

# Observations on the $\alpha$ -Amylolysis Pattern of Some Waxy Maize Starches from Inbred Line Ia453

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## ABSTRACT

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Maize starches of the endosperm mutants *waxy* (*wx*), *dull:waxy* (*duwx*), and *amylose-extender:dull:waxy* (*aeduwx*) from inbred line Ia453 lack amylose. However, in addition to high molecular weight (HMW) amylopectin, the *duwx* and *aeduwx* starches contained 40 and 80%, respectively, intermediate branched material of low molecular weight (LMW). As gelatinized, the amylopectin of the *wx* starch was easily hydrolyzed into small dextrans by the  $\alpha$ -amylase of *B. amyloliquefaciens*, but components of *duwx* and *aeduwx* possessed partial resistance to amylolytic attack. Residual material of intermediate size obtained by a 4-hr  $\alpha$ -amylolysis could

not be separated from LMW dextrans by fractional precipitation in methanol. It is suggested that this material possessed a more regularly branched structure, in which the D-glucosyl chain segments were too short to allow  $\alpha$ -amylase action. The granular starches of *duwx* and *aeduwx* genotypes were initially considerably more resistant than the *wx* sample to  $\alpha$ -amylase attack. This was possibly due to an altered structure in the amylopectin component or the high content of intermediate material in the former granules.

The structure of starch granules and their components is of major importance for the functionality of the starch. Plants of different mutant genotypes possess starch with unique properties (Yuan et al 1993, Yuan and Thompson 1998, Klucinec and Thompson 1999, Villwock et al 1999) of interest in industrial applications. The altered structure of the starch components affected by different mutations is also of interest for the understanding of the very complex biosynthesis (Ball 1995, Martin and Smith 1995) and structure of the starch granules (Gallant et al 1997, Waigh et al 1999). Considerable work on starches from different mutant plants has therefore been performed through the years, though details of the fine structure of the starch components remain incomplete.

A range of maize endosperm mutations is known. The *waxy* (*wx*) mutation results in a starch composed solely of amylopectin, whereas *amylose-extender* (*ae*) gives an increased amylose content (Boyer and Liu 1985, Ikawa et al 1978) and longer average chain lengths in the amylopectin component (Ikawa et al 1978, Baba and Arai 1984, Yun and Matheson 1993). In addition, the *ae* mutation is associated with the synthesis of intermediate branched polyglucans with amylopectin-like chains, though with a clearly different chain length distribution and a low molecular weight and, thus, a different fine structure (Wang et al 1993a,b). Intermediate material is also found in other mutants such as *dull* (*du*), and in double combinations of the mutations (Wang et al 1993a,b). The exact structure of the intermediate material, which remains uncertain, seems to be dependent not only on the type of mutation, but also on the inbred line of the maize plant. Thus, the *wx* mutation in combination with other mutations within the inbred line Oh43 was reported to be free from intermediate material (Wang et al 1993b). However, several other inbred lines, among which inbred line Ia453 was included, possessed intermediate material in combinations with the *wx* mutation (Boyer and Liu 1985).

Intermediate material was included in the supernatant fraction together with the amylopectin component after precipitation of amylose in 1-butanol (Wang et al 1993a). Recently, Klucinec and Thompson (1998) were able to separate intermediate material from amylopectin in *ae* and *aedu* starches by precipitation of the former in a mixture of 1-butanol and isoamyl alcohol. They suggested that the intermediate material had a branched structure sim-

ilar to that of amylopectin, but with structural features (including more long chains) that resulted in altered physical behavior.

The genetic background also affects the susceptibility of starch granules to the attack by  $\alpha$ -amylases and glucoamylases. The reason for this is partly explained by the different types of crystallites that are formed by the starch components (Planchot et al 1997, Williamson et al 1992). The *ae* mutation results in granules of the B-crystalline type (Jane et al 1999), and some of the amylose is also complexed with lipids as V-crystallites (Shi et al 1998). These granules are more resistant to enzymatic attack compared with their normal counterparts (Gallant et al 1972, Knutson et al 1982). Among a range of starches from different genotypes and inbred lines, the *sugary* (*su*) mutation, which also gives an increased amylose content, possessed the best susceptibility to enzymatic attack (Fuwa et al 1978a,b). The majority of several double- and triple-mutations, including the *wx* genotype (*aewx*, *duwx*, and *aeduwx*), within the maize inbred Oh43 also possessed granules that were better digested by glucoamylase than the nonmutant starch (Fuwa et al 1979).

In this work, we studied the attack by bacterial  $\alpha$ -amylase on three *waxy* starches, two containing both amylopectin and intermediate material, and all without amylose, from the inbred maize Ia453. The hydrolysis of the gelatinized starches was followed by gel-permeation chromatography. The initial stages of the digestion of granular starches were followed by pumping the enzyme through a thin bed of the granules in a column. Mixtures of dextrans that were solubilized from the granules at different stages were collected and partly characterized.

## MATERIALS AND METHODS

### Starch and Enzymes

Maize of inbred line Ia453 with endosperm mutations *waxy* (*wx*), *dull:waxy* (*duwx*), and *amylose-extender:dull:waxy* (*aeduwx*) were used. The plants were grown at the Rock Springs Agricultural Farm at The Pennsylvania State University in 1989 and 1992. Equal amounts of mature kernels were pooled from 10 ears grown in each year, and the starch granules were isolated as described previously (Boyer and Liu 1985, Boyer et al 1976). The  $\alpha$ -amylase of *Bacillus amyloliquefaciens* (Boehringer-Mannheim) had an activity of 475 U/mg when measured in 0.01M NaOAc buffer, pH 6.5, at 25°C, using soluble starch (Merck) as substrate (5 mg/mL). The activities of sweet potato  $\beta$ -amylase (Boehringer-Mannheim) and isoamylase of *Pseudomonas amyloclavata* (Hayashibara Shoji) were 2,500 U/mL and 655,000 U/mL, respectively, according to the suppliers. The  $\beta$ -amylase preparation contained a low activity of  $\alpha$ -glucosidase that, on prolonged incubation, transformed a

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minor part of maltose into glucose. This did not, however, affect the apparent result because glucose was not resolved from maltose in the gel-permeation chromatograms.

