with waxy-maize (Bertoft, 1989b) and potato amylopectin (Zhu and Bertoft, 1996), this was mainly caused by a shortening of the external chains, though the i.c.l. was also reduced. In fractions 11.1 and 11.2 it was only half of the original value.

The average number of chains (n.c.) was slightly higher than found earlier for comparable fractions from waxy-maize amylopectin (Bertoft, 1989b), which again suggested

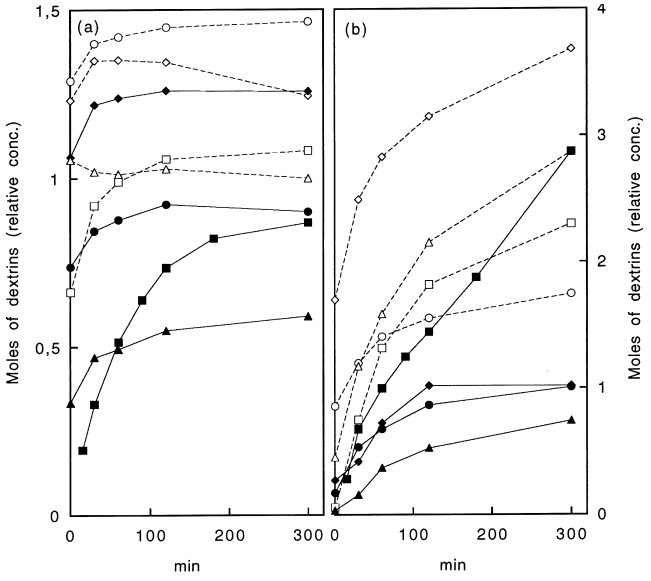

Fig. 3. Molar concentration of dextrins with (a) d.p. > 30 and (b) d.p. < 30 in alpha-amylolysis mixtures of ϕ,β-LD as a function of time; WRS (■), fractions 2.1 (▲), 10.1 (●), 10.2 (◆), 7.2.2 (□), 3 (△), 11.1 (○) and 11.2 (◇).

the WRS was more tightly branched. Fraction 13 possessed the highest β-limit value (63%), which was caused by the comparatively long external chain fragments and very short internal chains. The n.c. in this fraction was only 1.5. Because branched dextrins are composed of two or more chains, fraction 13 was a mixture of branched and linear dextrins and, thus, the e.c.l. and i.c.l. values represented the average of this mixture.

3.3. Preparation of ϕ,β-LD

Because the alpha-amylase of *B amyloliquefaciens* simultaneously attacks the external and internal chains, the positions in the gel-permeation chromatograms of any special group of intermediate α-dextrins are difficult to detect. If the external chains are removed, the intermediate dextrins possess more specified sizes at the different stages of the hydrolysis reaction (Bertoft, 1989a). ϕ,β-LDs were therefore prepared by successive phosphorolysis and beta-amylolysis of the WRS and the selected fractions of α-dextrins. The yields of these preparations ranged from 73% to 97% of the theoretically expected values, based on their ϕ,β-limit values, with the exception of fractions 11.2 and 13 that were obtained in lower yields (Table 2). The average d.p.-values were higher than those suggested from the ϕ,β-limit values, because some amount of the low-molecular-weight material was lost together with the glucose 1-phosphate and maltose during the preparation of the samples. Thus, fraction 13, that was obtained in a yield of only 40%, possessed a d.p.-value of 16, which was higher than the original d.p. of 12. As discussed before, this fraction also contained linear dextrins that were completely hydrolysed by the phosphorylase and beta-amylase.

3.4. Characterization of ϕ,β-LD by a second alpha-amylolysis

The molecular-weight-distributions of the fractions of ϕ,β-LD are shown in Fig. 2a and b. The WRS ϕ,β-LD was of high molecular weight and fractions 13–7.2.2 possessed nearly gaussian distributions. Fraction 2.1 possessed a double-peak at d.p. 350 and 500. The peak positions of the fractions indicated the average d.p. of groups of intermediate dextrins that originated from the WRS and they were named according to size as cI–bI in analogy with our earlier studies.

When the WRS ϕ,β-LD was treated with the alpha-amylase, the initial reaction was similar to the hydrolysis of the original WRS, with the exception that the low-molecular-weight fraction dI was not produced in the absence of the external chains (Fig. 2a, c and e). After 5 h, when the rate of the reaction was slow, the main products were around d.p. 80 together with smaller dextrins having a d.p. < 30 (designated d). Contrary to an earlier similar study on waxy-maize starch β-LD (Bertoft, 1989a), the positions of the peaks of intermediate hydrolysis products were difficult to establish. At some time intervals, the chromatograms possessed some peaks and shoulders, whereas in others they seemed to overlap each other, resulting in more smooth-shaped curves.