### Debranching of Starch Samples

Starch granules were dissolved on a boiling water bath in 90% (v/v) dimethylsulfoxide (DMSO) to a concentration of 50 mg/mL and then diluted with water to 10 mg/mL. An aliquot (0.9 mL) was diluted with 0.1M NaOAc buffer, pH 3.5 (0.1 mL), and treated with diluted (10×) isoamylase (2  $\mu$ L) for 5 hr at room temperature. The sample was then heated on a boiling water bath for 10 min to inactivate the enzyme. An aliquot (300  $\mu$ L) was diluted with an equal volume of water and then analyzed by gel-permeation chromatography on Superdex 75. The pH was adjusted to 4.8 with 0.2M NaOAc in another aliquot (300  $\mu$ L), and the volume was adjusted to 600  $\mu$ L with 0.1M NaOAc buffer, pH 4.8, before treatment with  $\beta$ -amylase (10  $\mu$ L) at room temperature overnight. Only maltose was found by gel-permeation chromatography in these control samples, which showed that the debranching was complete.

An aliquot (300  $\mu$ L) of the original starch sample was also diluted twice in the NaOAc buffer, pH 4.8, and treated with  $\beta$ -amylase without debranching with isoamylase.

### $\alpha$ -Amylolysis of Gelatinized Starch

Starch granules were dissolved in DMSO as above, then diluted in 0.01M NaOAc buffer, pH 6.5, and treated at 25°C with  $\alpha$ -amylase using a final concentration of 0.03 U/mL and 10 mg of substrate/mL. Reaction was stopped at intervals in small aliquots (1 mL) with 5M KOH (0.1 mL) and analyzed by gel-permeation chromatography on Sepharose CL 2B and Sepharose CL 6B.

### Fractionation of $\alpha$ -Dextrins in Methanol

$\alpha$ -Amylolysis was also made in large batches under conditions identical to those above but using 10 g of starch granules. The reaction was stopped after 4 hr and the intermediate  $\alpha$ -dextrins were precipitated with 5 volumes of methanol, washed with methanol and then acetone, and air-dried. The dextrins were then fractionated into series of precipitates using increasing methanol concentrations as described by Bertoft and Spoof (1989). The size distribution of the dextrins was analyzed on Sepharose CL 6B.

### $\alpha$ -Amylolysis of Starch Granules

Starch granules (2.5 g) were digested with a diluted  $\alpha$ -amylase solution (0.002 U/mL) by a method described earlier (Bertoft et al 1993a) that prevented a secondary hydrolysis of the solubilized dextrins in solution, thus making it possible to analyze the com-

position of the dextrans that were originally solubilized from the granules. Briefly, the granules were packed as a thin layer ( $\approx$ 2 mm) into the lower compartment of a special column (4.6 cm diam.) and the enzyme in NaOAc buffer, pH 6.5, was pumped (0.33 mL/min) through the starch at 25°C. The enzyme was then trapped onto an anion-exchanger (DEAE-Sepharose fast flow, Pharmacia) in the upper compartment of the column, whereas the solubilized dextrans were collected in fractions (18 mL). The rate of the hydrolysis was followed by analyses of the total carbohydrate content (Dubois et al 1956) in the fractions. Those representing 1% (25 mg) intervals (0–1%, 1–2%, etc.) were pooled and concentrated to 10 mg/mL. The buffer salts were removed by gel-permeation chromatography in two steps on a large column (2.5  $\times$  35 cm) and then a small column (1.4  $\times$  6 cm) of Sephadex G-10 (Pharmacia). Approximately 2% of the total carbohydrate content was lost in this step (mostly maltose). The samples were then concentrated to 5 mg/mL and to an aliquot (0.55 mL) was added 0.1M NaOAc buffer, pH 4.8, (0.05 mL) and  $\beta$ -amylase (10  $\mu$ L). The reaction took place at room

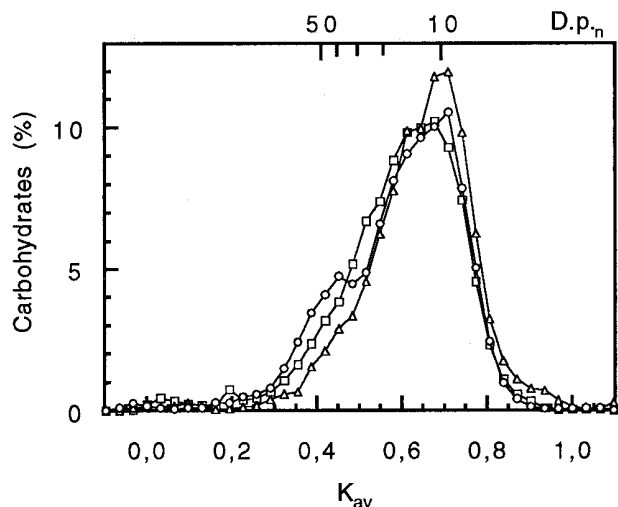


Fig. 1. Chain length distribution on Superdex 75 of isoamylase debranched wx (○), duwx (Δ), and aeduw (□) maize starches from inbred line Ia453.