The ϕ,β-LD of fractions 2.1–13 were also treated a second time with the diluted alpha-amylase solution. Two types of hydrolysis patterns were found among the fractions, which were therefore divided into two main groups designated A and B. Peaks and shoulders that indicated the positions of characteristic groups of dextrins were given the postscripts a and b, respectively (Fig. 2).

The fractions containing large-molecular-weight dextrins from the original 1- and 3-h alpha-amylolysis mixtures (fractions 2.1, 10.1 and 10.2) belonged to group A. After 1 h of the second alpha-amylolysis, fraction 2.1 possessed a similar size-distribution as that obtained from the WRS ϕ,β-LD (Fig. 2c). During the subsequent hydrolysis it remained, however, much more resistant, so that at 5 h (Fig. 2e) the peak at d.p. 350 (dextrin cVIa) still remained as a major constituent together with a new peak found at d.p. 200 (dextrin cVa). In addition a weak shoulder at d.p. 80–100 was indicated, but only low amounts of the smaller dextrins (d) were formed. The composition of fraction 2.1 was thus obviously different to the overall composition of the amylopectin.

Fraction 10.1, that originally possessed a peak at d.p. 200 (cVa), was only slightly attacked by amylase. Two peaks at d.p. 150 and 100, corresponding to dextrins cIVa and cIIIa, respectively, were indicated in the chromatogram after 1 h. At 5 h a nearly identical chromatogram was obtained, except for a very small increase in the low-molecular-weight

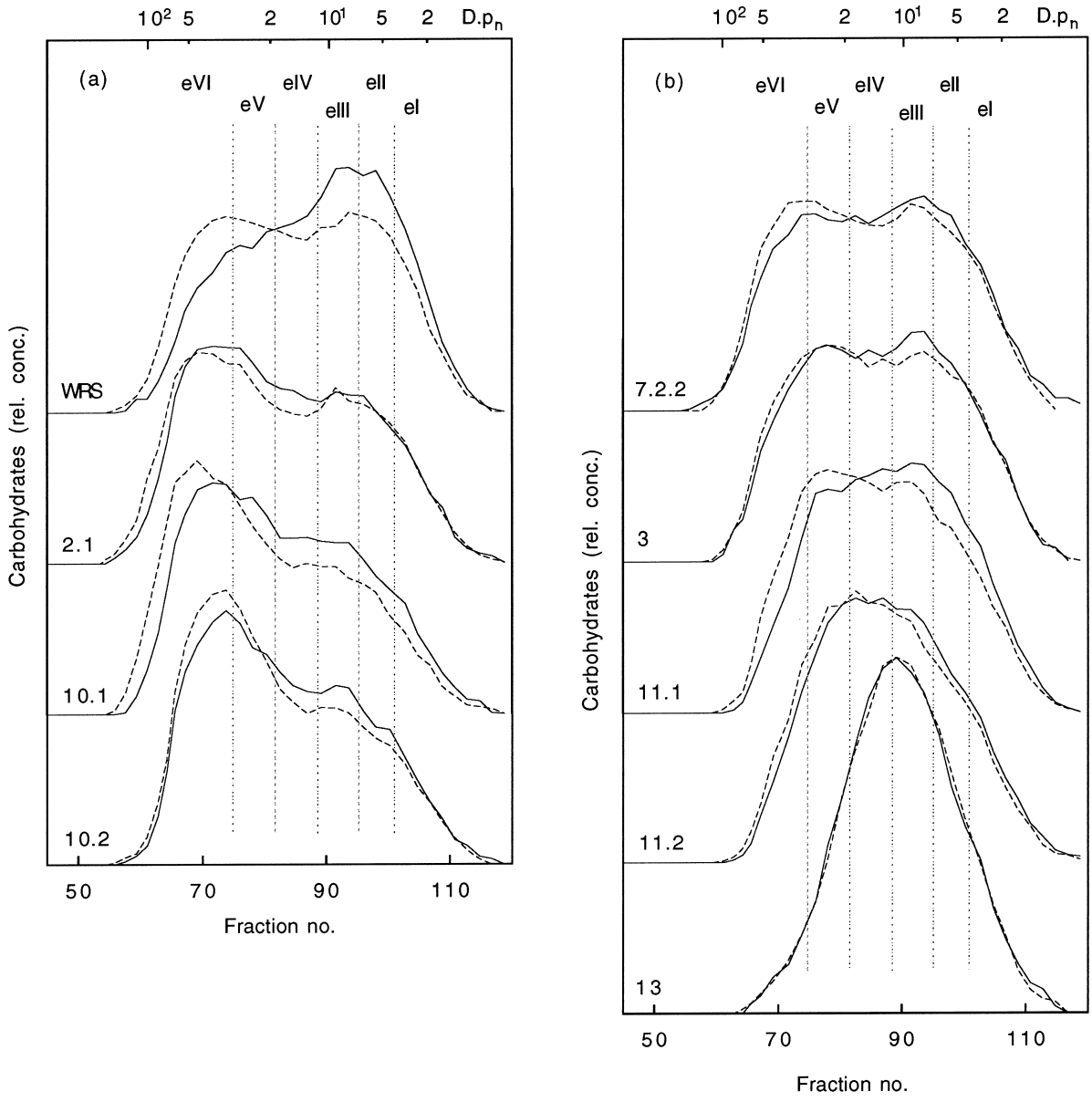

Fig. 4. Fractionation on Superdex 75 of hydrolysis mixtures of ϕ,β-LD after extensive alpha-amylolysis for 1 (--) and 3 h (—); (a) WRS and fractions possessing dextrin profiles of group A, (b) fractions possessing profiles of group B. The chromatograms were divided into groups of dextrins designated eI-eVI.

material. Fraction 10.2, which mainly contained dextrin cIIIa, was also only slowly attacked, resulting in a shift of the peak position to d.p. 75 (dextrin cIIa).

In Fig. 3 the changes in the hydrolysis mixtures on a molar basis are shown. The composition of fraction 2.1 was slowly changed up to 5 h, whereas that of fractions 10.1 and 10.2 remained practically unchanged after 2 h of hydrolysis, when dextrins cIIIa–cVa constituted the main products. Most probably, therefore, they represented unit clusters from the amylopectin.

The fractions that belonged to group B possessed a different hydrolysis pattern with peaks at slightly different positions than those of group A. Fraction 7.2.2 had its peak position at d.p. 175 (dextrin cVb, Fig. 2b) and was after 1 h (Fig. 2d) hydrolysed into dextrins of d.p. $\sim$ 90 (cIIIb) via a peak at d.p. 130 (dextrin cIVb, not shown). The further hydrolysis was slow, resulting in a shift of the peak to d.p. 65 (cIIb). On a molar basis there was no increase of dextrins with d.p. $>$ 30 after 2 h. The attack at these stages resulted mainly in the low-molecular-weight material (Fig. 3). Fraction 3, which possessed a peak around d.p. 80 (cIIIb), was only slowly attacked by the enzyme, giving an increase in the low-molecular-weight material and a shift of the peak position to d.p. $\sim$ 60 (cIIb). The molar amount of the dextrins with d.p. $>$ 30 was, however, nearly constant (Figs. 2 and 3). Fractions 11.1 and 11.2 were also comparatively resistant to the alpha-amylase attack. These fractions possessed peaks at d.p. 65 (cIIb) and 45 (cIb), respectively.

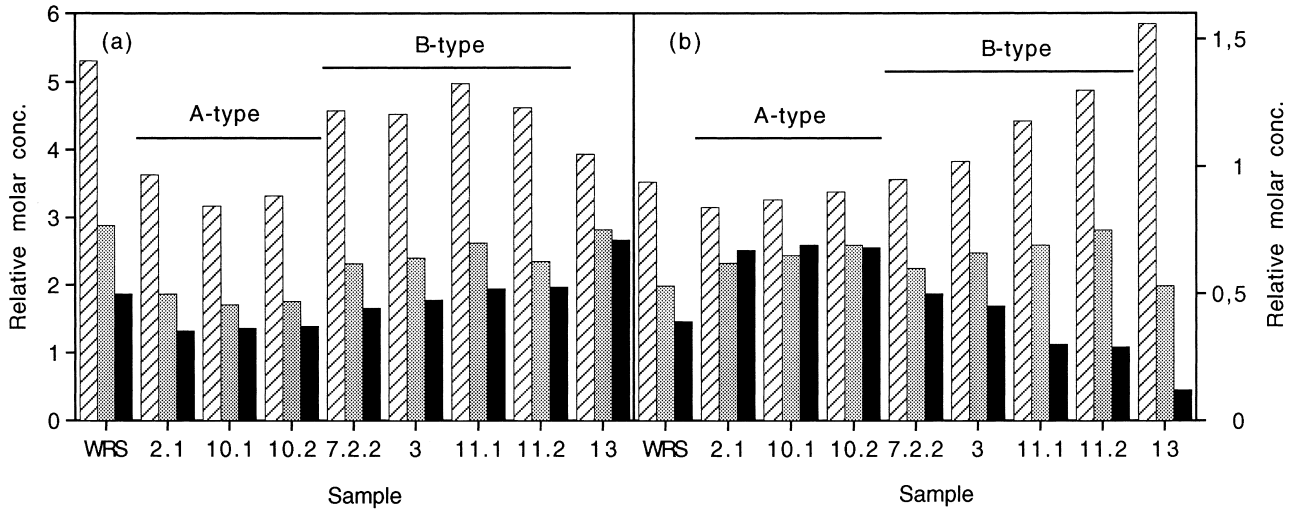

Fig. 5. Molar concentrations of groups of dextrins obtained from fractions of ϕ,β-LD after extensive alpha-amylolysis for 3 h: (a) dextrins eI (shaded bars), eII (grey bars) and eIII (black bars); (b) dextrins eIV (shaded bars), eV (grey bars) and eVI (black bars). Fractions possessing A- and B-type hydrolysis patterns are indicated.