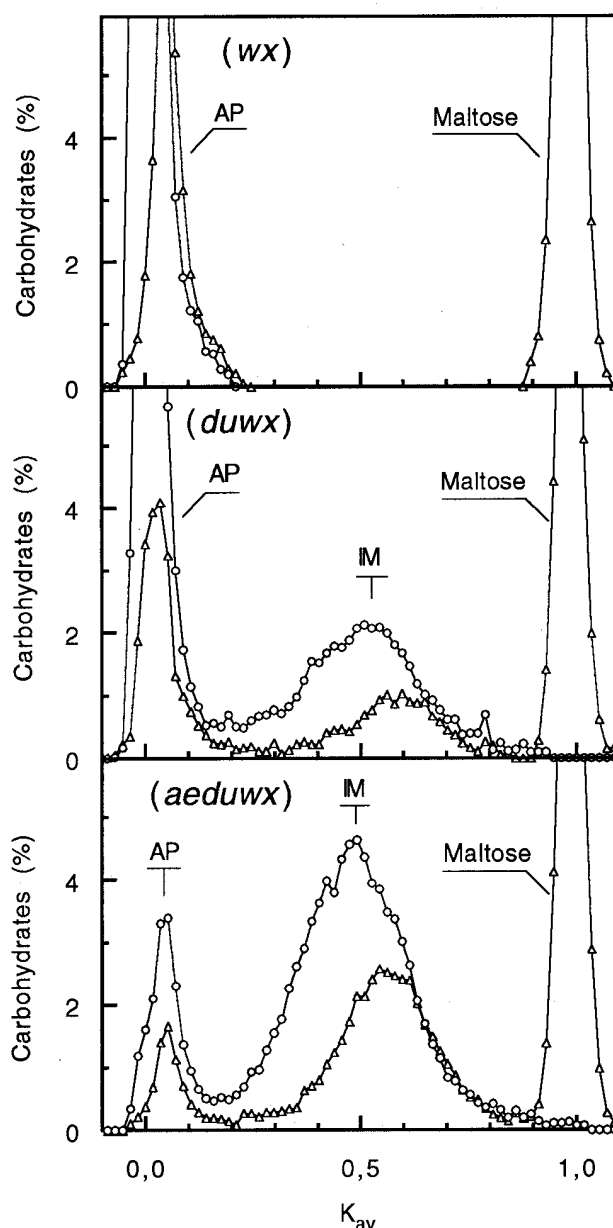


Fig. 2. Fractionation on Sepharose CL 2B of maize starches from inbred line Ia453 before (○) and after  $\beta$ -amylolysis (Δ) showing peaks for amylopectin (AP) and intermediate material (IM).

temperature overnight and was stopped by boiling in a water bath. The molecular weight distribution in the fractions before and after  $\beta$ -amylolysis was analyzed on Superdex 75.

### Gel-Permeation Chromatography

Three different gel-permeation matrices (Pharmacia) were used for analysis of molecular weight distribution. The column (1  $\times$  90 cm) of Superdex 75 was eluted at 0.5 mL/min with 0.1M KOH, the column (1  $\times$  90 cm) of Sepharose CL 6B at 0.5 mL/min with 0.5M KOH, and the column (1.5  $\times$  90 cm) of Sepharose CL 2B at 0.25 mL/min with 0.1M KOH. The samples (enzymatically treated or native starches dissolved in DMSO) were diluted to  $\approx$ 2 mg of carbohydrates/mL in  $\approx$ 0.5M KOH. Aliquots of 0.2 or 0.5 mL were applied on Superdex 75 and Sepharose CL 6B or on Sepharose CL 2B, respectively. Fractions (0.5 or 1 mL) were analyzed for carbohydrates with the phenol-sulfuric acid reagent (Dubois et al 1956). The columns with Superdex 75 and Sepharose CL 6B were calibrated with dextrans of known degree of polymerization (DP) as described previously (Bertoft 1991, Bertoft and Spooft 1989).

## RESULTS AND DISCUSSION

### Characterization of Waxy Starch Samples

Composition of unit chains of the three waxy mutant starches of the inbred line Ia453 was analyzed after debranching on a column of Superdex 75 (Fig. 1). The *wx* starch possessed a bimodal distribution with a group of long chains with DP  $\approx$ 40-50 and a major group of short chains with DP 10-20. The double mutant *duwx* sample had fewer long chains, which was in agreement with earlier findings (Yuan et al 1993, Wang et al 1993b, Fuwa et al 1987, Jane et al 1999). The single *ae* mutation was reported to strongly increase the proportion of long chains in both the amylopectin component and in the intermediate material (Wang et al 1993a). This was also the case in the combination with the *du* (Wang et al 1993a) or the *wx* mutation (Yuan et al 1993, Fuwa et al 1987, Jane et al 1999). However, in the triple mutant *aeduw*x sample, an increased amount of chains with DP >20 was found when compared to *duwx*, but the proportion of long chains was much lower than in the *wx* starch (Fig. 1). Thus, it seemed that the combination of the *du* and *wx* mutations counteracted the effect of the mutation at the *ae* locus.