A small shift to lower d.p. was the result of the production of the low-molecular-weight material. Fraction 13, finally, was the most resistant fraction (Fig. 2). Because of the resistance of the dextrins with d.p. $\leq$ 90, dextrin cIIIb seemed to represent the unit cluster of the fractions of group B. Table 2 summarizes the groups of dextrins found and their estimated peak positions.

3.5. Extensive amylolysis of ϕ,β-LD

To obtain a more complete picture of the composition of the fractions on the sub-cluster level, they were treated with a 100 × more concentrated alpha-amylase solution (3 U/mL). This resulted in an extensive hydrolysis into mixtures of dextrins which after 3 h were nearly resistant to further attack. The gel-permeation chromatograms were obtained from Superdex 75 (Fig. 4) and divided into six parts designated eI–eVI, corresponding to groups of dextrins with sizes up to d.p. $\sim$ 80. The products from these final stages of the alpha-amylolysis were tightly branched.

The smallest single-branched dextrin known to be produced by the alpha-amylase of *B. amyloliquefaciens* is 6^2-α-maltosyl-maltotriose (French et al., 1972; Hughes et al., 1963). Three types of single-branched hexaose dextrins were identified as products from WRS β-LD (Umeki and Yamamoto, 1972). Such dextrins were probably found within group eII (d.p. 5–6). The larger dextrins eIII–eVI (with d.p.-ranges 7–12, 13–19, 20–30, and $>$ 30, respectively) contained probably two or more branches. If the smallest branched dextrin has d.p. 5, the dextrins with d.p. $<$ 5 (eI) were linear and obtained by a repetitive attack on longer internal chains.

The branched dextrins eII–eVI could be regarded as small sub-cluster structures representing units of blocks

Table 3
Characterization of ϕ,β-limit dextrins by extensive α-amylolysis

Sample	Type of structure[a]	D.p. of whole mixture[b]	D.p. of blocks[c]	Number of blocks[d]	Density of blocks[e]
Amylopectin	Mixed	8.4	12.8	—	—
2.1	A	11.2	16.8	14.5	5.3
10.1	A	11.8	17.1	5.8	5.3
10.2	A	11.4	16.7	4.1	5.5
7.2.2	B	9.4	14.4	8.4	6.1
3	B	9.2	13.8	4.2	6.4
11.1	B	8.5	12.7	3.1	6.7
11.2	B	8.9	13.1	2.3	6.7
13	B (?)	8.6	11.5	1.2	7.7

[a] Based on the profiles of hydrolysis products.
[b] From gel-permeation chromatography on Superdex 75 (Fig. 4a and b).
[c] D.p. of branched blocks eII–eVI.
[d] Average number of branched blocks eII − eVI in the dextrins.
[e] (Number of blocks)/(d.p. of fraction) × 100.

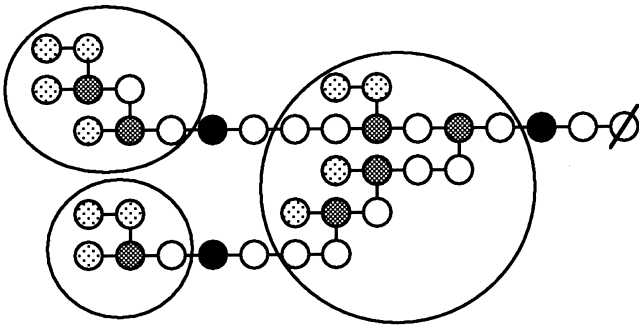

Fig. 6. Hypothetical ϕ,β-LD showing a unit cluster composed of the branched building blocks eII-eVI (encircled). Black circles represent D-glycosyl residues that bind to the building blocks, grey circles are residues involved in branches and dotted circles are residues in external chain stubs. The reducing unit is to the right. The internal chain segments between the blocks are eventually attacked a second time by alpha-amylase to give the short linear dextrins eI.

that build up the units of clusters in the amylopectin. The two main groups of fractions were clearly distinguished on the basis of their profiles of building blocks. All fractions of group A possessed very similar profiles with large amounts of dextrins eVI and eV (Fig. 4a). The fractions of group B possessed more narrow profiles with lower molecular weight. The profiles of fractions 7.2.2 and 3 showed two major groups with mostly eV and eIII, whereas eIV was enriched in fractions 11.1 and 11.2 (Fig. 4b). Fraction 13 possessed a narrow distribution with a single peak at d.p. ~ 12.

The WRS ϕ,β-LD, which represented the original macromolecule, showed a profile that was different from any of the isolated fractions. The small branched dextrins

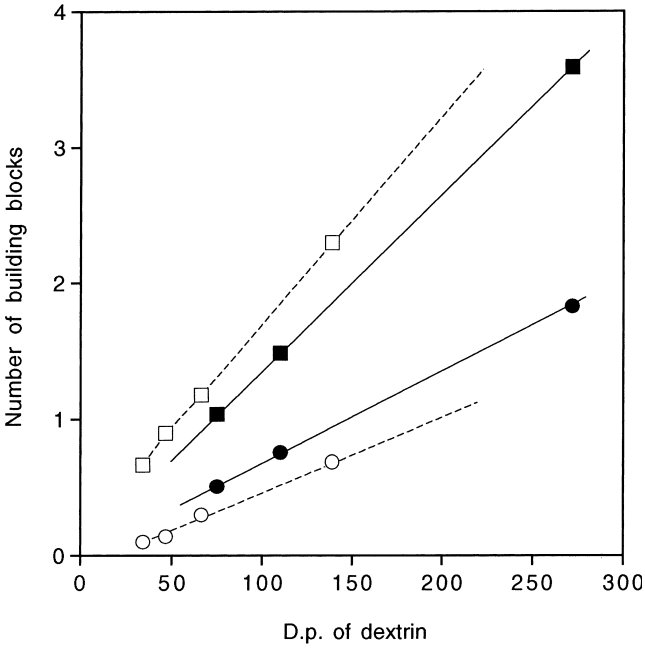

Fig. 7. ''Standard curves'' on the number of building blocks versus d.p. of ϕ,β-LD of type A fractions (black symbols) and type B fractions (white symbols): □, dextrin eIII; ○, dextrin eVI.

eII and eIII constituted the major products. Possibly, therefore, the amylopectin contained areas of only one or a few branches (''mini-cluster'') in addition to the larger (''true'') clusters.

The molar concentrations of dextrins eI–eVI are compared in Fig. 5. The WRS ϕ,β-LD gave rise to the highest amounts of the linear dextrins eI, showing that the amylopectin originally contained a lot of longer internal chains available for repetitive attack. These dextrins were also found in larger amounts in the samples of group B than in those of group A. Within each group, however, all fractions possessed approximately equal molar concentrations of eI. All samples of group A possessed also similar proportions of the branched building blocks eII–eVI. Within group B, dextrins eIII and eIV constituted a series of increased molar concentrations with decreasing average d.p., whereas that of eVI decreased.

Owing to the large production of eI, the WRS ϕ,β-LD possessed the lowest average d.p. of the mixtures of the extensive amylolysis (Table 3). The average d.p. of the branched building blocks (eII–eVI) in WRS ϕ,β-LD was 12.8, which was similar to the values found for samples 11.1 and 11.2 of group B. The larger dextrins in this group (samples 3 and 7.2.2) had blocks of d.p. ~ 14, whereas the d.p. of the blocks in group A was ~ 17. The average number of blocks in the ϕ,β-LD samples was calculated as (moles of branched blocks)/(moles of dextrins in the sample). The dextrins of sample 2.1 contained on the average 14.5 blocks (Table 3) and samples with lower d.p. possessed lower average number. Sample 13, which possibly belonged to group B, seemed to constitute mostly single building blocks with an average d.p. of 11.5.

The density of the branched blocks in the samples was defined as (the number of blocks)/(average d.p. of the sample) × 100. As illustrated in Fig. 6, this is the same as the proportion of D-glucosyl residues that bind a building block within a unit cluster. These residues should be found at the internal chains that were attacked during the extensive alpha-amylolysis. The density of building blocks was different in the two structural groups (Table 3). The samples of group A possessed similar densities with 5.3%–5.5% of the D-glucosyl residues being involved in the binding to a building block. The densities in group B were higher and increased with decreasing d.p. from 6.1% to 6.7%. Sample 13 had the highest density of 7.7%.

3.6. Composition of building blocks in structural units

The composition of building blocks within the individual groups of dextrins cIb–bIa was approximately estimated by construction of ''standard curves'', in which the number of each type of branched block was drawn versus the average d.p. of the samples. Examples of ''standard curves'' for dextrins eIII and eVI are shown in Fig. 7 and similar curves were constructed for the other blocks. In all cases the blocks belonging to the samples of group A possessed linear

Table 4
Number and types of building blocks in groups of dextrins produced by α-amylolysis of ϕ,β-limit dextrins

Group of dextrin	eVI	eV	eIV	eIII	eII	eI (linear)	eII-eVI (branched)	eI: (eII-eVI)
A-type								
bIa	3.7	3.3	4.5	7.2	10.4	38.3	29.1	1.32: 1
cVIa	2.3	2.1	2.9	4.5	6.5	24.0	18.3	1.32: 1
cVa	1.3	1.2	1.7	2.7	3.7	13.4	10.6	1.26: 1
cIVa	1.0	0.9	1.3	2.0	2.7	9.9	7.9	1.25: 1
cIIIa	0.7	0.7	0.8	1.4	1.7	6.3	5.3	1.19: 1
cIIa	0.5	0.5	0.7	1.0	1.3	4.7	4.0	1.18: 1
B-type								
cVb	0.9	1.1	1.7	2.9	4.1	15.6	10.7	1.46: 1
cIVb	0.6	0.8	1.3	2.1	3.0	11.3	7.8	1.45: 1
cIIIb	0.4	0.5	0.8	1.5	2.0	7.6	5.2	1.46: 1
cIIb	0.2	0.4	0.6	1.0	1.4	5.3	3.6	1.47: 1
cIb	0.2	0.3	0.5	0.9	1.1	4.1	3.0	1.37: 1