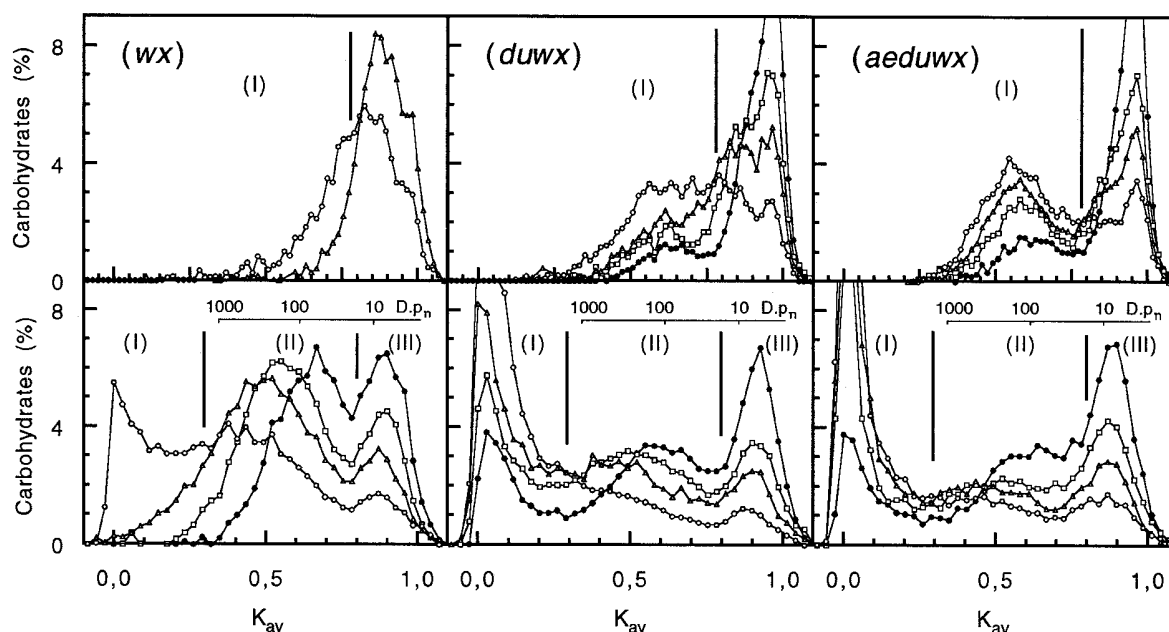
Long chains typical for amylose were absent in all three samples.

The molecular weight distributions of the starches were analyzed on a column of Sepharose CL 2B (Fig. 2). The material of the *wx* mutant eluted completely at the void volume of the gel, suggesting a typical HMW amylopectin. The *duwx* and *aeduw*x samples possessed a LMW material in addition to material at the void volume. In normal maize starch, such material is typically amylose (Boyer and Liu 1985). Wang et al (1993b) analyzed a series of mutant maize starches from inbred line Oh43, in which branched material with molecular weights similar to amylose also was found. They therefore characterized this component as an intermediate material. However, all waxy starches that they analyzed were free of the intermediate component (Wang et al 1993b). In this investigation,  $\approx$ 40% of the starch material was intermediate in the *duwx* mutant of the inbred line Ia453, whereas the major part (80%) of the *aeduw*x starch was of this type (Table I).

The  $\beta$ -amylolysis limit of the *wx* sample was 57% (Table I), which was within the typical range for amylopectins (Manners 1989). The *duwx* starch possessed a slightly higher limit value, suggesting longer external chain segments, whereas the *aeduw*x starch had a lower limit. From the reduction in the areas of the peaks in the gel-permeation chromatogram (Fig. 2), the  $\beta$ -limit value of the amylopectin component of *aeduw*x was estimated to 60% and that of the intermediate material was 50%. It therefore appeared that the latter material, which was the major component in the sample, possessed comparatively short external chain segments. In the *duwx* sample, both components possessed similar, increased limit values.

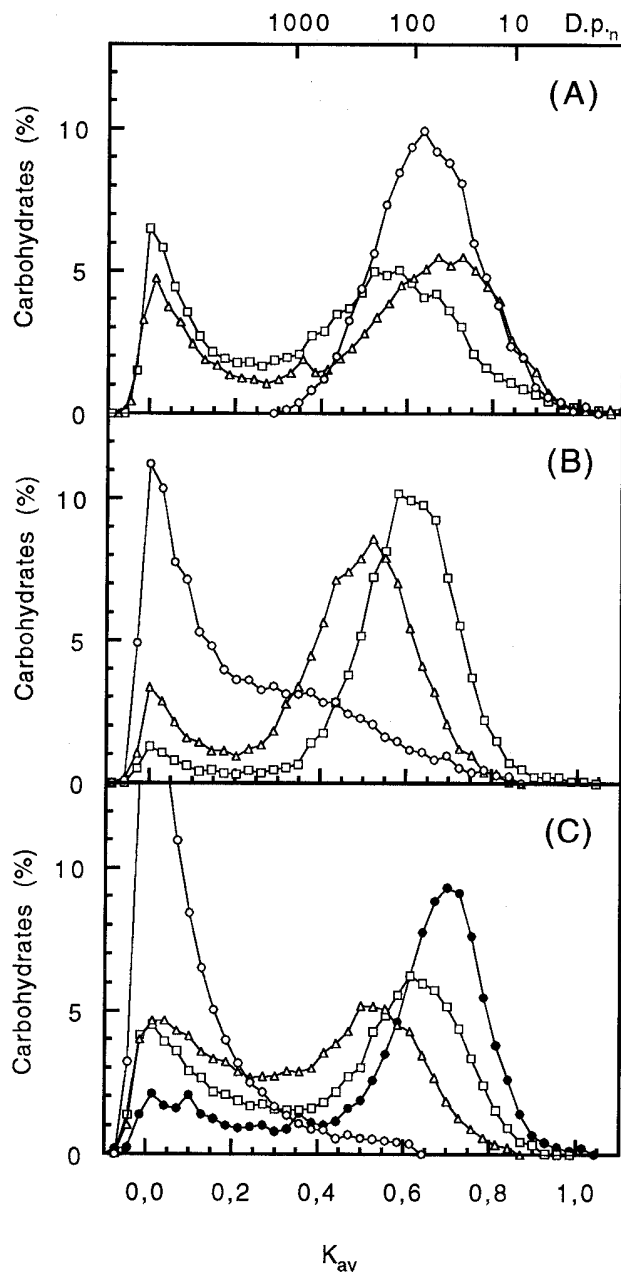
### $\alpha$ -Amylolytic of Gelatinized Starch

The hydrolysis of gelatinized *wx* starch with the  $\alpha$ -amylase of *B. amyloliquefaciens* was followed by gel-permeation chromatography on both Sepharose CL 2B and Sepharose CL 6B (Fig. 3). The column with the latter gel was calibrated with branched dextrans (Bertoft and Spooft 1989) and divided into three fractions representing (I) DP > 1,500, (II) DP 1,500-20, and (III) DP < 20. Dextrans included in fraction I were resolved on the Sepharose CL 2B column. All of the amylopectin, that originally eluted at the void volume of the Sepharose CL 2B gel, was already degraded into smaller dextrans that mostly were included into the Sepharose CL 6B gel within 0.5 hr of treatment. At 1 hr of hydrolysis, all dextrans



**Fig. 3.** Fractionation on Sepharose CL 2B (upper panel) and Sepharose CL 6B (lower panel) of the hydrolysates obtained after the action of  $\alpha$ -amylase on gelatinized maize starches from inbred line Ia453 for 0.5 ( $\circ$ ), 1.0 ( $\Delta$ ), 1.5 ( $\square$ ), and 4.0 hr ( $\bullet$ ). Chromatograms were divided into fraction I (DP > 1,500), II (DP 1,500-20), and III (DP < 20).

were included into the latter gel and the reaction rate decreased, which indicated that long internal chain segments that are easily attacked by the enzyme (Robyt and French 1963) were no longer present. At this stage, the intermediate dextrans mostly represent individual clusters or groups of clusters from the amylopectin (Bertoft 1989, Bertoft et al 1999). Simultaneously, with dextrans of intermediate sizes, a group of LMW dextrans was produced (fraction III). This contains mostly maltohexaose, which is independently produced by attack at the external chains (Bertoft 1989). At 4 hr, the reaction rate was slow and the hydrolysis mixture therefore contained comparatively resistant dextrans completely included in fractions II and III. Overall, the reaction was very similar to that obtained earlier with waxy-maize starch (Bertoft 1989) and with amylopectins from waxy-barley (Bertoft and Åvall 1992), waxy-rice (Bertoft et al 1999), and potato (Zhu and Bertoft 1996).



**Fig. 4.** Fractionation on Sepharose CL 6B of (A) dextrins precipitated by 5 volumes of methanol from a 4-hr  $\alpha$ -amylolysis mixture of *wx* (○), *duwx* (△), and *aeduw* (□) maize starch samples; (B) dextrins obtained by subfractionation of *duwx* sample with methanol-water ratios of 0.9:1 (○), 1.3:1 (△), and 3.5:1 (□); (C) dextrins obtained from *aeduw* sample with methanol-water ratios of 0.5:1 (○), 1.3:1 (△), 2.5:1 (□), and 3.5:1 (●).

The *duwx* and *aeduw* starches were different from the *wx* sample. Though the HMW amylopectin component was hydrolyzed rapidly, it appeared that the intermediate component in both samples was only slowly attacked (Fig. 3). After the initial 1 hr of hydrolysis,  $\approx 45\%$  of the *duwx* and  $\approx 55\%$  of the *aeduw* sample still contained dextrans of sizes similar to the intermediate material, and after 4 hr,  $\approx 18$  and  $21\%$ , respectively, remained in the samples. The origin of this material was most probably the intermediate component, though it could not be excluded that new resistant material of this size also was formed from the amylopectin. Although obtained in lower amounts, the small dextrans that were included into the Sepharose CL 6B gel (fraction II) possessed size-distributions of the same type as those obtained from the *wx* starch at different time intervals. The LMW fraction (III) was, however, produced at similar rates and amounts in all three samples. Because this fraction mainly is produced as a result of the exo-attack pattern at external chains (Bertoft 1989), it suggested that these parts of both the amylopectin and the intermediate material were equally available to the  $\alpha$ -amylase. At 4 hr of hydrolysis, most of the external chains were expected to be extensively shortened (the production of fraction III was very slow at this stage) and approaching the length obtained after  $\beta$ -amylolysis. Based on the  $\beta$ -limit values (Table I), we calculated that the amount of intermediate material should have been reduced to  $\approx 15\%$  in the *duwx* sample if it was attacked only at the external chains by the  $\alpha$ -amylase. The observed  $\approx 18\%$  of intermediate material remaining at 4 hr of hydrolysis indicated that this indeed was the case. A pure external attack pattern would reduce the intermediate material to  $\approx 40\%$  in the remaining mixture of the *aeduw* sample. Because only  $\approx 21\%$  was found, endo-attack had also occurred at the internal chains of this material, suggesting that some parts were less densely branched than in the corresponding material of the *duwx* starch. The fact that the remaining intermediate material after  $\alpha$ -amylolysis was almost similar in the two samples, even though the original amount in the *aeduw* starch was double that found in *duwx*, also suggested less resistance to the  $\alpha$ -amylase.

#### Precipitation of $\alpha$ -Dextrans in Methanol

Branched intermediate  $\alpha$ -dextrans can be separated from the LMW material by precipitation in 5 volumes of methanol (Bertoft and Spooft 1989). Larger batches of the 4-hr hydrolysis mixtures were prepared with all three starches and the precipitates were free from fraction III when analyzed by gel-permeation chromatography (Fig. 4A).