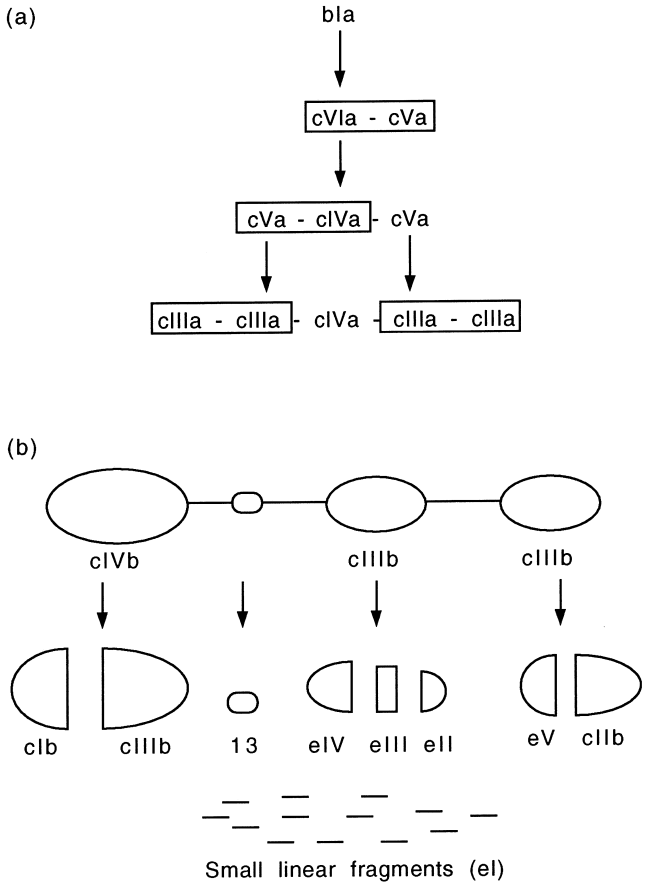

Fig. 8. Proposed model of the structural architecture of waxy-rice amylopectin. (a) The A-type structure is regularily built up to larger units from units of clusters of defined sizes (cIIIa, cIVa). Dextrin cVa is built up from two cIIIa, but it can also exist as a large unit cluster. Arrows trace the subpieces (in boxes) obtained from larger dextrins during initial stages of alpha-amylolysis. (b) The B-type structure contains unit clusters of less defined sizes (cIIIb, cIVb) irregularily combined into larger dextrins. Alpha-amylase attack (arrows) results in a range of dextrins, including small branched and linear fragments originating from inter-cluster segments. Attack occurs also at internal chains connecting the building blocks within the clusters, thereby slowly reducing their size (into cIb and cIIb).

relations that were different from those of group B. The d.p. given in Table 2 was then used to estimate the composition of building blocks in each group of dextrins (Table 4). Thus, the group of the large dextrin bIa, belonging to the A-type of dextrins found in sample 2.1, was built up of approximately four units of block eVI and three units of eV. The smaller blocks were found in increased number and in total bIa contained $\sim$ 29 branched blocks. The linear dextrins (eI) constituted $\sim$ 38 in number and the ratio of linear to branched pieces [eI : (eII–eVI)] was therefore 1.32 : 1. If, as suggested before, dextrins eI were obtained by a repetitive attack at internal chains between clusters or between the units of branched blocks within the clusters, this ratio showed that the repetitive attack occurred a little more than once on average.

The smaller groups of dextrins of the A-type constituted a series of lower ratio of linear to branched blocks (Table 4). The dextrins of the B-type had higher ratios ($\sim$ 1.46 : 1), which did not depend on their size (with the exception of cIb that possessed a slightly lower ratio of 1.37 : 1, though it remained higher than those found for the A-type dextrins).

Dextrin cIVa, which on the basis of the slow alpha-amylolysis rate (Figs. 2 and 3) represented a cluster of the A-type, was built up of $\sim$ 1 unit of eVI, eV and eIV, 2 of eIII and 3 of eII (Table 4). Dextrin cIIIa, also a unit cluster, could be combined with another similar unit to build up a structure with the size and composition equal to dextrin cVa. Thus, the latter dextrin would be built up of two units of clusters. The slow alpha-amylolysis suggested, however, that there also existed unit clusters of the size of cVa. Dextrins cIVa and cVa could further be combined into dextrin cVIa, to which an additional cVa could be bound giving bIa. Thus, it was possible to describe the structure of type A dextrins as the regular composition of units of clusters drawn in Fig. 8a, which is analogous to those suggested for other amylopectins (Bertoft, 1991b; Bertoft et al., 1993b; Bertoft and Åvall, 1992).