A further subfractionation by size of the hydrolysis products of amylopectin can be done by precipitation with a series of increasing methanol concentration. The precipitate of the *wx* sample gave a series of fractions of decreasing sizes (not shown) comparable to other amylopectin samples that we have studied (Bertoft and Spooft 1989, Zhu and Bertoft 1996, Bertoft et al 1999). However, the separation of the precipitates of the *duwx* and *aeduw* samples was very poor (Fig. 4B and C). The dextrans with sizes similar to those of the *wx* sample were separated by size, but large dextrans that represented the more resistant material were obtained in all fractions. Dextrans of these sizes (DP >1,000) from amylopectins

**TABLE I**  
Composition and  $\beta$ -Amylolytic Limits of *wx*, *duwx*, and *aeduw* Maize Starches of Inbred Line Ia453

| Starch       | Content (%)     |                 | $\beta$ -Limit (%) |    |                    |
|--------------|-----------------|-----------------|--------------------|----|--------------------|
|              | AP <sup>a</sup> | IM <sup>b</sup> | AP                 | IM | Whole <sup>c</sup> |
| <i>wx</i>    | 100             | -               | 57                 | -  | 57                 |
| <i>duwx</i>  | 60              | 40              | 63                 | 62 | 64                 |
| <i>aeduw</i> | 20              | 80              | 60                 | 50 | 54                 |

<sup>a</sup> Amylopectin.

<sup>b</sup> Intermediate material.

<sup>c</sup> Amount of maltose.

would normally precipitate in methanol and water ratios <1:1. It seemed, therefore, that the different behavior of the intermediate material in methanol was due to a polymeric structure of a type different from the amylopectin component.

### Hydrolysis of Granular Starches with $\alpha$ -Amylase

The initial stages in the solubilization of the native starch was studied by pumping a diluted  $\alpha$ -amylase solution through a thin bed of starch granules in the lower compartment of a column as previously described (Bertoft et al 1993a). The enzyme was trapped on an ion-exchanger (DEAE-Sephrose) and the neutral dextrans were thereby separated from the enzyme before a secondary hydrolysis of the solubilized material occurred. The dextrans were collected ( $\approx 18$  mL/fraction and hour) and fractions representing each successive percentage of solubilized material were pooled and studied by gel-permeation chromatography on Superdex 75.

All three granular samples were attacked with different rates by the  $\alpha$ -amylase (Fig. 5). The rate of the hydrolysis of the *wx* sample was fast in the initial 50 hr. When  $\approx 4\%$  of the starch had been solubilized, the rate decreased gradually. At the end of the experiment (at 260 hr),  $\approx 9\%$  of the granules had been solubilized. Though starches from different sources are solubilized at very different rates (Leach and Schoch 1961, Fuwa et al 1977, Dettori-Campus et al 1992, Gallant et al 1992), the pattern of solubilization was similar to that for other starch granules earlier tested with this method (Bertoft and Manelius 1992, Bertoft et al 1993b, Manelius and Bertoft 1996, Manelius et al 1997). The two other starches possessed atypical patterns. The hydrolysis rate of the *duwx* starch granules was initially very slow. After this stage, the rate became nearly constant, and after 260 hr, the granules had solubilized to the same extent as the *wx* sample. The *aeduwx* granules were considerably more resistant to the  $\alpha$ -amylase. The starch was attacked with a constant, slow rate, and at the end of the experiment only 2% of the granules had been solubilized.

The susceptibility to fungal glucoamylase attack of a range of mutant maize starch granules from inbred line Oh43 was investigated by Fuwa et al (1979). In contrast to our results, they found that the endosperm mutations *duwx* and *aeduwx* were highly digestible. In their investigation, the starch granules were solubilized to much higher levels within a few hours by a high enzyme concentration. In this investigation, the initial stages of the enzymatic attack were studied using a very diluted  $\alpha$ -amylase preparation. The *du* mutation apparently made the granules more resistant to the  $\alpha$ -amylase attack only initially. If the reaction had proceeded for longer times, the *duwx* granules probably would have been more extensively hydrolyzed than the *wx* granules (Fig. 5). The reason

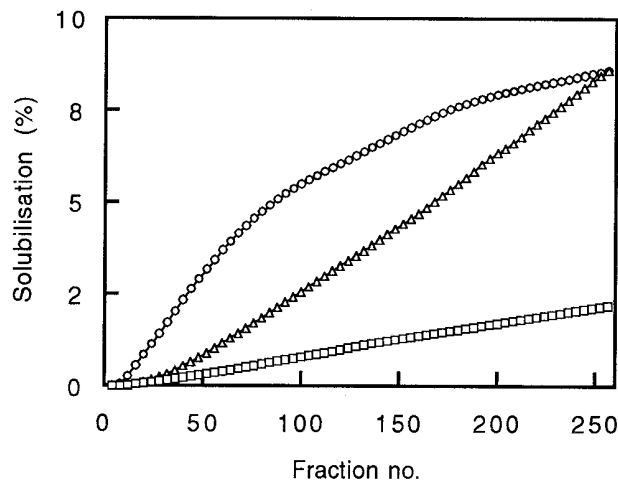


Fig. 5. Solubilization by  $\alpha$ -amylase of starch granules from *wx* (○), *duwx* (△), and *aeduwx* (□) maize of inbred line Ia453;  $\approx 1$  fraction/hr was collected.

for the slow initial attack is not clear. However, the enzyme has to adsorb to the granule surface before any catalytic activity could occur, and possibly an altered surface structure interfered with the adsorption. Also, in this investigation the inbred Ia453 was used, in which the *duwx* mutation contained a lot of enzyme resistant material that was not associated with the similar mutation of the Oh43 inbred background (Wang et al 1993b).

The *ae* mutations produce high-amylose starch granules with a B-type crystallinity that is highly resistant to enzymatic degradation (Gallant et al 1972; Fuwa et al 1978a,c). As gelatinized, the *ae* starch is, however, attacked at the same rate as normal maize starch (Fuwa et al 1978a). In combination with the *wx* mutation, the starch does not possess amylose and the situation is thus different. The *aewx* granules are also of B-type crystallinity (Shi and Seib 1995, Jane et al 1999) but were reported to be susceptible to amylase like most other *wx* combinations with A-type crystallinity (Fuwa et al 1979). In triple combination with the *du* and *wx* mutations, B-type crystallinity was reported from inbred W64A (Shi and Seib 1995). This was in agreement with a higher proportion of long chains (DP > 20) in the amylopectin that generally are associated with the B-type crystallinity (Hizukuri 1985, Jane et al 1999). In our investigation the *aeduwx* of inbred Ia453 possessed increased amounts of chains with DP  $\approx 20$ –35 only, whereas DP > 35 were found in lower amounts compared to the *wx* mutant (Fig. 1). Fuwa et al (1979) showed that *aeduwx* starch granules from inbred Oh43 were as highly susceptible to glucoamylase attack as were *duwx* granules. In contrast, the similar endosperm mutation of inbred Ia453 resulted in highly resistant granules (Fig. 5). The unusual high concentration of intermediate material was possibly a reason for the resistance. The effect of this material on the crystallinity and fine structure of the starch granules is not known, but as shown above, it appeared to be highly resistant to the enzyme as gelatinized. An altered structure of the amylopectin component could also result in a different granular structure and high enzymatic resistance, which is characteristic for B-type starch (Planchot et al 1997). In comparison, the better digestibility of the *duwx* sample suggested different structures of both the granules (A-type) and the components in that starch. Preliminary microscopy observations showed that both *duwx* and *aeduwx* starches contained some granules that possessed nonbirefringent areas under polarized light (not shown). The frequency of these granules was higher in *aeduwx* than in *duwx*.

### Composition of Dextrans Solubilized from Granular Starches

The molecular weight distributions of the dextrans solubilized from the starch granules at different stages are shown in Fig. 6. The changes in the distribution of the dextrans from the *wx* sample followed a pattern common to other starches: the initially solubilized dextrans were of higher molecular weight than those solubilized later (Bertoft and Manelius 1992, Bertoft et al 1993b, Manelius and Bertoft 1996, Manelius et al 1997). In the first percentage of solubilized carbohydrates, a major group of dextrans possessed DP

TABLE II  
Percentage Molecular Weight Distribution and  $\beta$ -Amylolysis Limits of Dextrans Solubilized from Granular *wx*, *duwx*, and *aeduwx* Maize Starches of Inbred Line Ia453

| Starch        | Stage of Solubilization, % | Degree of Polymerization |        |     | $\beta$ -Limit (%) |
|---------------|----------------------------|--------------------------|--------|-----|--------------------|
|               |                            | >100                     | 13–100 | <13 |                    |
| <i>wx</i>     | 1                          | 30                       | 35     | 35  | 55                 |
|               | 2                          | 17                       | 39     | 44  | 65                 |
|               | 8                          | 5                        | 46     | 49  | 69                 |
| <i>duwx</i>   | 1                          | 12                       | 35     | 53  | 69                 |
|               | 2                          | 5                        | 37     | 58  | 69                 |
|               | 8                          | 3                        | 36     | 61  | 66                 |
| <i>aeduwx</i> | 1                          | 2                        | 37     | 61  | 75                 |
|               | 2                          | 1                        | 44     | 55  | 72                 |

values of  $\approx 13$ –500, and in the second percentage, the amount of the largest dextrans was already reduced. Successive stages possessed only minor changes (not shown), and when 7–8% of the granules had solubilized, the group had a range of DP 13–100. In an earlier study (Bertoft and Manelius 1992), with a commercial waxy-maize starch sample, the dextrans that were initially solubilized covered a considerably broader range in which the largest dextrans had values of DP  $> 5,000$ . Thus, it seemed that different samples of waxy-maize starch granules are attacked differently and might possess unique properties. In addition to the larger dextrans, a separate group of carbohydrates at DP  $\approx 6$  was produced at all stages. Because a similar group was obtained from the external chains of the gelatinized starch, it probably originated from the same parts (external chains) of the components within the granular starch.

The fractions collected from the *duwx* starch granules contained considerably fewer larger dextrans, whereas the group at DP 6 was obtained in higher amounts than from the *wx* sample (Fig. 6). Only a small shift toward lower molecular weight at the later solubilization stages was obtained. This small change in size distribution agreed with the nearly constant solubilization rate of the granules. Because of the low digestion rate of the *aeduw*x sample, only the two first percentages of released material were collected. The size distribution in these fractions possessed slightly fewer larger dextrans than the corresponding fractions from the *duwx* starch.

The fractions were also partly characterized by treatment with  $\beta$ -amylase. In Fig. 6, the profiles of the distribution of the  $\beta$ -limit dextrans from the first percentage of solubilized dextrans are shown. All samples possessed limit dextrans and therefore most of the dextrans with DP  $\geq 13$  were branched. There was no indication of any specific group of dextrans of LMW other than the maltose peak, which confirmed the linear nature of at least the major part of the dextrans at DP 6. The percentage distribution of solubilized dextrans

with DP  $\geq 13$  (branched dextrans) and with DP  $< 13$  (mostly linear dextrans) is shown in Table II together with the  $\beta$ -limit values estimated as the amount of maltose produced by the  $\beta$ -amylase. In the *wx* sample, the proportion of branched dextrans decreased from 65% in the initial first percentage to 51% when 8% of the granules had been digested. The proportion of small linear dextrans therefore increased by 14%, which corresponded to a similar increase of the  $\beta$ -limit value. It appeared, therefore, that the structural nature of the branched dextrans (the average length of their external chains) remained the same at all studied stages of the hydrolysis. In the fractions from the *duwx* starch, the proportion of the material at DP 6 increased by 8% (from 53 to 61%) during the solubilization, whereas the  $\beta$ -limit values slightly decreased from 69 to 66%. This suggested an increased release of branched LMW dextrans from the granules at the successive stages of the solubilization. Contrary to the other starches, the two fractions obtained from the *aeduw*x starch suggested that the amount of small dextrans slightly decreased at the initial stages of the solubilization with a corresponding increase of dextrans at DP 13–100. The  $\beta$ -limit value, which was high already in the first percentage ( $\approx 75\%$ ), decreased slightly in the second percentage and corresponded to an increased release of branched material. The high  $\beta$ -limit values were either due to a large proportion of linear dextrans in the mixtures or to long external chains in the solubilized branched material. Thus, differences were not only found in the rate of the digestion of the starch granules, but also in the composition of the solubilized products from the three samples.