A regular structure for the B-type dextrins was more difficult to define, and possibly it did not exist. The model

drawn in Fig. 8b suggests that the units of clusters were of several sizes with structures preferentially built up like dextrins cIIIb and cIVb (Table 4), in which the building blocks eIV and eV constitute the framework. Some clusters are interconnected through bridges of very small branched blocks giving rise to the small dextrins in fraction 13 upon alpha-amylase attack. The enzyme could also attack internal chains within the clusters, thereby partly releasing building blocks of larger or smaller sizes, which slowly should reduce the size of the remaining clusters, as the events in Fig. 2 suggested. Some of these released parts were possibly isolated in fractions 11.1 and 11.2, whereas other parts were found in fractions not analysed further and/or lost during the preparation of the fractions.

3.7. Domain structure hypothesis

The existence of structurally different dextrins within the WRS sample was unexpected. Their origin is not known, but it is tempting to suggest that the amylopectin was built up of either intra- or intermolecular domains of different structures. A speculative drawing of their origin and isolation after the alpha-amylolysis is shown in Fig. 9. Starch granules are well known to be built up of alternating amorphous and semi-crystalline shells, commonly called ‘‘growth rings’’. The semi-crystalline shell is further organized into amorphous and crystalline lamellae, in which the amylopectin contributes to the principal architecture (Jenkins and Donald, 1995; Oostergetel and Bruggen, 1993). The semi-crystalline shells are also organized into blocklet structures (Gallant et al., 1992) and recently a skeletal structure was reported in wheat starch granules (Seguchi and Kanenaga, 1997). Because waxy starches also possess growth rings (Manners, 1989), it is clear that the amylopectin fraction participates in both types of structures. Possibly, different types of fine structures organized as domains provide a basis for the physically different forms that occur within the granules. This, in turn, suggests that different forms of the starch synthesizing enzymes (Smith et al., 1997) could be periodically active during their development.

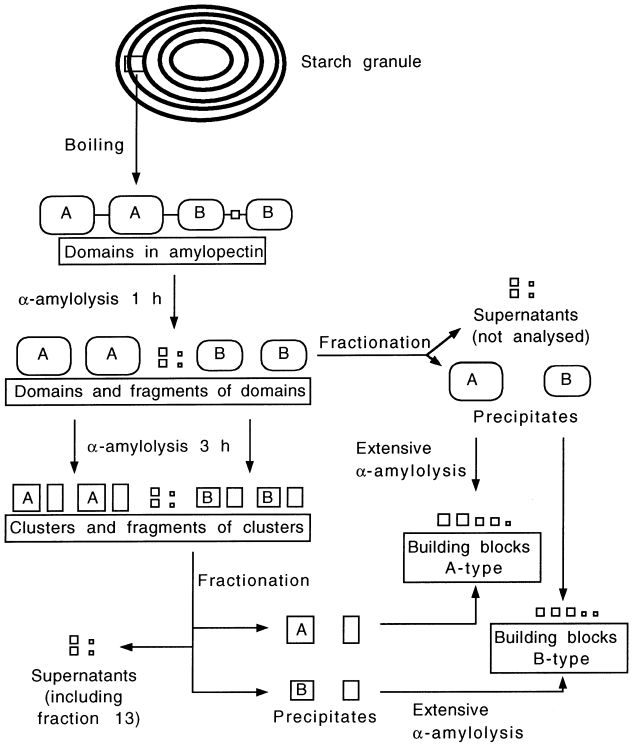

Fig. 9. Speculative drawing that traces different domains in the amylopectin to growth rings of alternating amorphous and semi-crystalline regions in the starch granule. The domains exist as either intra- or intermolecular structures. Initial alpha-amylolysis results in dextrins composed of larger A-domains and smaller B-domains, which are, more slowly, hydrolysed further into units of clusters and their fragments. The products of the hydrolysis are isolated by fractional precipitation in methanol according to size and domain origin and their compositions of building blocks are finally analysed by extensive amylolysis.

Whether starches of other sources also are organized in domains, or whether it is a unique property of waxy-rice, is not known. We proposed earlier, however, that the amylopectin of potato starch possesses phosphorylated domains (Zhu and Bertoft, 1996). The composition of unit chains in the fractions of the WRS is now being investigated.

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References

Akai, H., Yokobayashi, K., Misaki, A., & Harada, T. (1971). Structural analysis of amylopectin using *Pseudomonas* isoamylas. *Biochim. Biophys. Acta*, *252*, 427–431.

Bertoft, E. (1989). Investigation of the fine structure of amylopectin using alpha- and beta-amylase. *Carbohydr. Res.*, *189*, 195–207.