It was not possible to trace the exact origin of the solubilized dextrans. As discussed above, a major part probably derived from the amylopectin component because this component was apparently less resistant to amylase attack. With the exception of the first percentage digested from the *wx* granules, the  $\beta$ -limit values of

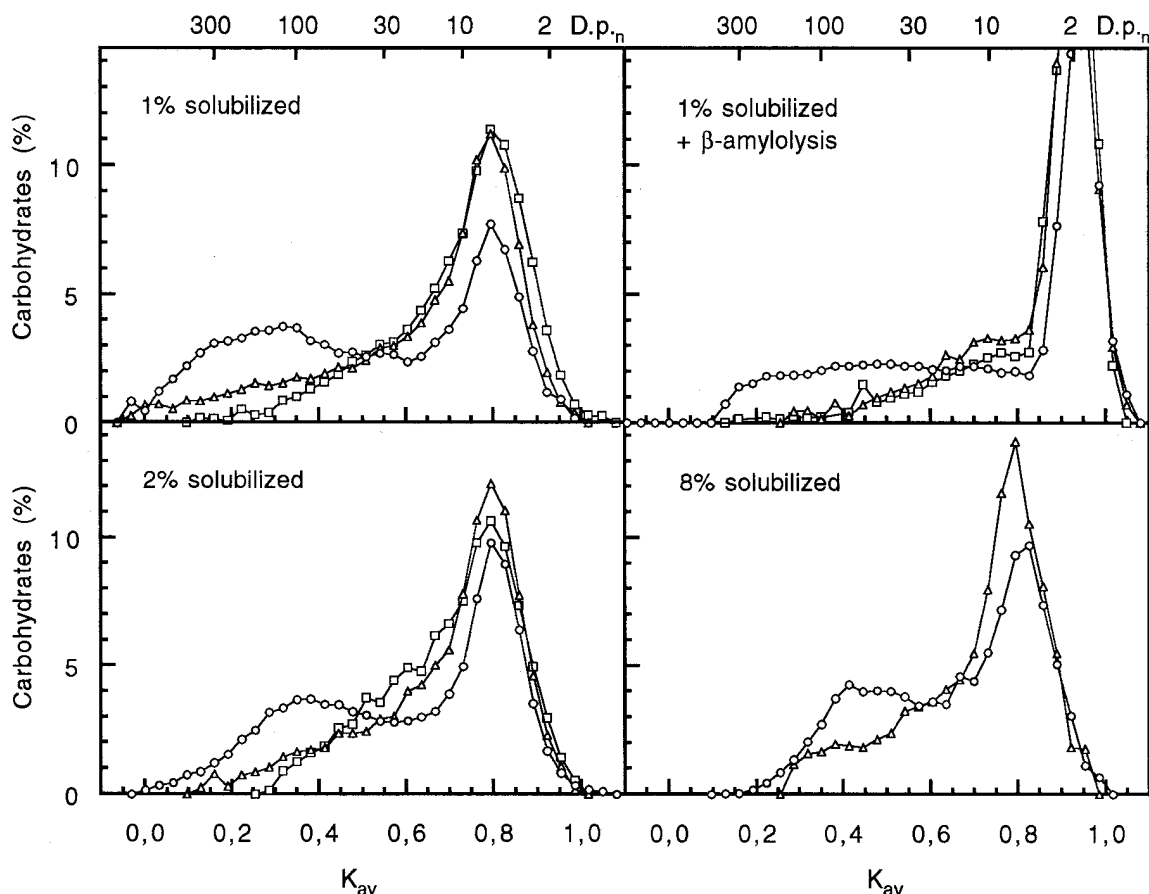


Fig. 6. Fractionation on Superdex 75 of dextrans solubilized at different stages from starch granules of *wx* (○), *duwx* (△), and *aeduw*x (□) maize and the composition of initial first percentage after treatment with  $\beta$ -amylase.

the solubilized material were higher at all stages than those found for the original starches (Table I). Possibly, this was partly due to linear fragments that originated from the external chains of the intermediate material.

## CONCLUSIONS

The three *wx* mutant starches of maize inbred line Ia453 were all free from amylose, but the double and triple mutants *duwx* and *aeduw*x contained a LMW, branched intermediate material in addition to amylopectin. This material possessed chain lengths of the types also found in the amylopectin component. Both samples possessed material that was highly resistant to  $\alpha$ -amylase attack. Probably this corresponded to the intermediate material, though it was also possible that it originated from an amylopectin component of altered structure. The enzyme contains nine subsites at its active site, and only if all subsites are filled with D-glucosyl residues from the substrate, the reaction is expected to be effective and fast (Robyt and French 1963). This strongly suggested that longer internal chain segments between the branches, comparable to those found between clusters of branches in normal amylopectin, did not exist in the resistant material. Therefore, it seems that the material possessed a more regularly branched structure in which the chains contained sufficient density of branching to prevent  $\alpha$ -amylase action. The altered behavior in methanol-water mixtures could also be explained by a different kind of structural architecture. The high content of intermediate material in *duwx* and, especially in *aeduw*x, should have an influence on the semicrystalline structure of the starch granules and thereby on the susceptibility to  $\alpha$ -amylase attack. The major part of the dextrans that solubilized from the granules probably originated from the amylopectin component.

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