Bertoft, E. (1989). Partial characterization of amylopectin alpha-dextrins. *Carbohydr. Res.*, *189*, 181–193.

Bertoft, E. (1991). Chains of intermediate lengths in waxy-maize amylopectin. *Carbohydr. Res.*, *212*, 245–251.

Bertoft, E. (1991). Investigation of the fine structure of alpha-dextrins derived from amylopectin and their relation to the structure of waxy-maize starch. *Carbohydr. Res.*, *212*, 229–244.

Bertoft, E., Manelius, R., & Qin, Z. (1993). Studies on the structure of pea starches. Part 1: Initial stages in α -amylolysis of granular smooth pea starch. *Starch/Stärke*, *45*, 215–220.

Bertoft, E., Qin, Z., & Manelius, R. (1993). Studies on the structure of pea starches. Part 3: Amylopectin of smooth pea starch. *Starch/Stärke*, *45*, 377–382.

Bertoft, E., & Spoof, L. (1989). Fractional precipitation of amylopectin alpha-dextrins using methanol. *Carbohydr. Res.*, *189*, 169–180.

Bertoft, E., & Åvall, A. -K. (1992). Structural analysis on the amylopectin of waxy-barley large starch granules. *J. Inst. Brew. London*, *98*, 433–437.

Beutler, H. -O. (1984). Maltose. *Methods Enzyme Anal.*, *6*, 119–126.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F.

(1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem., 28*, 350–356.

French, D., Smith, E. E., & Whelan, W. J. (1972). The structural analysis and enzymic synthesis of a pentasaccharide alpha-limit dextrin formed from amylopectin by *Bacillus subtilis* alpha-amylase. *Carbohydr. Res., 22*, 123–134.

Gallant, D. J., Bouchet, B., Buléon, A., & Pérez, S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. *Eur. J. Clin. Nutr., 46*, S3–S16.

Hanashiro, I., Abe, J. -i., & Hizukuri, S. (1996). A periodic distribution of chain length of amylopectin as revealed by high-performance anion-exchange chromatography. *Carbohydr. Res., 283*, 151–159.

Hizukuri, S. (1985). Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules. *Carbohydr. Res., 141*, 295–306.

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins and its significance. *Carbohydr. Res., 147*, 342–347.

Hughes, R. C., Smith, E. E., & Whelan, W. J. (1963). Structure of a pentasaccharide α-limit dextrin formed from amylopectin by Bacillus subtilis α-amylase. *Biochem. J., 88*, 63p–64p.

Jane, J. -l., Kasemsuwan, T., Leas, S., Zobel, H., & Robyt, J. F. (1994). Anthology of starch granule morphology by scanning electron microscopy. *Starch/Stärke, 46*, 121–129.

Jenkins, P. J., & Donald, A. M. (1995). The influence of amylose on starch granule structure. *Int. J. Biol. Macromol., 17*, 315–321.

Manners, D. J. (1989). Recent developments in our understanding of amylopectin structure. *Carbohydr. Polym., 11*, 87–112.

Morrison, W. R., Milligan, T. P., & Azudin, M. N. (1984). Relationship between the amylose and lipid contents of starches from diploid cereals. *J. Cereal Sci., 2*, 257–271.

Oostergetel, G. T., & Bruggen, E. F. J. v. (1993). The cyrstalline domains in potato starch granules are arranged in a helical fashion. *Carbohydr. Polym., 21*, 7–12.

Robyt, J., & French, D. (1963). Action pattern and specificity of an amylase from Bacillus subtilis. *Arch. Biochem. Biophys., 100*, 451–467.

Seguchi, M., & Kanenaga, K. (1997). Study of the three-dimensional structure of wheat starch granules stained with remazolbrilliant blue dye and extracted with aqueous sodium dodecyl sulfate and mercaptoethanol solution. *Cereal Chem., 74*, 548–552.

Smith, A. M., Denyer, K., & Martin, C. (1997). The synthesis of the starch granule. *Annu. Rev. Plant Physiol. Plant Mol. Biol., 48*, 67–87.

Thoma, J. A., Brothers, C., & Spradlin, J. (1970). Subsite mapping of enzymes. Studies on *Bacillus subtilis* amylase. *Biochemistry, 9*, 1768–1775.

Umeki, K., & Yamamoto, T. (1972). Enzymatic determination of structure of singly branched hexaose dextrins formed by liquefying α -amylase of *Bacillus subtilis*. *J. Biochem., 72*, 101–109.

Waffenschmidt, S., & Jaenicke, L. (1987). Assay of reducing sugars in the nanomole range with 2,2′-bicinchoninate. *Anal. Biochem., 165*, 337–340.

Zhu, Q., & Bertoft, E. (1996). Composition and structural analysis of alpha-dextrins from potato amylopectin. *Carbohydr. Res., 288*, 155–174.